Norbert Pallua
Christoph V. Suschek
Editors

Tissue Engineering

From Lab to Clinic

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Preface

Although efforts to generate bioartificial tissues and organs for human therapies date back to the 80s, these efforts have only come closer to reality in the past 10 years. The possibility of such bioartifical tissues has been fueled by major advances in cell and molecular biology and the development of more sophisticated cell-culture technologies. The term *Tissue Engineering* has come to a broader application in the last five years and now encompasses the many interdisciplinary fields of knowledge that are crucial to generate or regenerate tissues or even whole organs. Tissue engineering has the potential to revolutionize health care, improving the treatment options and quality of life for millions of people worldwide, yet, saving enormous financial resources in terms of health care costs. One of its defining characteristics is that it draws upon and requires close collaboration among scientists in many diverse specialties. Cell and molecular biologists, biomaterials engineers, advanced imaging specialists, robotics engineers, and developers of equipment such as bioreactors, where tissues are grown and nurtured, are all part of the process of tissue engineering. Another characteristic of tissue engineering is that the field has brought together researchers worldwide in international collaborative efforts. Tissue engineering as a promising research is underway in all parts of the globe. The emergence of tissue engineering has coincided with the emergence of the Internet, making far-reaching collaborations more possible than ever before.

The topics in *Tissue Engineering: From Lab to Clinic*, addressed by world-renowned authorities, were selected to cover the spectrum from basic development to clinical application. Pertinent information on cell isolation and expansion, both in animals and humans, provides guidance for the clinical scientists interested in this area. Written by leading experts in the field, each chapter offers a detailed state-of-the-art overview of the respective field of research, the author's visions regarding his research area, as well as its limitations. This book provides a conceptual framework that includes the entire necessary background material in all areas of tissue engineering.

Tissue Engineering: From Lab to Clinic is intended to be a reference for first-year to senior-level graduate courses in Tissue Engineering, in departments of bioengineering, and for students who perform research in tissue replacement and restoration. Additionally, this book attempts to give guidance for students in biology, medicine, and life-sciences, working with primary and complex cell biology. This book, seeks to provide both undergraduate and graduate students with the scientific foundation of

tissue engineering. We therefore hope to provide a reference to biomedical engineering students, bioengineers, biological sciences graduate students, as well as their teachers, to managers and scientists in the biotech industry, and academic researchers.

Norbert Pallua
Christoph V. Suschek

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Part I

Basics and Principles of Tissue Engineering

Micro- and Nanotechnology in Tissue Engineering

1

Daniela Coutinho, Pedro Costa, Nuno Neves, Manuela E. Gomes, and Rui L. Reis

1.1 Introduction

Tissue engineering (TE) is a rapidly growing scientific area [129] that aims to create, repair, and/or replace tissues and organs by using combinations of cells, biomaterials, and/or biologically active molecules [42, 119]. In this way, TE intends to help the body to produce a material that resembles as much as possible the body's own native tissue. By doing so, TE strategies promise to revolutionize current therapies and significantly improve the quality of life of millions of patients.

The classical TE strategy consists of isolating specific cells through a biopsy from a patient, growing them on a biomimetic scaffold under controlled culture conditions, delivering the resulting construct to the desired site in the patient's body, and directing the new tissue formation into the scaffold that can be degraded over time [42, 119].

Most of the presently existing TE techniques rely on the use of macrostructured porous scaffolds, which act as supports for the initial cell attachment and subsequent tissue formation, both in vitro and in vivo [88, 102, 113]. This kind of approach has been successful to a certain extent in producing relatively simple constructs relying on the intrinsic natural capability of cells and tissues to self-regenerate, remodel, and adapt. For this reason, cells have been the most significant factor in the generation of the tissue itself [33]. However, this natural capability of cells for adapting to its surrounding environment has limitations and that is the main reason why TE has not been able to generate complex thick tissues so far [47]. In fact, one of the most important drawbacks of the currently available constructs in TE approaches is related to the lack of means to generate effective oxygen and nutrient dispersion pathways that can reach a whole construct homogenously and, therefore, enable the functionality/viability of the construct upon implementation.

In order to generate constructs capable of accurately mimicking/replacing structures as defined and organized as complex tissues and organs, novel kinds of scaffolds and devices have lately been developed, which potentially allow obtaining a fine control over the cellular positioning, organization, and interactions [36]. For this, much has contributed the continuous technological development in the areas of micro- and nanotechnologies, both in terms of production methods and in analysis tools [107]. Developments in these areas may allow a finer control over the architecture of scaffolds, making them no longer simple substrates for cellular adhesion and proliferation, but most importantly, active agents in the process of tissue development [37]. Micropatterning integrated in a TE approach is a result of the combination of micro- and nanofabrication techniques with materials science and surface engineering, which results in a deep exploration of the microenvironment where cells are embedded [37, 90]. In TE, micro- and nanotechnologies can also be applied to fabricate biomimetic scaffolds with increased complexity to promote, for example, vascularization, also enabling to perform a series of high-throughput experiments (Fig. 1.1).

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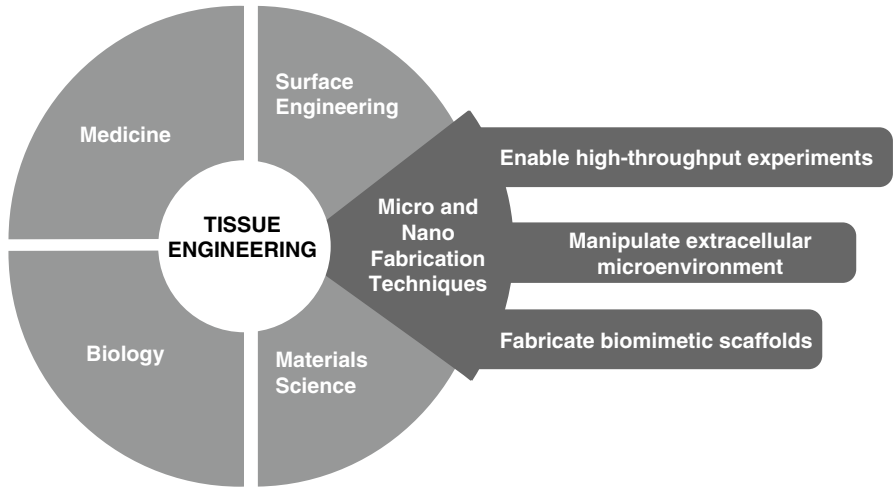


Fig. 1.1 Schematic representation of the different areas of expertise involved in a tissue engineering (TE) strategy. Highlight to the inputs brought by micro- and nanofabrication techniques

The main aim of this chapter is to shed new light on the use of micro- and nanotechnologies in TE strategies, highlighting the need for new technologies to obtain an in-depth understanding of the microenvironment at the implantation site. This chapter also reviews some of the most widely used and most promising micro- and nanoscale technologies in TE strategies. Subsequently, the most advanced applications of micro- and nanofabrication techniques in different areas of TE will be described, showing their enormous potential for being applied to the improvement of the life quality of patients. Although the described techniques are usually applied for developing 2D structures, successful attempts to move towards 3D structures by means of micro- and nanofabrication techniques will be explored. Finally, the application of micro- and nanotechnologies in the development of cell/tissue culture systems (microbioreactors) will also be briefly discussed.

1.2 Aim of the Discipline

Micro- and nanotechnologies consist of the design, characterization, production, and application of structures, devices, and systems by controlling their shape and size at micrometric or nanometric scales. The properties of materials at atomic, molecular, and macromolecular scales are significantly different from those at a larger macroscopic scale, and for this reason, micro- and nanosciences have been receiving increasingly scientific, industrial, and social attention. The development of those areas may revolutionize and change the world as we know it and as we see it. Materials science and health are among the various areas that may mostly benefit from the evolution of micro- and nanotechnologies.

The major challenge encountered in biomaterials science is the issue of biocompatibility. Given its vital role, the control of the biocompatibility has become a major challenge and a main focus of research in the process of developing new materials for TE applications. Micro- and nanotechnologies, previously used in other research areas, are now integrated into the optimization of interactions occurring at the interface between materials and cells. By providing the possibility to tailor features such as micro- and nanotopographies, these novel technologies allow for the development of new micro- and nanostructured materials possessing unique properties allowing the development of solutions for the most complex end-applications.

Micro- and nanotechnologies can be regarded and designed from two different perspectives: the top–down and the bottom–up approach. As these terms suggest, the top–down approach can be pictured as large entities creating small devices while the bottom–up approach consists of small entities generating large devices. Both the perspectives are valid and interconnected and can lead to the development of new solutions for unaccomplished needs in several fields.

1.3 State of the Art

1.3.1 The Need for Micro and Nanotechnologies in Tissue Engineering Strategies

One of the major motivations for the increasing effort spent on designing and developing nanostructured materials for TE strategies is that natural tissues and the associated extracellular matrices are in fact composed of nanostructured materials [3, 69, 104] (Fig. 1.2). These highly organized and cooperative micro- and nanobuilding blocks assemble in a controlled way to ultimately build healthy tissues. When an implant first contacts its host environment, proteins are the first biological entities to interact with the surface of the biomaterial [10, 76]. In fact, the surface of the material gets covered by a layer of proteins, just a few seconds after implantation in a competitive process. Since cells do not interact with naked surfaces, the proteic layer adsorbed onto the material's surface will interact with the cell receptors at the cell membrane surface [110]. Thus, the presence of specific molecules is mandatory for a suitable attachment and proliferation of cells onto the surface of a given substrate [48]. Proteins are the key elements creating a bridge between the nonbiological surface of materials and cells [23]. Figure 1.3 represents, in a simplified way, the cascade of events occurring at a biomaterial implantation site. The adsorption of proteins onto the surface of biomaterials from a surrounding environment is a rather complex process. The adsorptive behavior of proteins is highly dependent on various surface properties such as chemistry, charge, topography, wettability, and surface energy [20, 89]. These parameters of the implant's surface will determine not only the amount of proteins adsorbed but also their type and conformation. The protein net charge and charge distribution, size, conformation, and hydrophobic domains are some of the characteristics of proteins that might have a profound effect on the implant surface properties and could ultimately influence the cell response to the implant's surface [10, 23, 76, 96]. Therefore, while adsorbing to surfaces, these nanoscale entities can either retain their original conformation or may have it altered in response to the environment conditions [23]. This surface-specific adjustment can result in the presentation of different aminoacidic regions to cells [23].

The initial contact between anchorage-dependent cells and the biomaterial's surface involves various biological molecules, such as extracellular matrix (ECM) proteins, cell membrane proteins, and cytoskeleton proteins [137]. Interactions between these biological molecules and their specific receptors induce signal transduction, and consequently influence cell growth and differentiation [40, 115]. Thus, the surface micro- and nanostructures are of huge importance as they will first dictate the pattern of protein adsorption, ultimately influencing the cell behavior through the motifs presented by those adsorbed proteins to the

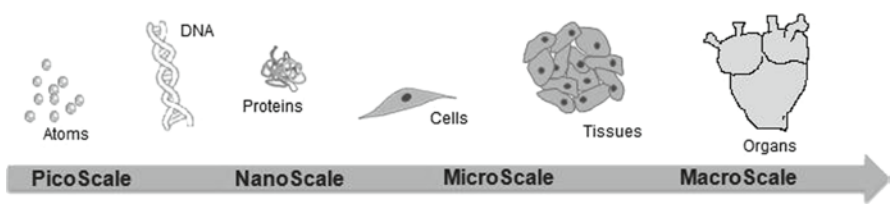


Fig. 1.2 Nano- and Microscales in Nature

Fig. 1.3 A glimpse at the cascade of events occurring at the implantation site

cells. Moreover, the cells themselves are also capable of directing their own position and orientation through a process called mechanosensing [112]. It has been shown that the cells can sense the topography of the surfaces to which they adhere and reposition themselves accordingly. Cells can respond both to the dimensions of the surface's roughness and also to its topography. Studies have revealed that cells can show a preference for adhering to surfaces within certain ranges of roughness, this factor being the determinant of the amount of cellular proliferation over that surface [6]. As for the surface's geometry, studies have shown that, when seeded in linearly grooved surfaces, cells tend to preferably adhere and grow longitudinally over the grooved lines [154]. The cell alignment is also favored in surfaces exhibiting grooves within certain width ranges [14, 31]. Figure 1.4 shows an example of a defined alignment of osteoblast-like cells (SaOs-2) on a patterned surface. Still, regarding geometry, studies have shown that cells tend to adhere preferably to randomly organized surfaces instead of geometrically organized ones. Thus, for the design of enhanced biomaterials to be used in TE strategies, it is of utmost importance to understand the cellular processes that lead to a more efficient tissue regeneration as well as those involved in the formation of new tissue avoiding triggering of undesired immunological or inflammatory responses. As referred, the interaction between polymeric biomaterials and biological elements is dictated by the processes occurring at the interface between these two phases. An approach to optimize the interaction of a biomaterial with its implantation site bioenvironment is by tailoring the properties of the material's surface with micro- and nanostructures that could elicit the desired behavior on the biological entities. For this, micro- and nanotechnologies can be used to overcome the limitations of the currently used techniques to fabricate biomaterials with effective micro- and nanosurface structures that could mimic as close as possible the micro- and nanobioenvironment.

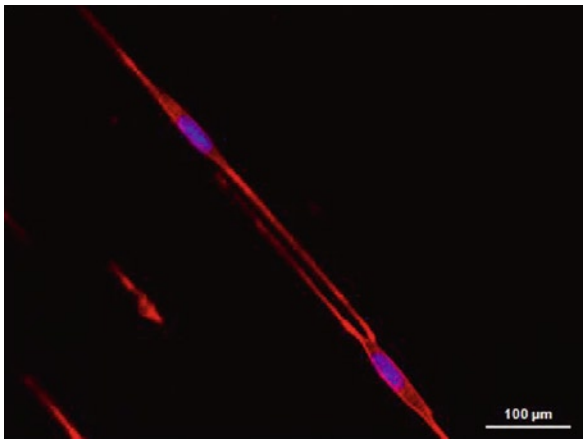


Fig. 1.4 Cell alignment of osteoblast-like cells (SaOs-2) on a patterned surface with the cytoskeleton stained with phalloidin and the nucleus counterstained with DAPI

1.3.2 Micro and Nanofabrication Methods

Micro and nanofabrication is the general term used for describing the processes of fabricating miniature micro or nanoscaled structures by using macroscale devices. The earliest microfabricated devices consisted of semiconductor integrated circuits and were fabricated by simple surface treatments or methods such as lithography or chemical vapor deposition [45, 117]. Apart from integrated circuits, which are mainly two-dimensional, these technologies are commonly used in the production of devices such as microelectromechanical systems (MEMS) [27, 118], laser diodes [156], flat panel displays [100], or fuel cells [61].

Different strategies have been utilized to apply two-dimensional micro- and nanofeatured devices to TE. Microfluidic devices can be produced through various surface treatments or lithographic methodologies. These methodologies consist of creating patterns on the surface of materials, into which cells can be cultured and positioned into specific locations by physical constraints. This physical constraint can also be used for controlling the interaction of cells with its surroundings, as with other cells. The same grooves that are used for cellular constraint can also be used for selectively feeding cells by perfusing with cell culture medium using microfluidic systems [58].

As mentioned above, micro- and nanofabrication methods can be roughly divided in two areas following radically different concepts: bottom–up or top–down (Fig. 1.5). The latter normally includes photolithography, microcontact printing, microtransfer molding, capillary force lithography, scanning probe lithography (SPL), and electrospinning methods, which will be discussed in the following sections.

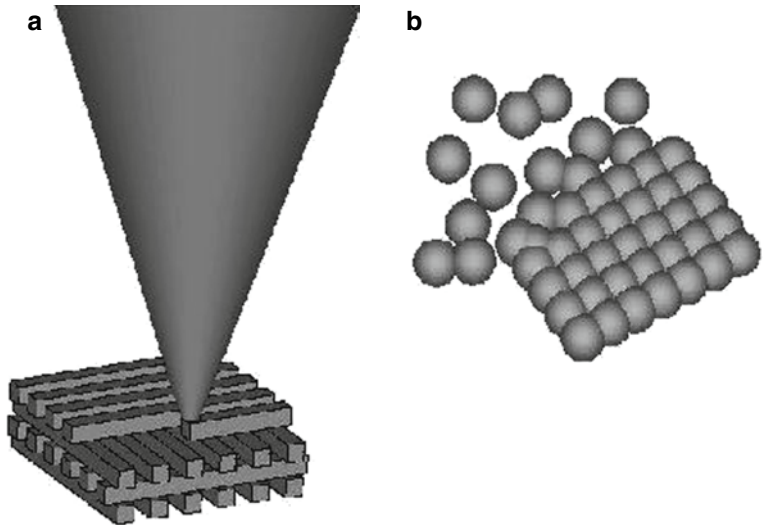


Fig. 1.5 Schematic diagram of the (**a**) *top–down* and (**b**) *bottom–up* nanotechnology approaches for TE

Both approaches require a great know-how on the surface properties of materials. Besides having insights into the molecular events through thermodynamic and kinetic processes (bottom–up), researchers must also possess the skills to understand device miniaturization and fluidics, which are associated with top–down fabrication strategies. Interestingly, bottom–up and top–down approaches have in some cases merged to create systems that are more effective for tissue regeneration purposes. For example, microfluidic devices have been used to act as vasculature systems and test the interaction of targeted particles with the cells [133]. Also, 3D structures have been developed by combining bottom–up and top–down approaches. Hydrogels were micromachined by means of a top–down approach and then these patterned building blocks were assembled to form a more complex structure through a bottom–up approach [35, 147]. The next subsections are devoted to presenting an overview of the most used techniques of each approach.

1.3.2.1 Bottom–Up Approach

The bottom–up approaches mainly rely on the natural assembly of atoms, molecules, or other nanosized building blocks such as peptide residues (dimensions typically of 2–10 nm [83]). Nature has already perfectly controlled micro- and nanoscale components by macromolecular recognition of various biological materials. Therefore, scientists are maintaining the trend of learning from nature how to design materials and systems using similar processes as the ones observed in nature [70]. The molecular assembly of building blocks by controlled reactions makes this technique more economic as compared to the conventional top–down methods [83].

Indeed, biologically inspired self-assembly holds great promise in the development of nanostructures [150]. For instance, research has shown that amphiphilic peptides can self-assemble to form hydrogels with great potential for TE strategies [150]. Moreover, self-assembled scaffolds functionalized with specific peptide moieties can be produced by incorporating these functional groups into the initial building molecule.

Of great interest are also the self-assembled-monolayers (SAMs), well established for the study of surface modification [114]. The SAMs are produced when a substance spontaneously forms a molecular monolayer on a surface [43]. Work reported by Shuguang Zhang and collaborators [151, 152] has shown that by observing the processes by which supramolecular structures are assembled in nature, the synthesis of novel synthetic materials can be developed. Thus, they have been able not only to coat surfaces by molecular assembly but also to develop nanofiber peptide and protein scaffolds with approximately 10 nm in diameter [150], which have proven to be suitable for applied studies in TE. Likewise, Frank Gu and collaborators [73] have successfully developed polymeric nanoparticles, which were formulated by the self-assembly of an amphiphilic triblock copolymer composed of end-to-

end linkage of poly(lactic-co-glycolic acid) (PLGA), polyethyleneglycol (PEG), and the A10 aptamer (Apt).

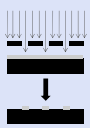
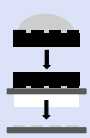
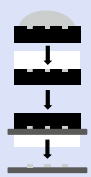
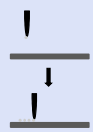
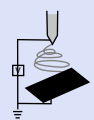
1.3.2.2 Top–Down Approach

Most of the techniques used in the development of micro- and nanopatterned surfaces for TE purposes follow this route. Frequently, their origin is in other fields of research such as microelectronics fabrication. Nevertheless, all of these techniques are based on the development of micro- and nanofeatures on the surface of a material by means of larger tools. In the next sections, we present an overview of the most relevant techniques used in micropatterning biological materials, as well as their advantages and limitations for this purpose. Photolithographic techniques are widely developed and optimized for patterning cells. Nonetheless, disadvantages of this technique, mainly regarding biocompatibility issues, have highlighted the interest on alternative techniques based on soft-lithographic methods, such as microcontact printing, microtransfer molding and molding in capillaries. Likewise, scanning probe lithographic methods based on atomic force microscopy (AFM) are emerging as useful techniques for cell patterning applications. Additionally, to these techniques, the development of micro- and nanopatterned surfaces by means of electrospinning method has demonstrated a notable importance in TE strategies, demonstrated by the exponentially increasing number of publications on this subject over the years. Tables 1.1 and 1.2 present a summary of the technologies described in the next sections, concerning its resolution, materials that can be used, as well as their most advantageous and disadvantageous features.

Photolithography

Photolithography has been one of the first techniques to be applied in the micropatterning of surfaces [135] for various areas including for the biomedical field.

Table 1.1 Summary of the nano- and microtechnologies most used

	Photolithography	Soft lithography				Electrospinning
		Microcontact printing	Microtransfer molding	Molding in capillaries	Scanning probe lithography	
Resolution	37 nm	35 nm–1 μm	250 nm	50 μm	20–30 nm	5 nm–1 μm
Scheme of the technique						
Most advantageous feature(s)	Precise control of the features	Simplicity Flexibility Allows for multipattern Applied to nonplanar surfaces	Applied to nonplanar surfaces Allows to generate 3D structures Pattern large areas	Allows to produce different patterns in a parallel fashion The use of wet patterns – broaden applications Creation of gradients	Precise control of the added groups	Structures similar to ECM High range of applicable materials
Most disadvantageous feature(s)	Polymers added with photosensitive compounds	Structural constraints of the stamp Low control of the ligand density	Microstructures have a thin film between the raised features	Patterned geometries limiting	Limited to a low range of patterned sizes	Poor mechanical properties
Reference	[41]	[64, 93]	[153]	[93]	[16]	[71]

Briefly, photolithography involves the placement of a precursor polymer solution and a UV light photoinitiator onto the substrate of choice, so that it forms a thin but uniform film (~1 μm thick) [1]. Photopolymerization is further performed under ultraviolet (UV) light using a mask to define selected exposure areas. Only the exposed regions of the prepolymer, which are a result of a patterned mask that is placed in between the light source and the precursor polymer solution, are submitted to photopolymerization. The surface is washed, leaving the patterned projections. Even though there are several variations of photolithographic methods, all rely on the use of a photoresistant mask with the desired pattern and the exposure of a precursor polymer solution to UV radiation.

Despite the enormous potential offered by this technology, the application of photolithography to patterning in biomedical applications presents some drawbacks, such as: (1) the high costs associated with the necessary equipment, namely, those related with the fabrication of the photoresistant mask, (2) the need for clean-room facilities, (3) the extra expertise required from the biologists, and (4) the use of chemical compounds which may be toxic to cells and induce denaturation of proteins.

Soft Lithography

As an attempt to overcome the limitations of photolithograpic methods, a set of techniques entitled soft-lithographic methods have been developed by Whitesides and his research group [93]. Like photolithography, these techniques also make use of a micropatterned surface to generate the pattern of interest in the desired substrate. In this case, an elastomeric mold is used to transfer the patterns into the surfaces, determining the term "soft." In soft lithography, a stamp is created by molding an elastomer, which is usually produced using polydimethylsiloxane (PDMS). It offers several advantages including being biocompatible and permeable to gases. Soft-lithographic methods mainly comprise microcontact printing, microtransfer molding, and molding in capillaries, also known as capillary force lithography.

The general advantages of the aforementioned methods comprise : (1) the lower costs of softer materials, (2) their potential biocompatibility, (3) PDMS surface can be replicated repeatedly from the microfabricated master, and (4) the biopatterning does not damage the stamp surface.

Microcontact Printing

Microcontact printing (μCP) was pioneered by Whitesides and his group in 1994 [136] and allows for patterning molecules onto a surface, resulting in control over the protein adsorption and cell behavior. Briefly, in this method, a stamp is produced by replica molding, which is usually made with PDMS. It involves inking the stamp with the substance to be transferred to the substrate. As the solvent evaporates, the molecules to be printed become deposited, and by removing the stamp from the surface, a pattern is created [37]. The first major application of this contact transfer-based method in the biomedical field consisted of the stamping of alkanethiolate inks, resulting in the formation of self-assembled alkythiol monolayers [87, 136]. Nevertheless, efforts have been made toward the use of different ink solutions [106] to pattern polymers [8, 132], DNA [67, 124], proteins [13, 32, 97, 105], or even cells [87, 116, 125]. However, the interactions between the stamp and the ink solution need to be controlled since PDMS is hydrophobic (Table 1.1).

The stamp being deformable allows detachment from the substrate without smearing the inked pattern. Moreover, because of its additive nature, it can be applied to nonplanar surfaces and develop multiprotein patterns [13]. Also, μCP is convenient for printing large areas (100 cm^2) in a single stamping step. Once the stamp is available, multiple copies of the pattern can be produced. Even so, it possesses some inherent disadvantages: (1) the density of the stamped material is not easily controlled [37, 106], (2) the elastomeric stamp might swell leading to a change on the pattern geometry [37], and also (3) contamination of the patterns with unpolymerized low molecular weight siloxane from the elastomeric stamp my compromise the process [101, 141].

Microtransfer Molding

Microtransfer molding (μTM) was described for the first time in 1996 [153] and is a technique which allows for generating rather complex 3D structures. In μTM, an elastomeric mold usually made of PDMS

Table 1.2 Examples of controlling cell microenvironment using micro- and nanotechnologies

Application	Micro-/ nanotechnology	Cell–microenvironment interaction	Material	Features	Cells	Effect observed	Reference
Bone	Photolithography	Cell orientation and behavior	–	330 nm deep and either 10, 25, or 100 μm in width	HOBs	Groove/ridge topographies are important modulators of both cellular adhesion and osteospecific function, and, critically, groove/ridge width is important in determining cellular response	[17]
	Photolithography	Cell behavior	Photocrosslinkable chitosan	500–500 or 100–100 μm	SaOs-2	The method utilized allowed to create patterned cell cocultures by using lysozyme or layer-by-layer surface switching	[51]
	Photolithography combined with electrochemical micromachining	Cell shape and behavior	Titanium	Cavities with 100, 30, and 10 μm diameters	MG63 osteoblast-like cells	Cell attachment depends on cavity spacing, cell growth and aggregation depends on cavity dimensions, and cell morphology depends on the presence of submicron-scale structural features	[155]
	Soft lithography	Cell orientation and behavior	Collagen	Groove Width: 27 mm; groove depth: 12 mm; ridge width: 2 mm	Mesenchymal osteoprogenitor cells	Cell alignment can be significantly affected by the topography of the carrier surface; appropriate surface topography can enhance bone formation	[12]
	Microcontact printing	Cell behavior	PHBV	1–10 μm groove width; 5–30 μm groove depth	Rat mesenchymal stem cell-derived osteoblasts	Microtopographies on PHBV can improve osseointegration when combined with chemical cues; microgrooves and cell adhesive-protein lines on PHBV can guide selective osteoblast adhesion and alignment	[53]
	Microcontact printing combined with hot-embossing imprint lithography	Cell orientation and behavior	Polyimide; fibronectin	8 μm wide grooves 4 μm deep (hot-embossing printing); 10 μm wide (microcontact printing)	MC3T3-E1 osteoblast-like cells	Although chemical patterns induced stronger alignment than mechanical topography when presented separately, mechanical topography dominated the alignment for all the chemical patterns when combined	[25]

	Electrospinning	Cell orientation and behavior	SPCL	Diameter of fiber 400 nm (average)	SaOs-2	The presence of the nanofibers induced the cell morphology to change into a more stretched and spread shape, increased cell activity, and viability	[127]
	Electrospinning	Cell orientation	PCL	Diameters of the fibers ranging between 200 nm and 1.2 μm	SaOs-2	The cells integrate with the surrounding fibers and migrate into the inner nanofibrous structure to form a three-dimensional cellular network	[78]
Cartilage	Replica molding	Controlled microenvironment	PDMS; glass coverslip	Channels 300 μm wide and 100 μm high	Chondrocytes isolated from articular cartilage	The method associated with an inverted microscope allowed to monitor real-time cell size changes; articular chondrocytes exhibited a trend of increasing changes in cell size with decreasing osmotic loading frequency	[24]
	Photolithography	Cell behavior	HA on PET	Channels 25 and 5 μm wide and with the same spacing	Articular knee chondrocyte	Micropatterned HA induced adhesion, migration, alignment, and differentiation of chondrocytes	[11]
	Photolithography	Cell shape and behavior	Agarose gel	15–65 μm wide; 40 μm deep	Chondrocytes isolated from avian sterna	Micropatterned polymer gels were subsequently applied as scaffolds for chondrocyte culture and proved effective in maintaining the key aspects of the chondrogenic phenotype (rounded cell morphology; production of type II collagen)	[94]
Vascular	Soft lithography	Controlled microenvironment	PDMS	10 μm wide, 3 μm deep, 10 μm distance between each groove	MSCs	Microgrooved patterns induced global gene expression changes (an increase in the smooth muscle marker calponin one, a decrease in cartilage matrix markers, and alterations in cell signaling)	[66]
	Soft lithography	Cell shape and behavior	PDMS and collagen	Groove and ridge widths of 330 nm each; depth of 100 nm	VSMCs	Nanopatterns could successfully guide VSMCs along the pattern axes, improving collagen construct strength	[44]
	Soft lithography	Cell behavior under shear stress	PU	95 μm wide and 32 μm deep	Endothelial cells	Endothelial cell retention is improved on micropatterned surfaces, which could help in reducing the thrombogenicity of implanted grafts	[34]

(*continued*)

Table 1.2 (Contiuned)

Application	Micro-/ nanotechnology	Cell–microenvironment interaction	Material	Features	Cells	Effect observed	Reference
	Soft lithography associated with melt molding and particulate leaching	Cell shape and behavior	PCL and PLGA	48 μm grooves; 5 μm deep; 12 μm spacing	VSMCs	VSMCs seeded into PLGA leached micropatterned PCL scaffolds maintain similar degrees of alignment as seen on nonporous micropatterned scaffolds (findings associated with promoting in vivo-like VSMC morphology, enhanced ECM production, and decreased proliferation)	[109]
	Microfluidic patterning	Cell shape and behavior	HA hydrogels		Cardiomyocytes	The linearly aligned myocytes detached from the surface and formed contractile cardiac organoids	[55]
	Microfluidic patterning	Cell-ligands	PEG	Gradient generation of tethered RGDS through microfluidics/photopolymerization process	HUVEC	The adhesive ligand gradients modulated the spatial distribution of attached endothelial cells	[57]
	Microfluidic patterning	Cell-ligands	PDMS	Width 50–1,000 μm and spacing between them 50–100 μm; patterned adhesion molecules: P-selectin, E-selectin, von Willebrand factor	Neutrophils, ChineseHamster ovary cells (CHO), platelets	The adhesion and rolling of three different cell types was controlled by patterning microfluidic channels with different ligands	[91]
	Electrospinning	Cell orientation and behavior	P(LLA-CL)	Diameters of the fibers ranging from 200 to 800 nm	SMCs	The SMCs attached and migrated along the axis of the aligned nanofibers and expressed a spindle-like contractile phenotype	[144]
	Microcontact printing	Cell behavior	PDMS	Width of 20 and 115 μm	Bovine aortic endothelial cells	The presented cell morphology may interfere with the mechanisms sensing the physical cues, therefore responding differently to shear stress	[74]

Neural	Microcontact printing	Cell–cell; Cell behavior	Silicon oxide	3–20 μm in line width; 3.5 nm high of the layer	Rat hippocampal neurons	The immobilized neurons showed resting membrane potentials comparable with controls and were capable of eliciting action potentials after 1 day of culture	[111]
	Microcontact printing	Cell orientation and behavior	Polystyrene; PDMS	4–6 μm wide lines	Rat embryonic cortical neurons	Cells comply well with the pattern and form synaptic connections along the experimentally defined pathways	[130]
	Replica molding	Cell shape and behavior	Poly(glycerol–sebacate) on sucrose-coated microfabricated silicon	2–5 μm in wavelength; depth of 0.45 μm	Bovine aortic endothelial cells	Cells cultured on substrates with smaller pitches exhibited a substantially higher frequency of cell alignment and smaller circularity index; this system showed to be appropriate for in vivo applications	[14]
	Electrospinning	Cell orientation and behavior	PLLA	Average diameter of 300 nm (concentration of 2% of PLLA) and 1.5 μm (concentration of 5% of PLLA)	NSCs	The rate of NSC differentiation was higher for PLLA nanofibers than that of microfibers and it was independent of the fiber alignment	[145]
	Electrospinning	Cell orientation and behavior	PCL	Fiber diameter around 250 nm	hESCs	When EBs were cultured onto PCL nanofibers, they were capable of differentiating into mature neural lineage cells including neurons, oligodendrocytes, and astrocytes. The aligned nanofibers could also direct the neurite outgrowth	[142]
Liver	Photolithography	Cell–cell (3D structure)	PEG hydrogels	500 μm in width	Hepatocytes	Three-dimensional photopatterned constructs were cultured in a continuous flow bioreactor for 12 days where they performed favorably in comparison to unpatterned, unperfused constructs	[126]
	Photolithography	Controlled microenvironment	PDMS	Through-holes (500 μm with 1,200-μm center-to-center spacing)	Primary rat hepatocytes; primary human hepatocytes; 3T3-J2 fibroblasts	The miniaturized, multiwall culture system for human liver cells with optimized microscale architecture was able to maintain phenotypic functions for several weeks	[59]

(*continued*)

Table 1.2 (Contiuned)

Application	Micro-/ nanotechnology	Cell–microenvironment interaction	Material	Features	Cells	Effect observed	Reference
	Photolithography combined with robotic microarraying of proteins	Controlled microenvironment	PEG	Collagen spots with 170 μm in diameter; 30–30 μm PEG wells	Rat hepatocytes	When exposed to micropatterned surfaces, hepatocytes interacted exclusively with collagen-modified regions, attaching and becoming confined at a single-cell level within the hydrogel wells	[2]
	Photolithography combined with anisotropic wet etching	Controlled microenvironment	Titania ceramic	Circles 200 μm in diameter; heights ranging from 50 to 135 μm	Rat liver hepatocytes	The cells mainly aggregate within the cavities, and only single cells or small clusters are attached to the rims between the pits	[95]
	Soft lithography	Controlled microenvironment	PDMS; polycarbonate	Branch pattern; main inlet and outlet channels are 2,650 μm; the channels progressively branch and decrease to 35 μm in width to form the smallest structures	Hepatoma cell line HepG2/C3A; primary liver cells	The design provides sufficient oxygen and nutrient mass transfer to support the viability and function of both a highly metabolic hepatoma cell line and primary rat hepatocytes	[22]
	Electrospinning	Cell–cell	PCLEEP	Diameters of the fibers ranging from 300 nm to 1.5 μm	Primary rat hepatocytes	Galactosylated PCLEEP nanofiber mesh exhibits the unique property of promoting hepatocyte aggregates within the mesh and around the fibers, forming an integrated spheroid–nanofiber construct	[29]
Stem cells	Photolithography	Cell orientation	Silicon	5 μm in width; 5 μm in spacing; 1.6 μm in depth	MSCs	The cells were shown to inhabit the grooves rather than ridges and exhibited an elongated shape, with unusually long processes	[149]
	Photolithography	Cell behavior	PLLA	3 μm in width; 100 μm in spacing; 1.5 μm in depth	Mouse bone marrow stromal precursors	The microtopography showed to influence the differentiation of cells to adipocytes, also affecting the rate of lipid production	[26]

Soft lithography	Cell shape and behavior	CCG blended membranes	200 μm in width and 80 μm in depth	hMSCs	Some of the cells formed a bridge-like structure between two adjacent ridges; the micropatterned CCG membranes could be used to regulate the distribution, alignment, proliferation, and morphology of hMSCs during cell culture in vitro	[148]
Soft lithography	Cell differentiation; controlled microenvironment	Cell-repellant PEG	150–40 μm diameter microwells	hESCs	The microwell technique could be a useful approach for in vitro studies involving ES cells and, more specifically, for initiating the differentiation of EBs of greater uniformity based on controlled microenvironments	[50]
Soft lithography	Cell-ECM	PDMS	3.5 mm in diameter bioreactors, arranged in a 4×3 array	hESCs	The microbioreactor array that combines the advantages of microarrays with those of bioreactors, provided means to study the growth and differentiation of hESCs under controlled conditions	[21]
Soft lithography	Controlled microenvironment	PDMS	Cylinders 200 μm in diameter and 120 μm in height	hESCs; MEFs	While exhibiting a similar viability and self-renewal profile as that of hES cells grown on flat surfaces, hES cells grown on microwell-patterned substrates show a greater level of homogeneity in aggregate size	[56]
Electrospinning	Controlled microenvironment	Blend of poly(DL-lactide-co-glycolide) and collagen I	Diameter of fibers ranging from 100 to 500 nm in diameter	BM-HSCs	Besides acting as an in vitro HSC culture system for supporting hematopoiesis , the constructs may also act as an efficient captor and carrier for HSCs	[75]
Electrospinning	Controlled microenvironment	PES	Fibers with an average diameter of 529 ± 14 nm	HSPCs	Aminated nanofiber mesh could further enhance the HSPC-substrate adhesion and expansion of CFU–GEMM forming progenitor cells	[28]

Legend: primary human osteoblasts (HOBs); Human osteogenic sarcoma cells (SAOS-2 cells); poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV); starch/polycaprolactone (SPCL); polycaprolactone (PCL); polydimethylsiloxane (PDMS); hyaluronic acid (HA); polyethylene-terephthalate (PET); mesenchymal stem cells (MSCs); bovine aorta cells (VSMCs); polyurethane (PU); polylactic-co-glycolic acid (PLGA); poly(ethylene glycol) (PEG); human umbilical vein endothelial cells (HUVEC); poly(L-lactid-co-ε-caprolactone) P(LLA-CL); human coronary artery smooth muscle cells (SMCs); poly(L-lactic acid) (PLLA); neural stem cells (NSCs); human embryonic stem (hESCs) cells; poly(e-caprolactone-co-ethyl ethylene phosphate) (PCLEEP); chitosan–collagen–gelatin (CCG); Human mesenchymal stem cells (hMSCs); murine embryonic fibroblasts (MEFs); hematopoietic stem cells (BM-HSCs); polyethersulfone (PES); hematopoietic stem/progenitor cells (HSPCs)

is produced by replica molding with the pattern of interest. A drop of the prepolymer solution is poured onto the mold and the excess gently removed [141]. The filled mold is placed in contact with the substrate, and the prepolymer solution is cured either by thermal or photochemical processes. After the procedure, the mold is peeled off leaving the pattern on the polymer. By stacking layers, 3D structures can be produced by means of μTM. Moreover, it presents some other useful characteristics [141, 153]: (1) it allows for patterning nonplanar surfaces, (2) it allows for fabricating structures in large surface areas (3 cm^2) [141] in a short period of time, (3) it is possible to rapidly replicate microstructures from the mold, (4) two or more components can be used to uniformly create microstructures, and (5) it is applicable to a wide range of materials. Depending on the applications, the main shortcoming of this technique is due to the thin films that are formed between the raised features [141].

Molding in Capillaries (Capillary Force Lithography)

Microfluidic patterning using capillaries, also known as capillary force lithography, allows for patterning a surface of a material from the flow of a solution [120–122]. Microchannels (or capillaries) are formed by the void spaces when the PDMS structure is placed on top of the substrate. Thus, in opposition to the aforementioned techniques, the material is added to the surface in the areas where the stamp is not in contact with the surface. Through capillary forces, these microchannels deliver the targeted fluid to the restricted areas of the substrate, allowing for a selective microfluidic patterning. The typical channel size is 50 μm, at which the fluids have a laminar flow with Reynolds Number ranging from 0.1 to 1 [93]. Therefore, if two streams coming from different inlets are to flow in a common channel, they will flow in parallel without turbulent mixing, and with diffusion only among both the fluids at the interface. This characteristic makes molding in capillaries a really interesting method for developing gradients. Lately, a great emphasis has been laid toward the generation of gradients by using microchannels [57]. Nevertheless, this technique does not simply allow to be used as a vehicle to deposit compounds, but also as a manner to cure itself into the surface or to remove a material.

Microfluidic patterning is very useful since it allows for selective protein immobilization and selective cell adhesion. Moreover, if gradient systems are used, it is possible to analyze the influence that two or more different drugs or other different compounds have on a single cell. Also, capillary force lithography is often used for immunoassays. The small volume of reagents required by this technique makes it a very powerful and unique tool for screening tests. However, the patterns are limited to the channel geometries [37].

Scanning Probe Lithography

Scanning Probe Lithography (SPL) embraces a range of techniques that involve the surface modification at the nanoscale by means of scanning probes such as AFM and scanning tunneling microscopes (STM) [41, 86]. By utilizing AFM and STM tips, SPL techniques enable to precisely pattern atoms or clusters of atoms onto a surface [41]. There are mainly three SPL subareas. The first one is entitled dip-pen nanolithography and allows for creating patterns of 15 nm. In this process, an AFM tip is inked with the material to be patterned, and while scanning a substrate, the tip transfers the material. For this, the humidity present plays an important role. Another area of SPL consists of the selective removal of a material from a surface. This is achieved by applying high pressure with the AFM tip onto the coated surface. Usually, the molecules that are removed from the surfaces are SAMs. Finally, the third area is based on the localized chemical modification of the surface, either by electrochemical anodization or by using a conductive AFM or STM tip.

1.3.2.3 Electrospinning

Electrospinning is a well-known and ubiquitous technique that enables the production of nanofibers and has been used by many researchers to make nanofibrous matrices for TE applications [46, 60, 71, 77, 79, 80, 84, 123]. The process of electrospinning results from the application of a high voltage electrostatic field operated between a metallic capillary of a syringe and a grounded collector. As a result of this electrostatic field, a solution drop deposited at the tip of the capillary tube elongates into long and thin fibers and projects over the grounded collector. The

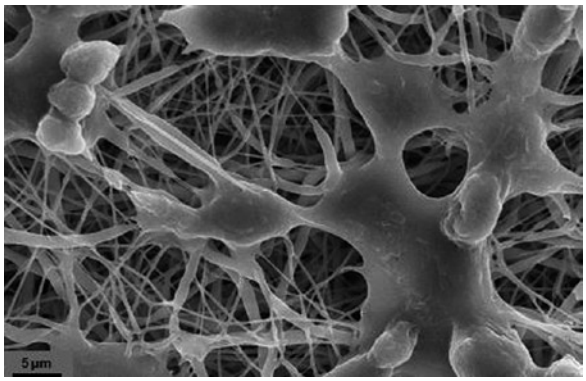


Fig. 1.6 Nanofiber mesh of polycaprolactone obtained by electrospinning cultured with SaOs-2

nanofibers produced by electrospinning are collected into a nonwoven web, which generally originate a random fiber orientation mesh with limited mechanical properties.

Scaffolds produced by this technique were shown to closely mimic the structure and morphology of native ECM that consists of fibrilar structures with nanosized diameters [68, 103, 143].

This technique also provides the possibility of producing nanofibrous scaffolds from native polymers such as collagen and elastin. TE constructs based on these materials produced by electrospinning have been widely reported [18, 80, 81], once they have been able to direct cell alignment and support cell growth (Fig. 1.6).

1.4 Clinical Applications

Clinical applications of the constructs developed by the synergies between the TE and the micro- and nanofabrication fields are still ahead of us. Even, the clinical applications of the products obtained using TE strategies are still limited to a very small number of approved products, such as collagen membranes for cartilage repair. Nevertheless, there are already some microfabricated devices on the market aimed for tissue regeneration. An example is MotifMesh™, obtained by the combination of micromachined layers of a biocompatible polymer that allow creating a controlled ECM.

Interestingly, Johnson et al. [49] stated that the strategic directions in TE leading to successful clinical applications would involve a closer interface between TE and micro- and nanotechnologies by enhancing cell biology and biochemistry. It is expected that the intense research effort focused on these technologies will produce significant contributions to overcome some known bottlenecks in TE. Inputs would be needed in the following areas: (1) cell behavior in different culturing conditions, (2) cell–cell interactions in cocultures, usually achieved by means of patterned surfaces, (3) cell-ECM communication by high-throughput platforms, or (4) the influence of several soluble factors on specific cells through microfluidic systems (Fig. 1.7). Although much work is still required, the ability to design and manipulate surface properties at the micro- and nanoscale has already shown to be useful for different applications in TE. However, most of the micro- and nanotechnologies proposed for TE applications have been focused on 2D structures. Besides the potential shown at a 2D level, micro- and nanotechnologies should also be extended and exploited to produce 3D structures. Support systems for tailoring cell behavior and for tissue regeneration developed by those technologies have shown positive research results; currently, efforts are directed toward mimicking the natural environment in tissues with increasing accuracy. Presently, microbioreactor systems are being developed that will enable tissue growth in a tightly controlled microenvironment. The next sections highlight the opportunities for biomaterials development offered by micro- and nanotechnologies with proteins and, subsequently, the

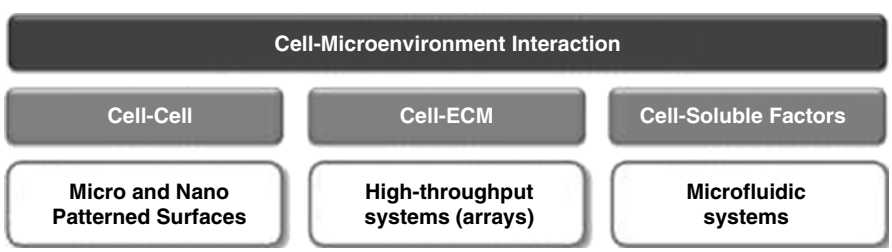


Fig. 1.7 Schematic representation of the micro- and nanoplatforms used for TE applications

cells aiming the regeneration of the above-mentioned tissues, both in 2D and 3D. An overview of the more recent and promising advances in the development of microbioreactors will also be presented.

1.4.1 Micro and Nanotechnologies in the Development of Enhanced Constructs for Tissue Engineering

In the development of constructs for TE strategy by means of micro- and nanotechnologies, researchers must pay attention to two important parameters: the design of the patterns should mimic as much as possible the native structural environment; and the biomaterial selected to be the groundwork of the patterned system should have appropriate characteristics for a specific application. During the last couple of decades, great efforts have been directed toward creating systems that could resemble the native environment of tissues. In fact, important areas of TE have already taken advantage of such structures to understand the phenomena occurring at the interface of biomaterial-biological entities, applying the concepts into a TE strategy.

Several materials have been developed with osteoinductive and osteoconductive surface topographies intended to lead to the formation of new bone in the case of fracture or disease. Further investigations have shown increased osteoblast functions, such as cell proliferation and activity, when cultured onto nanofeatured surfaces [65]. Moreover, reports have shown that the osteoclasts (bone-resorbing cells) activity [38, 134] was also influenced by the topographical micro- and nanosized cues. The coordination among osteoblasts and osteoclasts is of utmost importance for the effective maintenance of healthy bone. Therefore, results showing synergetic functions of osteoblasts and osteoclasts to ensure a healthy bone remodeling at the implant interface are of extreme importance. Recent studies have reported the successful application of microtopographies in improving the osteointegration and that microgrooves combined with chemical cues can guide selective rat mesenchymal stem cell-derived osteoblasts adhesion and alignment [53]. Further insights on the influence of patterns in the guidance of osteoblast cells were reported by S. Ber and colleagues, showing that through the appropriate choice of surface topography, both the cell alignment and bone formation by mesenchymal osteoprogenitor cells can be enhanced [12]. Polycaprolactone nanofiber meshes obtained from electrospinning have also shown enhanced cell attachment and proliferation when coated with biomimetic calcium phosphate (BCP) layer. [7]. In a different study, it is shown that the combination of nanofibers produced by electrospinning with microfibers originates scaffolds with enhanced characteristics for application in bone tissue regeneration [127]. The innovative structure of these scaffolds, inspired by ECM, simultaneously promotes cell adhesion through a nanonetwork and provides mechanical stability by means of the microfiber mesh. In another study, it was shown that the architecture of nano/microfiber-combined scaffolds elicited and guided the 3D distribution of endothelial cells without compromising the structural requirements for bone regeneration, demonstrating the potential of such structures to overcome the lack of vascularization that is associated with current bone TE constructs [108]. Figure 1.8 shows this scaffold system embedded in a collagen gel, allowing to visualize human umbilical vein endothelial cells (HUVECs) aligned both on the micro- and nanopolymeric fibers.

The advent of micro- and nanotechnologies associated with TE holds a great promise also for cartilage

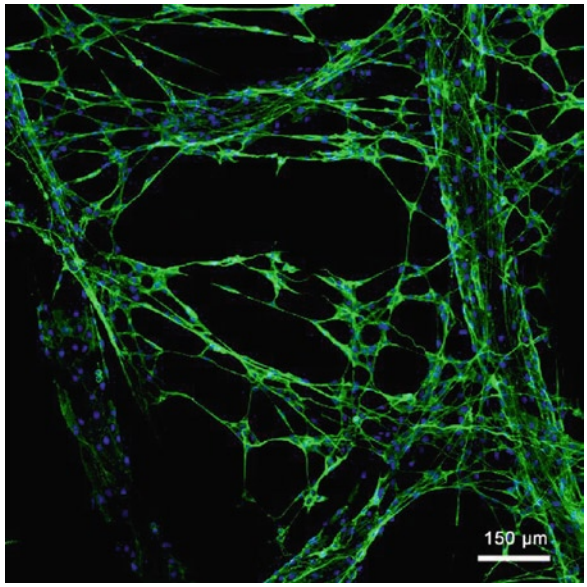


Fig. 1.8 Human umbilical vein endothelial cells (HUVECs) on collagen-nano and SPCL-microfiber-combined scaffold after 7 days of culture. HUVECs were stained with endothelial-specific marker platelet/endothelial cell adhesion molecule-1 (PECAM-1) and nuclei were counterstained with DAPI

tissue regeneration. Currently, researchers suggest that TE strategies combined with nanopatterned materials can be useful for obtaining functional regeneration of cartilage tissue. The rationale is based on mimicking as closely as possible the natural composition and properties of cartilage. A study reports that micropatterned hyaluronic acid (HA) surfaces induced higher adhesion, migration and alignment of knee articular cartilage chondrocytes when compared to homogenous surfaces. Moreover, the patterned surfaces were shown to promote cell differentiation into chondrocytes [11]. Similarly, Erik Petersen and his colleagues reported that cells cultured onto a microarray of micropatterned surfaces maintained their morphology and their ability to retain important phenotypic aspects of the chondrocytes [94]. Besides being applied into static cultures, micro- and nanotechnologies have also been applied for the understanding cell response to the dynamic changes of the extracellular osmolality. P. Grace Chao and colleagues have described a system that yielded new information regarding the dynamic osmotic loading of chondrocytes, which could also contribute with new insights about the mechanisms of cellular homeostasis of other cell types [24]. Also, electrospinning has been successfully applied to the development of nanofiber meshes of starch-compounded PCL to act as a scaffold for cartilage TE [4].

The behavior of vascular cells (such as endothelial and smooth muscle cells) has shown to be enhanced on micro- and nanostructured surfaces. Microfluidic networks have been developed to produce highly uniform flows that mimic physiological patterns [9]. Sachin C. Daxini and colleagues have devoted efforts into testing the hypothesis that by creating well-defined microtextured surfaces, low shear-stress regions could be created, which would help in retaining endothelial cells. They have shown that endothelial cell retention was significantly improved on micropatterned surfaces when compared to unpatterned ones, which would be beneficial for reducing the thrombogenicity of implanted vascular grafts [34]. Another study went deeper into the analysis of how endothelial cells respond to the shear stress caused by the blood flow. This study helped in understanding the process of endothelial mechanotransduction, suggesting that the design of new substitutes for vascular TE should not only consider the material and biological cues, but also the hemodynamic profiles, in order to improve tissue integration and regeneration [74]. An essential parameter in vascular TE is the recruitment of cells from the blood to the vascular wall in a situation of thrombosis or inflammation. In this context, Divya D. Nalayanda and colleagues have reported the development of microfluidic patterned surfaces that were successful in controlling the adhesion and rolling of endothelial cells under physiological flow [91].

The ability to direct cell attachment and orientation, with the possibility to create fluidically isolated compartments, points out to the distinctive advantage of micro- and nanotechnologies for neural tissue regeneration over the regular culture conditions. A very promising work that went deeper into the issue of synaptic signaling presented results that established that synapses forming on confining geometry of the micropattern are physiologically normal and capable of performing plastic modulations, demonstrating their usefulness as a model for signal processing by neuronal networks [130]. Essential parameters within a neural TE strategy are being further exploited by means of micro- and nanotechnologies, such as the cell orientation, allowing for mimicking the microcircuitry that is encountered in the native tissue. A work of Christopher Bettinger and coworkers reports the use of a flexible and biodegradable substrate of poly(glycerol–sebacate) with rounded features that could further elucidate the mechanism of cell alignment and contact guidance [14].

The liver is another well-studied system in TE since the existing systems cannot fully mimic the synthetic and metabolic liver system. One option is the development of liver-like structures by means of micro- and nanotechnologies. Recently, a very promising device was developed by scientists at the Harvard Medical School and the Massachusetts Institute of Technology that could eventually be used to create a functional liver organ. Basically, it involves the development of a network of microscopic tubes that branch out in a pattern similar to a vascularized system, to provide oxygen and nutrients to the liver cells. Indeed, 95% of the liver cells survived up to 2 weeks in this system [19]. Another system that combines microtechnologies and TE is the one described by Bhatia that consists of a miniaturized, multiwell culture system with a micropatterned architecture of collagen that has shown to maintain the phenotypic behavior of primary rat hepatocytes [59]. Recently, a microfluidics bilayer device with a physiologically based network was also described. The device showed to be able to maintain

hepatic functions of both human hepatoma cells and primary rat hepatocytes [22].

The application of micro- and nanotechnologies in the biological field has recently gone beyond the mainstream research for the engineering of a specific tissue. Nowadays, stem cells and biomaterials that support their adhesion, proliferation, and differentiation represent a very intense area of research. The use of micro- and nanotechnologies for stem cell adhesion, proliferation, and/or differentiation is a slightly different approach in comparison to the aforementioned tissue engineered approaches. By using stem cells, researchers aim the regeneration of not only one specific tissue but also a wide variety of tissues and organs. In fact, micro- and nanotechnologies are of extreme importance for studying stem cell differentiation since they allow generating a tightly tailored microenvironment according to the specificities of each tissue. Recently, Khademhosseini and collaborators have successfully developed cell-repellant poly(ethylene glycol) (PEG) wells that were used as templates for the formation of embryoid bodies (EB), which are cell aggregates of embryonic stem (ES) cells. This promising method showed that microwell techniques can be of great use for initiating the differentiation of EB under controlled microenvironments [50]. Another study showed the successful application of micro- and nanotechnologies for the maintenance of the undifferentiated state of human embryonic stem (hES) cells. The microwell-patterned surfaces have proven to be effective in generating almost perfect aggregates for further differentiation studies [56]. Bhatia and coworkers have been devoting a great effort into the development of high-throughput platforms for the analysis of the signals and mechanisms that regulate stem cell fate [15]. Recently, a platform for assessing the interaction of ECM bioentities and growth factors and their effect on stem cell fate was developed [39]. This high-throughput technology allows for the simultaneous analysis of 1,200 different experiments. The differentiation along cardiac lineage was assessed by means of a confocal microarray scanner. This technology represents a step further on the understanding of the microenvironment that dictates stem cell fate [39].

Besides the studies with ES cells, reports combining stem cells from other sources and micro- and nanotechnologies have shown really promising results. An example is the work described by D. Zahor and colleagues [149] who have induced topography-guided alignment of mesenchymal stem cells by culturing them on micropatterned silicon surfaces. Another study evaluated the efficacy of a micropatterned surface in differentiating multipotent mouse bone marrow stromal precursors into fat tissue [26]. The results showed the positive influence of the patterns in lipid production. Recently, the development of a biomimetic scaffold that could mimic a specific niche, where bone marrow-derived hematopoietic stem cells (BM-HSCs) could healthily proliferate and differentiate, was also reported [75].

1.4.2 Towards 3D Micro and Nanofabricated Structures

An effort has been made to push the domain of micro- and nanotechnologies from bi-dimensionality to three-dimensionality [82] extending the applicability of these technologies. This transition to 3D structures can be achieved using micro- and nanotechnologies to buildup three-dimensional structures by stacking multiple two-dimensionally microfabricated layers. This kind of technology has been widely used in TE strategies for developing micro- and nanogrooved three-dimensional microfluidic devices for the replication of several kinds of tissues [131]. Despite the high resolution achievable through those techniques, the basic two-dimensional nature of the substrates used has not yet allowed to fully replicate the real three-dimensional structure of tissues, and consequently, to replicate their full functionality.

Some of the above-mentioned techniques have already been successfully applied in the development of 3D supports for cell growth. For example, a method that combines the chemistry of bottom–up approaches with the engineering of top–down approaches was successfully employed to the development of 3D hydrogel structure [35]. Indeed, of the many synthetic materials being explored, hydrogels are among the most widely adopted for 3D cell cultures, due to their high water content and mechanical properties. Top–down TE approach allows for constructing 2D patterned hydrogels, either by photolithography or soft lithography methods. A bottom–up approach is used further for the modular assembly of the small building blocks. A good example of this combined process is the packing

of rod-shaped collagen microgels seeded with HepG2 hepatocytes and cocultured with endothelial cells at the surface [128]. Langer and collaborators developed structures with defined shapes by micromolding photocrosslinkable hydrogels. Several cell types were encapsulated and the architecture tightly controlled [54, 147]. Using a different approach with photopolymerizable PEG hydrogels, it is possible, with tailored chemistry and architecture, to generate a 3D structure that can further support hepatocyte survival and liver-specific function [126].

Conceptually, 3D microfabricated objects should be created with three-dimensional resolution and ideally be freely manipulated (i.e., fabricated without any necessary attachments to the substrates). One of the challenging issues in TE strategies is still the uniform distribution of cells within 3D-scaffolds. As an attempt to surpass this challenge, the effect of three-dimensional, porous poly(L-lactide-co-glycolide) (PLGA) scaffolds modified with poly(ethylene oxide) (PEO) on cell behavior within a bone TE strategy was studied. This work showed that patterned regions of low and high cell adhesion were demonstrated on scaffolds fabricated with 1 mm thick stripes of PEO and non-PEO regions, respectively [62]. Another very promising work was recently reported [55], which is based on using microfluidic patterning for the development of 3D cardiac organoids. After seeding, the cardiomyocytes elongate along the HA patterns that serve as inductive templates for organoid assembly. This study has further shown that only after 3 days of culture, the cardiomyocytes detach from the surface and start to show a contractile behavior.

Many groups are now investigating the possibility of creating 3D objects through rapid prototyping techniques which consist of building up layer upon layer of material by printing methodologies. This approach is already successfully used in large-scale industrial rapid prototyping by using techniques such as inkjet printing, stereolithography, selective laser sintering, or fused deposition modeling. Such technologies are capable of generating objects with high geometric freedom and possessing highly complex three-dimensional architectures. Prototyping techniques based on the material selective deposition are also capable of building structures from actual living tissues/cells. The technology, so-called bioprinting, offers the ability to deposit cells and other biomolecules in a rapid layer-by-layer method, allowing creating three-dimensional tissue-like structures. A complex three-dimensional microfluidic system was fabricated with PDMS by rapid prototyping using two-level photolithography and replica molding. This method allows generating complex patterned microfluidic systems [5].

The 3D bioprinting technology is currently being studied for possible use in TE applications where organs and body parts are intended to be built using inkjet techniques [85]. The above-mentioned techniques, although capable of being fast and economic, are mostly limited by the scale factor since, in most cases, they are not capable of applying high printing resolutions.

Ultrasmall features may be achieved by the 3D microfabrication technique of 2-photon polymerization. In this approach, the desired 3D object is traced out in situ (in the interior of a photopolymerizable liquid gel) by a focused laser beam. Unlike the commonly used lasers, the laser source used in this methodology is based on a visible light, ultrashort pulse laser. The gel is cured to a solid only in the places where the laser is focused, due to the nonlinear nature of photoexcitation. When the process is finished the remaining gel is simply washed away. Feature sizes with 700 nm can be produced by this method, which also allows the production of complex structures including moving and interlocked parts [52]. Another important feature of this technology is the nature of the polymerizable gel which can be made of a range of proteins such as fibrin, collagen, and albumin which are very abundant in native human tissues.

1.4.3 Towards In Vivo Microenvironment: Microbioreactors

Several recent studies demonstrate the importance of mimicking in vitro certain critical aspects present in the native environment of tissues to be regenerated. Therefore, besides the basics of TE strategies, scaffolds, and cells, it is of utmost importance to provide the cell-scaffold constructs with the appropriate microenvironment that will encourage effective organization among the elements of a TE strategy. The most widely used type of culturing system operates in static culture conditions. However, it is known that it may cause nonhomogenous distribution of cells and nutrients and does not allow mimicking the flow stresses that are present in vivo. Bioreactors are systems that have shown to be successful in surpassing these challenges in culture

systems. Bioreactors used in TE strategies not only allow the growing of cells to higher densities but can also be used as extracorporeal devices for liver and kidney diseases. Moreover, in these systems, the biological and biochemical processes are closely monitored and the culturing conditions, such as the pH, temperature, pressure, shear stress, nutrients supply, and waste removal, are tightly controlled.

Microbioreactors, also known as microfluidic bioreactors, offer further advantages for cellular applications. Besides providing large surface-area-to-volume ratio, they can offer microscale controllable fluid circuits [72, 139]. Indeed, microreactors have shown promising results in applications where conventional bioreactors have failed, since they act not as a mere culturing system, but as a device for studying the mechanisms occurring at the tissue microenvironment. This allows designing better materials and/or culturing systems. For instance, a perfusion-based, micro 3D cell culture platform was designed and fabricated, based on SU-8 lithography and PDMS (polydimethylsiloxane) replication processes and used to study the loading of cell/agarose constructs. Moreover, this system was found to be particularly useful for cell culture-based drug screening systems [138]. In fact, cell culture assays, combined with microbioreactors are now emerging as enabling tools for high-throughput cytotoxicity assays, since they allow for high controllability of operation and on-line monitoring/sensing [146]. A different system demonstrated the long-term culture (more than 2 weeks) of mammalian (human foreskin fibroblasts, HFF) cells in a microbioreactor under constant perfusion and the importance of understanding the relationship between design parameters (channel size, oxygen lever supply, shear stress, flow rate) and cell behavior (cell growth, cell morphology, perfusion rate) in microscale culture system [63]. In another approach, a microbioreactor with the size of a glass slide was used for studying the mechanisms involved in culturing in vitro human embryonic stem cells (hESCs) [30]. A different microbioreactor with a PDMS-treated surface was developed based on mass transport simulation. The elastomeric surface was treated with a surfactant in order to diminish the non-specific protein adhesion, keeping the culture conditions steady. This system helped in understanding the kind of environment that provides better chondrocytes stable culture conditions over the culturing period [140]. Within a liver TE strategy, two types of PDMS microbioreactors containing a membrane, used as a scaffold for the attachment of cells, were developed. In this system, the cells were immersed in the culture medium, expressing much higher functions and better mimicking the hepatocytes in vivo. After 15 days of culture, the primary adult rat hepatocyte cultures have shown good cell attachment and reorganization, revealing to be promising tools for future liver TE [92]. Also, aiming at promoting liver tissue regeneration, Linda Griffith and coworkers have developed a microbioreactor for perfused 3D liver culture. The reactor dimensions were designed in such a way that the flow rates meet the estimated values of oxygen demand and also provide a shear stress at or below the physiological one. For 2 weeks, primary rat hepatocytes rearranged to form tissue-like structures, indicating that this system approximates the perfusion and architectural properties of an in vivo hepatic tissue [98, 99]. Recently, an array of twelve microbioreactors was reported [21]. With this technology, high-throughput assays can be performed, by assessing many factors that regulate cell behavior. The microbioreactor was fabricated by soft lithography and each individual microbioreactor is perfused by a culture medium. Moreover, this system allows for cells to be cultured onto the substrate or encapsulated in hydrogels, being the results followed by automated image analysis. As a proof-of-concept, researchers have cultured C2C12 cell line, primary rat cardiac myocytes, and hESCs, illustrating the utility of the microbioreactors array for controlled studies.

A variety of large-scale bioreactors have been developed and optimized for TE strategies. Nevertheless, microfluidics has lead to the advent of a new generation of bioreactors aimed at mimicking in vitro the native microenvironment of tissues. Despite the already performed research in this area, microbioreactors are a relatively new direction in TE, still leaving a plenty of room for new developments in human 3D models for studying the underlying mechanisms in cellular microenvironments.

1.5 Expert Opinion

Micro and nanotechnologies have been emerging as useful tools both for developing structures for TE strategies and for studying in vitro the in vivo microenvironment

of tissues. Also, micro- and nanofabrication methodologies are of huge importance, since they enable the study and the development of solutions for problems associated with nanoscale phenomena, both at the production and at the analysis level.

The micro/nano enabling technologies allow developing biodegradable scaffolds to be used within a TE strategy with a tight control over the nanostructure of the materials. Those technologies allow controlling the mechanical, chemical, and biological properties, mimicking closely the details and characteristics of native tissues. Furthermore, complex platforms may be created including micro- and nanopatterned substrates to be developed into 3D nanostructured scaffolds with high level of architectural control. Also microbioreactors will allow a precise regulation in vitro of the native extracellular microenviroment. Those micro- and nanotechnologies open the possibility to effectively enable the study of the cell behavior in physiologically mimetic conditions and also to control and direct the cell fate.

1.6 Five-Year Perspective

The efficacy of biomaterials within the presently pursued TE clinical strategies is still in its early stage. A better design of biomaterials at the nanoscale is believed to be critical for improving its efficiency when implanted, by matching the detailed nanoscale features of natural human tissues. For this statement to be confirmed and validated experimentally, the role of micro- and nanofabrication technologies is undeniably of great importance. It is expected that similar to the burst that micro- and nanofabrication technologies gave to the microelectronics field, theTE strategies will also rapidly evolve with the synergies that are being established with micro- and nanotechnologies.

Despite the challenges that still lie ahead, significant evidence has been reported showing the huge impact that micro- and nanofabrication tools can have in the development of enhanced TE strategies. This cooperative interaction between the fields of TE and micro- and nanotechniques is a new area of research, but has already shown a great potential to enhance constructs design and tools for a better understanding/modeling of the interface between implanted materials/cells and their surroundings at a target implantation site. The outputs from the research on micro- and nanotechnologies are expected to provide a large amount of information regarding the phenomena occurring at a nanoscale level in the human body and at the interface between the biomaterial and the native microenviroment. These insights will provide the researchers with the needed tools to design and fabricate culture platforms that match tissue-specific parameters. Ultimately, this knowledge will allow the development of more functional and complex biomaterial scaffolds and better tissue-regeneration structures that will be tailored to produce tissue substitutes for specific clinical applications.

1.7 Limitations/Critical View

As mentioned earlier, there are some limitations on the way micro- and nanotechnologies are currently facilitating the study and development of future TE applications. These limitations result mainly from the relatively less intensive exploitation of these technologies in the context of TE. Like other established research fields, micro- and nanotechnologies need to be developed through a process of adaptation and optimization to provide sufficiently effective and affordable solutions for TE-related applications.

Currently, although being available technologies capable of building structures with micro- and nanoarchitectures, these are mostly applied to build either micro or nanosized devices. The current major challenge of micro- and nanotechnologies in TE is the extension of the micro- and nanoarchitectures to macroscale devices which are large enough for substituting/regenerating tissues. To build those devices, the production technologies need to improve into faster and more effective procedures, without compromising the level of detail of the structures produced.

Most devices that are currently being developed have small dimensions and relatively low geometrical complexity. Every tissue requires a particular architecture which can also differ according to the geometrical location. Most tissues need a coordinated combination of different cell and tissue types. Thus, the architecture of the developed devices will need to account for the arrangement and interactions of those different kinds of cells. An important kind of arrangement is the one for the vascularization of tissues. By

applying vascular-like structures in the inner architecture of scaffolds/constructs, it will be possible to obtain thick functional tissues.

Due to the massive complexity that is possible to obtain through the micro- and nanostructured macrodevices, data processing must be a concern. Thus, new software and hardware solutions will need to be developed for handling the necessarily enormous amount of information involved in the process. Also other informatics tools will need to be developed for the actual generation of such complex structures, since they are too complex for being developed and designed in a way other than through automated design tolls. It is for this reason that it is also necessary to do a thorough analysis of the natural building principles used by nature during tissue generation in order to accurately achieve these nature-mimicking algorithms.

1.8 Conclusion/Summary

This chapter reviewed the recent developments regarding the use of micro- and nanofabrication techniques as enabling technologies to tailor the characteristics of constructs aimed for TE. Technologies such as photolithography and soft lithography, adapted from microelectronics, began in the last couple of decades to provide opportunities for the TE area. Despite the already demonstrated potential of micro- and nanotechnologies in some areas of the TE (bone, cartilage, vascular, neural, liver TE, and stem cell research), the developments are still in an early stage. In fact, currently, although some of the described technologies are indeed capable of building structures with micro- and nanoarchitectures, these are mostly applied to build either micro or nanosized devices. The current major challenge of micro- and nanotechnologies in TE is therefore the extension of the micro/nano architectures to macroscale devices which are large enough for substituting/regenerating tissues. To build those devices, the production technologies need to be improved in order to become faster and more effective and at the same time not compromising the level of detail of the structures produced.

Nevertheless, given that the technology behind micro- and nanofabrication techniques is already well established, the creation of innovative biomaterials is developing at a rapid pace, and the input from tissue and cell biology is continuously increasing, we believe that we are just on the verge of new opportunities that result from the synergies of the development of those areas.

Throughout the review, some issues for future research are identified, hoping that technology can develop into those expanding areas. It is strongly expected that micro- and nanotechnologies will have a major impact on TE, as they once had on microelectronics. Thus, key contributions regarding cell–microenvironment interactions are envisioned. Moreover, taking advantage of these new insights about the in vivo microenvironment, we expect the development of systems that could actually engage in healthy tissue regeneration.

Suggested Readings with Abstracts

Engel E, Michiardi A, Navarro M, et al. Nanotechnology in regenerative medicine: the materials side. Trends Biotechnol. 2007;26(1):39–47.

Regenerative medicine is an emerging multidisciplinary field that aims to restore, maintain, or enhance tissues and hence organ functions. Regeneration of tissues can be achieved by the combination of living cells, which will provide biological functionality, and materials, which act as scaffolds to support cell proliferation. Mammalian cells behave in vivo in response to the biological signals they receive from the surrounding environment, which is structured by nanometre-scaled components. Therefore, materials used in repairing the human body have to reproduce the correct signals that guide the cells towards a desirable behavior. Nanotechnology is not only an excellent tool to produce material structures that mimic the biological ones but also holds the promise of providing efficient delivery systems. The application of nanotechnology to regenerative medicine is a wide issue and this short review focuses only on the aspects of nanotechnology relevant to biomaterials science. Specifically, the fabrication of materials, such as nanoparticles and scaffolds for TE, and the nanopatterning of surfaces aimed at eliciting specific biological responses from the host tissue will be addressed.

Falconnet D, Csucs G, Grandin HM, et al. Surface engineering approaches to micropattern surfaces for cell-based assays. Biomaterials. 2006;27(16): 3044–63.

The ability to produce patterns of single or multiple cells through precise surface engineering of cell culture substrates has promoted the development of cellular bioassays that provide entirely new insights into the factors that control cell adhesion to material surfaces, cell proliferation, differentiation, and molecular signaling pathways. The ability to control the shape and spreading of the attached cells and cell–cell contacts through the form and dimension of the cell-adhesive patches with high precision is important. Commitment of stem cells to different specific lineages depends strongly on cell shape, implying that controlled microenvironments through engineered surfaces may not only be a valuable approach toward fundamental cell-biological studies but also of great importance for the design of cell culture substrates for TE. Furthermore, cell patterning is an important tool for organizing the cells on transducers for cell-based sensing and drug discovery concepts. From a material engineering standpoint, patterning approaches have greatly profited by combining microfabrication technologies, such as photolithography, with biochemical functionalization to present to the cells biological cues in spatially controlled regions where the background is rendered nonadhesive (“nonfouling”) by suitable chemical modification. The focus of this review is on the surface engineering aspects of biologically motivated micropatterning of two-dimensional (flat) surfaces with the aim to provide an introductory overview and critical assessment of the many techniques described in the literature. In particular, the importance of nonfouling surface chemistries, the combination of hard and soft lithography with molecular assembly techniques, as well as a number of less well-known but useful patterning approaches, including direct cell writing, are discussed.

Emanuele Ostuni, George Whitesides M, Shuichi Takayama, Xingyu Jiang, Donald Ingber E. Soft lithography in biology and biochemistry. Annu Rev Biomed Eng. 2001;3:335–73.

Soft lithography, a set of techniques for microfabrication, is based on printing and molding using elastomeric stamps with the patterns of interest in bas-relief. As a technique for fabricating microstructures for biological applications, soft lithography overcomes many of the shortcomings of photolithography. In particular, soft lithography offers the ability to control the molecular structure of surfaces and to pattern the complex molecules relevant to biology, to fabricate channel structures appropriate for microfluidics, and to pattern and manipulate cells. For the relatively large feature sizes used in biology (≥50 μm), production of prototype patterns and structures is convenient, inexpensive, and rapid. Self-assembled monolayers of alkanethiolates on gold are particularly easy to pattern by soft lithography, and they provide exquisite control over surface biochemistry.

Khademhosseini A, Langer R, Borenstein J, et al. Microscale technologies for tissue engineering and biology. Proc Nat Acad Sci USA. 2006;103(8):2480–7.

Microscale technologies are emerging as powerful tools for TE and biological studies. In this review, we present an overview of these technologies in various TE applications, such as for fabricating 3D microfabricated scaffolds, as templates for cell aggregate formation, or for fabricating materials in a spatially regulated manner. In addition, we give examples of the use of microscale technologies for controlling the cellular microenvironment in vitro and for performing high-throughput assays. The use of microfluidics, surface patterning, and patterned cocultures in regulating various aspects of cellular microenvironment is discussed, as well as the application of these technologies in directing cell fate and elucidating the underlying biology. Throughout this review, we will use specific examples where available and will provide trends and future directions in the field.

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Biomimetic Scaffolds in Tissue Engineering

2

Ian O. Smith and Peter X. Ma

2.1 Introduction

Tissue engineering is a dynamic field in which our collective knowledge of medicine, life sciences, and engineering are brought together and applied synergistically toward the design of new materials, devices, and techniques in regenerative medicine [17]. Biomimetics is defined as the application of methods and systems, found in nature, to technology and engineering. Within tissue engineering, especially in scaffold design, biomimetics is a potential path to success as cell response in vivo is a vital factor for the effectiveness of a treatment. During the early years of biomaterials development, during the so-called "first generation," the goal was straightforward. Hench defines it in his 1980 paper, *biomaterials,* as "to achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host" [12]. This statement still applies in today's research, but due to the constant evolution of our understanding of how tissues work and form, the biomimetic features that we attempt to produce have evolved as well.

The current generation of biomaterials for tissue engineering aims to influence cellular behavior through various means [13]. These include the tailoring of the specific aspects of scaffold design, the choice of the material or scaffold architecture, cell-based therapies, and factor-based tissue engineering. Our group has conducted work in all of these areas, but for the purpose of this discussion we focus on scaffold materials and design. Specifically, we discuss our work in the field of mimicking the naturally occurring extracellular matrix (ECM) and how this is a promising approach to effectively tailor cell response and to successfully engineer replacement tissues.

2.2 Aims of Biomimetics in Tissue Engineering

The overarching aim of the scaffold design for tissue engineering is to mimic the naturally occurring ECM (Fig. 2.1), through its structure or chemistry, in order to elicit a more desirable cell response [20]. Sometimes, these techniques also are able to improve upon certain features of the ECM through surface treatment and the inclusion of controlled release components, and to improve the mechanical properties over less-biomimetic scaffold designs [20]. By incorporating the architectural and chemical aspects of the ECM, these scaffolds promote cell attachment and proliferation, provide biological cues, allow sufficient nutrient transfer, and provide mechanical and biological features to influence cell function. Therefore, the naturally occurring ECM is a benchmark of sorts, up to which the scaffold design is held.

This approach is used in developing scaffolds for tissue engineering of several tissue types. These include hard tissue, such as bone (trabecular scaffolds, nanofibrous scaffolds) and bone/ligament junctions (triphasic scaffolds), as well as soft tissue, including eye (limbal-corneal junction scaffolds), nervous (neural regeneration through the use of neural progenitor cells [NPC]), and vascular tissues (hydrogels for angiogenesis).

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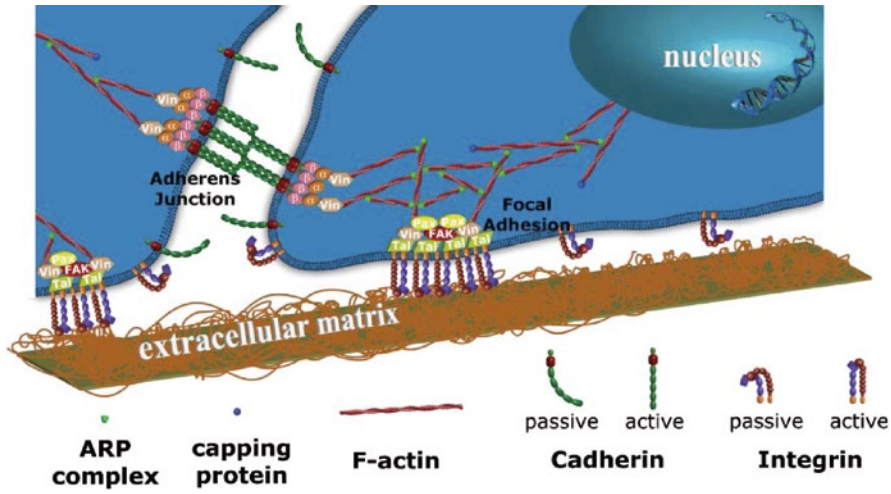

Fig. 2.1 Schematic of cells binding to the extra-cellular matrix, illustrating the components involved. Image reproduced with permission from Girard, Cavalcanti-Adam, Kemkemer and Spatz, 2007, The Royal Society of Chemistry. [1]

2.3 State-of-the-Art Biomimetic Materials

Biomimetics is an area of great interest for bone tissue engineering. Biomimetic scaffold design in this field can be divided into three basic areas. These are nanofibrous polymer scaffolds, composite scaffolds, and surface-modified scaffolds. Each of these three groups tackles the task of mimicking the ECM in its own way. The use of nanofibers in polymer scaffolds is a way to mimic a specific structural feature of the ECM. The aim here takes advantage of the fact that synthetic nanofibers can be made on a similar size scale (50–500 nm) to naturally occurring collagen I fibers, found in abundance within the natural ECM. These fibers may play an important role in regulating cell function prior to and during the formation of tissues [6, 9]. For example, the nanofibrous matrix consisting of collagen fibers serves as a site for the nucleation of apatite crystals during the formation of woven bone [7]. These fibers are on the scale of 50–500 nm, a size range that can be attained using current techniques. The most common technique for forming polymer nanofibers is electrospinning [23]. This method uses an electrical field to draw a thin stream of a polymer solution toward a collector, forming a roughly two-dimensional sheet of fibers. This method has been employed to create fibers of several materials, including PLLA, PLGA, PVA, gelatin, collagen, and others. The method has been successful in consistently fabricating nanofibers, but is currently limited in that it produces them only in thin constructs. Thicker three-dimensional arrangements can be created by stacking these sheets [29], but the resulting networks lack any sort of well-defined macroporosity or interconnectivity, which are necessary in tissue engineering. More recently, attempts have been made to shape electrospun fibers into three-dimensional interconnected porous networks [22], but with little success as yet.

A novel and effective technique has been developed for creating a nanofibrous, interconnected, macroporous polymer scaffold for tissue engineering, which overcome the limitations of electrospinning [21]. Figure 2.2 is a scanning electron micrograph of an example of this type of scaffold. Figure 2.2a illustrates the interconnected porous network, while Fig. 2.2b is a higher-magnification image of the pore wall, showing its nanofibrous nature. This method employs thermally induced phase separation (TIPS), combined with porogen casting and leaching, to achieve this result. The TIPS portion of the procedure is initiated when a polymer is dissolved in a solvent and this solution is placed at a temperature to induce phase separation. The solvent crystals are then removed by sublimation and the remaining polymer lyophilized to yield a network of nanofibers. By varying the polymer concentration and the type of solvent(s) utilized, various scaffold structures are obtainable. To further tailor the scaffold structure to our needs, these processing parameters are combined with porogen casting and leaching. First, a pore mold is created by binding paraffin spheres into a matrix by heating them in an oven at 37°C. Then the polymer solution is poured into the space between the spheres and TIPS is completed at a lower temperature. After removing the

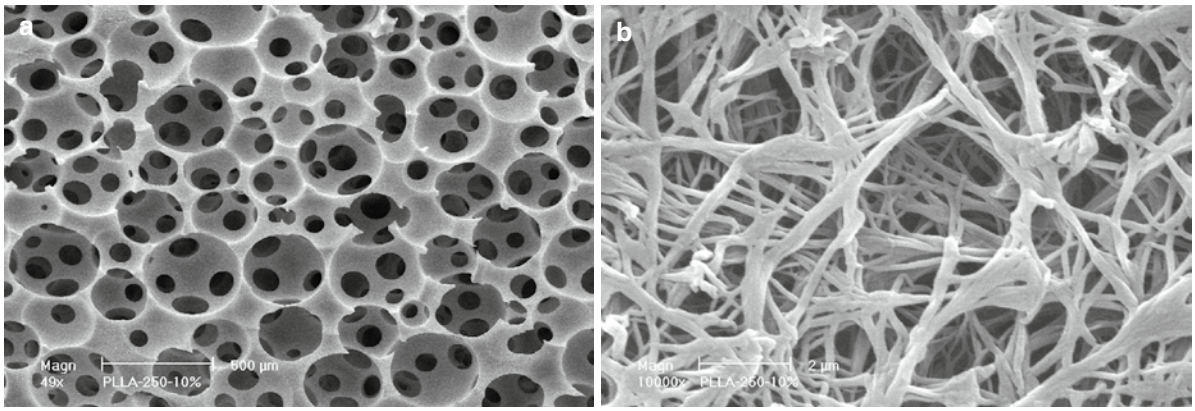

Fig. 2.2 SEM micrograph of macroporous and nanofibrous scaffold prepared by thermally induced phase separation sugar sphere template leaching technique, at (**a**) low (×50) and (**b**) high (×10,000) magnifications (reprinted with permission from Wei and Ma, 2006, Wiley) [34]

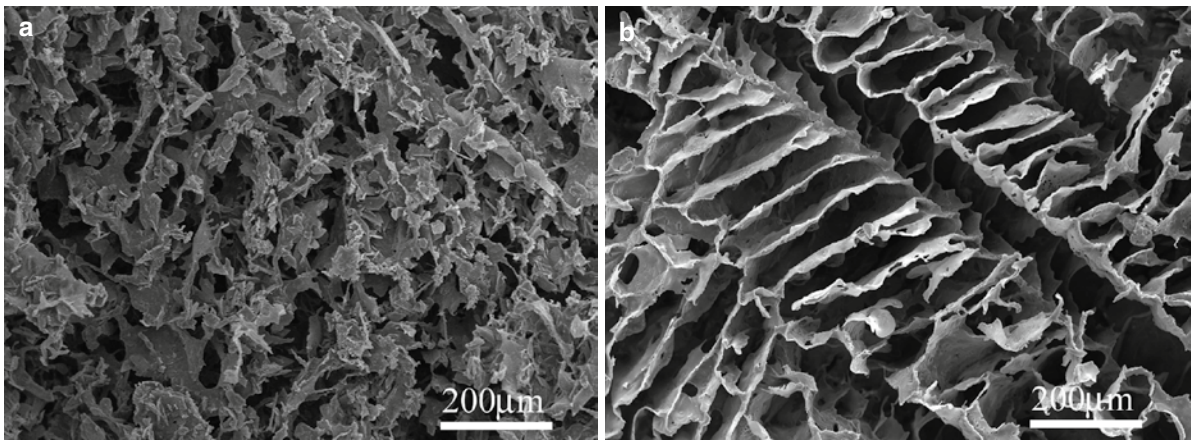

Fig. 2.3 SEM micrographs of (**a**) PLLA/HAP foam (PLLA/HAP: 50/50) and (**b**) PLLA foam, prepared by thermally induced phase separation technique (reprinted with permission from Zhang and Ma, 1999, Wiley) [37]

spheres by selective solvent leaching, all that remains is the nanofibrous network, now incorporated into a macroscopic network of interconnected pores [21].

Another technique used to fabricate nanofibrous polymer scaffolds is molecular self-assembly. This method utilizes noncovalent interactions that occur between macromolecules to build a more complex structure [25, 36]. This technique allows for the control of functionality, but does not lend itself to the ability to form macroporous interconnected scaffold structures, and structures formed using self-assembly do not possess mechanical and degradation properties suitable for many tissue engineering applications. Molecular self-assembly does show promise, but further work must be done to address the limitations.

A second approach for creating a biomimetic scaffold similar to the ECM is to combine multiple components into composite structures. An example of this is to incorporate nanocrystalline hydroxyapatite particles into the structure of a polymer scaffold. This arrangement is meant to mimic the stage of bone growth when osteoblasts have deposited apatite crystals onto the collagen fibers of the ECM and then begin to form the woven bone [35]. Early attempts to incorporate HA into polymer matrixes involved mixing the crystals into the polymer solution prior to casting [33]. This method was met with limited success, as the crystals reduced the ability of the polymer to be formed into the desired architecture. Figure 2.3 is a comparison of a PLLA scaffold formed using TIPS, with another formed with

the same method, but including HA crystals. Additionally, these early attempts used the available HA on the scale of several hundred microns, which is well above the scale of naturally occurring apatite crystals in bone (several nanometers) [37]. More recently, the benefit of using nanoscale apatite crystals has been verified for both bioactive materials, such as HA, and bioinert materials, including Alumina, comparing cell response to variations in grain size (grain size is a concept in ceramics where a grain is a single crystal among many in a polycrystalline solid) [30–32]. The newer method of incorporating apatite into a polymer scaffold is by growing it biomimetically [38, 39]. This method uses simulated body fluid (SBF), a solution that contains a combination of ions at the same concentrations or above those found in vivo. A polymer scaffold is placed into SBF and allowed to incubate for a given time. During this time, apatite crystals nucleate onto the surface of the scaffold. The number of these crystals can be varied by changing the length of incubation time. An example of a scaffold fabricated using this method is shown in Fig. 2.4.

Following this progression, the next step in the evolution of the biomimetic composite scaffold is to incorporate these two approaches into one scaffold that mimics both the collagen fibers found in the native ECM, as well as the nanocrystalline apatite which nucleates on those fibers. One potential method to achieve this is to expose a nanofibrous network to SBF, and to grow the crystals directly. This has been achieved but with only limited success. Electrospun fiber mats have been successfully treated and crystals grown, but these mats have the same inherent issue as nontreated electrospun scaffolds; their two-dimensional structure does not allow them to be used as effective tissue engineering scaffolds. A possible solution to this issue is to progress to three-dimensional macroporous nanofibrous scaffolds, fabricated using the modified TIPS method. This has been done with some success, with initial deposition on the fibers near the pore surfaces [34]. Figure 2.5 is a SEM micrograph of the surface of a scaffold fabricated using TIPS, showing apatite crystals formed after 4 and 30 days in SBF. After the longer incubation time, the deposition has become too great at the pore surface, effectively creating a solid-walled ceramic scaffold. Further work must be done to better understand the mechanisms behind the interactions between the SBF ions and the scaffold. This will allow us to tailor the processing conditions to promote ion transport throughout the nanofibrous network, resulting in the even distribution of apatite particles.

Besides aiming to reproduce the ECM structure, biomimetics also aims to reproduce the ECM chemistry. The ECM contains within its structure, certain molecules that are recognized by cells and that promote adhesion, proliferation, and differentiation. One example is the Arg-Gly-Asp (RGD) peptide, a sequence well known for encouraging cell adhesion. Such bioactive sites are not inherently present in materials typically used in scaffold design. Therefore, a prominent strategy in tissue engineering is to modify the scaffold materials, so that they elicit a more desirable response

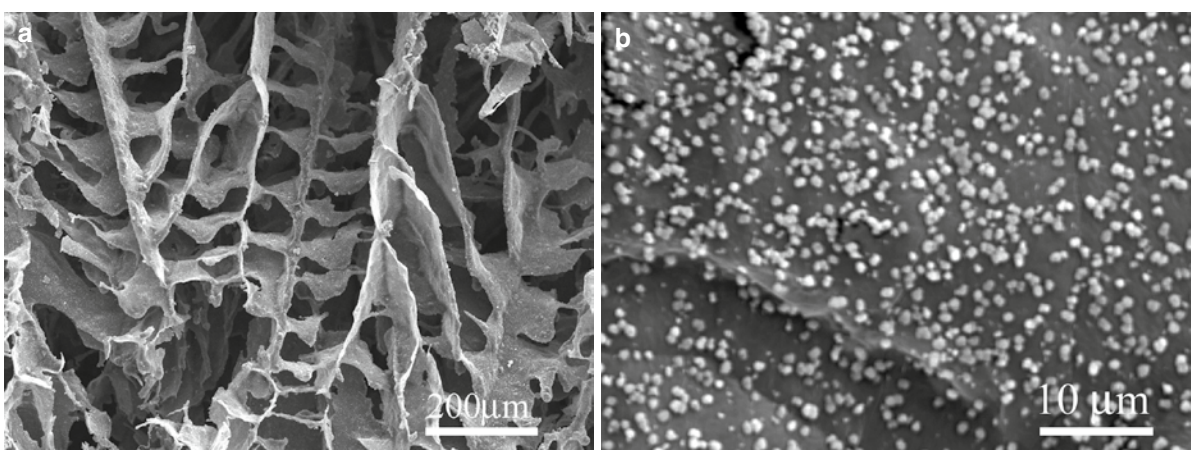

Fig. 2.4 SEM micrographs of a PLLA foam, fabricated using TIPS and incubated in simulated body fluid (SBF) for 30 days at (**a**) low magnification (×100) and (**b**) high magnification (×10,000)

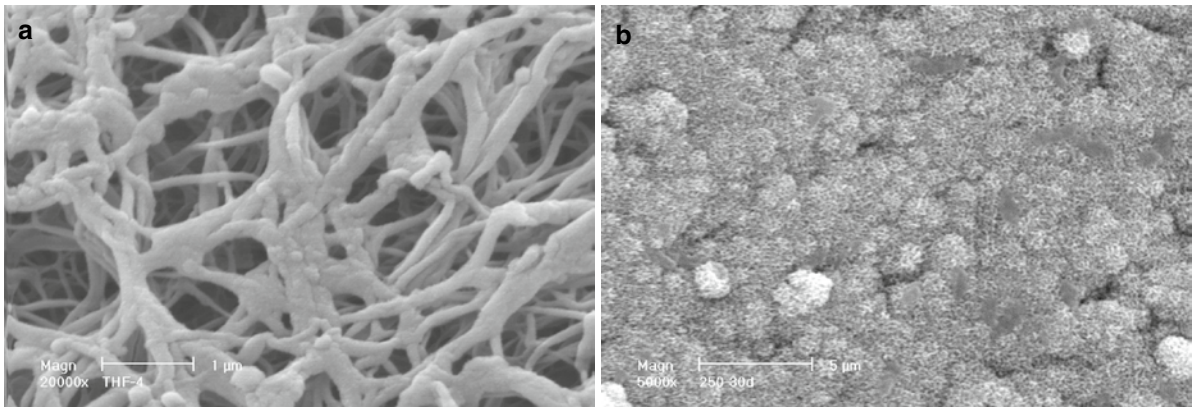

Fig. 2.5 SEM micrograph of macroporous and nanofibrous scaffold prepared by thermally induced phase separation sugar sphere template leaching technique, (**a**) after 4 days in SBF and (**b**) after 30 days in SBF (reprinted with permission from Wei and Ma, 2006, Wiley) [34]

from their host cells. Since the cell–scaffold interaction takes place at the scaffold surface, these modifications are performed at that site, normally after the scaffold has been fabricated. Some examples of surface treatment include grafting the aforementioned peptide sequence, or other groups, onto the polymer chains using copolymerization or grafting reactions [2, 3]. Surface hydrolysis and plasma treatment are two techniques used to introduce functional groups to the scaffold surface [8, 14, 24]. All of these methods have been successful only in cases where the scaffold thickness is small, making them less-than ideal for application to 3D scaffold surfaces. To combat this issue, two newer techniques have been introduced.

Electrostatic layer-by-layer self-assembly utilizing alternating adsorption of cationic and anionic species is able to create polycation-polyanion polyelectrolyte complexes one layer at a time on a molecular scale [4, 11]. Nanofibrous PLLA scaffolds fabricated using this technique incorporate gelatin onto the surface of PLLA scaffolds, in order to better mimic the composition of native ECM collagen. The amount of gelatin coated on the PLLA surface was controlled by the number of self-assembled layers and this method does not affect the material morphology and mechanical properties of the underlying scaffold. The inclusion of gelatin introduces a component which further mimics the natural ECM and, as it is denatured collagen, its use also avoids typical concerns associated with animal and cadaver sources.

In another strategy, gelatin spheres acted both as a porogen and a surface-modification agent [19]. To fabricate porogen-induced surface-modified nanofibrous PLLA scaffolds, the gelatin spheres were packed into Teflon molds and heat-treated in a saturated water vapor atmosphere for designated times, forming a mold of the pore space. PLLA solution was then poured drop-wise into each gelatin sphere assembly and the molds placed into a freezer to allow phase separation and entrapment of the gelatin. Gelatin was then leached out by placing the scaffolds into distilled water, the scaffolds frozen at −18°C, and then freeze-dried.

By using a solvent mixture of H_2O/THF, the gelatin was successfully entrapped onto the surface of the PLLA scaffolds. This allowed the PLLA molecules to diffuse onto the surface of the gelatin spheres, entangling with the gelatin molecules. These entrapped gelatin molecules remained stable on the polymer surface when placed in H_2O at a temperature above its gelation temperature.

There are several advantages for using this method, including the fact that it proceeds in a single step, where gelatin spheres act as both porogen and surface-modification agent. Secondly, this method can be applied to various scaffold materials, so long as the correct solvent mixture is used. Finally, when compared to the incorporation of functional groups by copolymerization, this method maintains the bulk properties of the underlying scaffold material.

The examples of biomimetic materials in scaffold design discussed thus far have pertained to bone. Other areas of tissue engineering where biomimetic materials are used include both hard and soft tissues. The junction between soft tissue and bone, including the subchondral bone/cartilage and bone/ligament is a challenge for reconstruction. For example, the anterior cruciate ligament (ACL) integrates with the bone through a fibrocartilage interface, which could potentially be regenerated using a stratified scaffold which mimics the naturally occurring transition found at that interface [28]. A triphasic scaffold is used, with a phase for ligament formation, a phase for the interface, and a phase for the bone. Fibroblasts, chondrocytes, and osteoblasts were cultured on this scaffold, and tissue formation evaluated over time in vivo. These scaffolds supported cellular interaction and tissue infiltration. This approach is promising, but this type of scaffold system must be further optimized.

The limbal-corneal epithelium of the eye is another tissue–tissue junction that has been studied for scaffold application. The cross-linked recombinant human collagen scaffold was studied as a carrier for limbal cells, and the refractive index and tensile strength of the scaffold were found to be comparable to human cornea [5]. The scaffold was found to be a possible substrate for growing cells for clinical transplantation.

The regeneration of the central nervous system is a challenging task. One approach is to use NPC, which can be cultured on substrates serving as temporary ECM in vivo. Scaffold modification with bioactive components, including proteins, peptides, and growth factors can tailor NPC response [27]. Laminin, a neurite promoting ECM protein, has been incorporated onto electrospun PLLA nanofibers in an attempt to promote PC12 cell viability and neurite outgrowth [16].

For vascular tissue engineering, the presence of ECM components within the scaffold promotes the growth of endothelial cells and in vitro formation of vascular conduit. A hybrid scaffold of polycaprolactone (PCL) film coated with fibrin, gelatin, fibronectin, angiogenic growth factors, and proteoglycans promoted the adhesion and proliferation of human umbilical vein endothelial cells (HUVECs) [26]. These results were an improvement over nontreated PCL scaffolds.

The application of angiogenic factors and adhesion sequences, as well as local gene therapy, is one technique for improving angiogenesis in ischemic tissues. This technique employs 3D hydrogel matrices that provide these adhesion sequences and/or release angiogenic growth factors such as PDGF [15], VEGF-A(165), and bFGF, as well as plasmid DNA [10] to transfect the surrounding cells and improve angiogenesis. These 3D matrices can be placed at the site of injury and act as a scaffold and release system to promote angiogenesis. For example, the inclusion of PLGA microspheres for the release of PDGF in a nanofibrous scaffold increased blood vessel number and in vivo gene expression of chemokines in the CXC family [15].

2.4 Clinical Applications

As discussed earlier, biomimetic materials are utilized in the design of engineering scaffolds in the replacement of a number of different tissues. Each of these tissue types not only has a unique set of requirements, based on the characteristics of the local cell population and properties of underlying ECM, but also specific requirements for the new tissue once it is in place. These requirements are of great importance when considering how these scaffolds can be used in a clinical setting. For example, bone is a tissue that has very specific structural requirements. Load-bearing applications require an implant that possesses suitable mechanical properties. Unfortunately, current scaffolds do not possess such properties and are limited primarily to nonload-bearing applications such as cranio-maxillofacial sites. Advances in neural regeneration offer potential application toward the reunion of nerve conduits through guided regeneration. Vascular tissue engineering has been employed with limited success in the placement of tissue-engineered arteries.

The effectiveness of tissue engineering using biomimetic materials in clinical applications is limited by the properties of those materials. By continuing to refine the materials and scaffold design, more and more clinical applications will be made available.

2.5 Expert Opinion

The authors work on polymeric scaffolds, with applications in bone tissue engineering. From the point of view of bone tissue engineering scaffold design, biomimetics is a vital component of a successful approach. Naturally occurring ECM provides a template onto which cells attach and proliferate, influences cell function and differentiation, and allows the

sufficient diffusion of nutrients throughout. Scaffolds that mimic the ECM have similar characteristics and, additionally, can be designed to more effectively deliver the chemical cues in the short and long term. Design nanofeatures should be combined with macro- and microstructures, such as in the nanofibrous, interconnected macroporous polymer scaffold described in the section on *State-of-the-Art Biomimetic Materials*, earlier in this chapter. This scaffold provides a path for vascularization and cellular ingrowth by mimicking the interconnected structure of trabecular bone, while possessing a nanoscale structure consisting of interwoven polymer fibers similar in scale to collagen fibers (50–500 nm in diameter). This structure had promoted increased adsorption of key proteins, leading to improved cell attachment, increased proliferation, and enhanced differentiation and function. From this, additional steps can be taken to further improve cell response. These include the addition of nanospheres for regional drug or growth factor delivery, surface engineering using self-assembly techniques, porogen-induced surface modification, and the inclusion of apatite crystals within the polymer nanofiber network. These areas of modification offer a myriad of potential improvements in an already successful tissue engineering scaffold. This system will likely yield further exciting results in the very near future.

2.6 Five-Year Perspective

The next few years will almost certainly see a further movement toward mimicking and improving on the physical and chemical components of the ECM for scaffold design. Additionally, a growing understanding of life sciences and regenerative biology through the use of stem cells, combined with these continuing rapid developments in materials science and engineering capacity (nanofabrication, phase separation, and self-assembly), signify significant strides forward in the near future.

2.7 Limitations/Critical View

When designing and engineering with an ultimate goal of mimicking and improving upon the natural structure and chemistry of the ECM, we are always playing a game of catch up. While tissue engineers have had success in promoting cell response and the formation of new tissue, the task of replacing a fully functional tissue is a difficult one. The current status of this technology is not good enough and we are continuing to develop creative ways to overcome the limitations. It should be noted, though, that biomimimetics is not simply copying the ECM. In many cases, the ECM itself is not the ideal structure to be mimicked. Future work must take into account that the regenerative process should be accelerated in tissue engineering, and scaffold design is continuously evolving to adopt the advantageous aspects of the ECM, while improving upon its structure when possible, to promote cell adhesion, proliferation, differentiation, and neotissue genesis [20].

2.8 Conclusion

In summary, biomimetics in tissue engineering has brought about considerable positive progress. The design, fabrication, and implementation of biomimetic scaffolds for tissue engineering are at the cutting edge of the field. The inclusion of nanophase design elements in scaffolds is one approach to mimicking the ECM and affecting cell response at the cell–material interface. The inclusion of these elements through either scaffold processing or surface modification leads to more effective polymer, ceramic, and composite scaffolds. These scaffolds result in improved protein adsorption and cell response in vitro and in vivo. Therefore, biomimetics is an area of great interest within tissue engineering, and the future will almost certainly see it being utilized in the design of the next generation of tissue engineering treatment options.

Suggested Reading

Langer R, Vacanti JP. Tissue engineering. Science. 1993; 260(5110):920–6.

The loss or failure of an organ or tissue is one of the most frequent, devastating, and costly problems in human health care. A new field, tissue engineering, applies the principles of biology and engineering to the

development of functional substitutes for damaged tissue. This article discusses the foundations and challenges of this interdisciplinary field and its attempts to provide solutions to tissue creation and repair.

Ma PX. Biomimetic materials for tissue engineering. Adv Drug Deliv Rev. 2008;60:184–98.

Tissue engineering and regenerative medicine is an exciting research area that aims at regenerative alternatives to harvested tissues for transplantation. Biomaterials play a pivotal role as scaffolds to provide three-dimensional templates and synthetic extracellular matrix environments for tissue regeneration. It is often beneficial for the scaffolds to mimic certain advantageous characteristics of the natural extracellular matrix, or developmental or wound healing programs. This article reviews current biomimetic materials approaches in tissue engineering. These include the synthesis to achieve certain compositions or properties similar to those of the extracellular matrix, novel processing technologies to achieve structural features mimicking the extracellular matrix on various levels, approaches to emulate cell–extracellular matrix interactions, and biologic delivery strategies to recapitulate a signaling cascade or developmental/wound healing program. The article also provides examples of enhanced cellular/tissue functions and regenerative outcomes, demonstrating the excitement and significance of the biomimetic materials for tissue engineering and regeneration.

Hench LL, Polak JM. Viewpoint: third-generation biomedical materials. Science. 2002;295(5557):1014–7.

Whereas second-generation biomaterials were designed to be either resorbable or bioactive, the next generation of biomaterials is combining these two properties, with the aim of developing materials that, once implanted, will help the body heal itself.

Anderson JM. The future of biomedical materials. J Mater Sci Mater Med. 2006;17(11):1025–8.

The purpose of this communication is to present the author's perspectives on the future of biomedical materials that were presented at the Larry Hench Retirement Symposium held at Imperial College, London, in late September 2005. The author has taken a broad view of the future of biomedical materials and has presented key ideas, concepts, and perspectives necessary for the future research and development of biomedical polymers and their future role as an enabling technology for the continuing progress of tissue engineering, regenerative medicine, prostheses, and medical devices. This communication, based on the oral presentation, is meant to be provocative and generate discussion. In addition, it is targeted for students and young scientists who will play an ever-increasing role in the future of biomedical materials.

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Natural and Synthetic Scaffolds

3

Patricia S. Wolfe, Scott A. Sell, and Gary L. Bowlin

3.1 Introduction

Tissue engineering is an interdisciplinary field aimed at the application of the principles and methods of engineering and life sciences toward the fundamental understanding of structure–function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue functions [8, 38, 56, 57, 78, 111]. Typically, this involves collaborative efforts between materials scientists, cell and molecular biologists, immunologists, surgeons, and engineers to create replacement tissues that will be accepted by the body and promote native extracellular matrix (ECM) production. This requires the use of materials that do not activate catabolic pathways in the body, ultimately leading to fibrous encapsulation or destruction of the material [25, 78, 104, 111].

Natural and synthetic ECM analogues have played a vital role in the field of tissue engineering since the early 1980s [8, 17, 119]. Improvements in the fabrication process as well as scaffold structure continue to occur with hopes of finding an ideal scaffold for each specific tissue engineering application. The overall function of the biodegradable scaffold is to create a three-dimensional microenvironment that will provide the necessary support for the transplanted or host cells to induce normal physiologic regeneration and function. Ideally, the scaffold should mimic the native ECM it is going to replace. In order for this to happen, several design considerations should be taken into account including fabrication, structure, biocompatibility, and biodegradability [17]. Many different materials have been used to create scaffolds for different tissue engineering applications, each one offering different features and characteristics. More information will be given later in this chapter about these design considerations and the different materials used to fabricate various ECM analogues.

Fabrication techniques for scaffolds range from traditional engineering methods such as solvent casting and particulate leaching, to computer-aided design (CAD) technologies consisting of techniques such as 3D printing and solid-freeform fabrication (SFF). Other processes include textile techniques such as electrospinning and weaving, and decellularization of tissues [11]. This chapter focuses on some of these fabrication processes in greater detail.

Although tissue engineering scaffolds have come a long way since they were first introduced, there are currently very few products on the market to show for their success. As for the products that are available, even though they have made a remarkable impact in the medical industry and have improved the quality of life for many, there are still several limitations and drawbacks, which are discussed in Sect. 4.

3.2 Aim of the Discipline

3.2.1 Tissue Engineering ECM

The use of isolated cells or cell substitutes is the most direct tissue engineering approach, typically using autologous or allogenic cells as therapeutic agents. This allows for the replacement of cells in areas of damaged tissue, ultimately using the cell's ability for

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replication to promote tissue repair and resume function [57, 78]. These cells can be differentiated and tissue specific (i.e., injecting chondrocytes into cartilage [40]) or can be undifferentiated, generic stem cells that could be injected into areas of damaged tissue and allowed to differentiate as needed [18, 104]. The upside of such an approach is the avoidance of surgery, as well as the ability to manipulate cells as needed *in vitro* prior to their implantation into the body. However, the major drawback of this approach is the time required to culture a usable number of cells. Without the presence of a large universal cell bank, cells must be taken from a donor and cultured to a usable number prior to the implantation in their eventual recipient [57, 104].

Another approach to tissue engineering is the use of either precellularized or acellular ECM analogues. The use of matrix analogues may be the most challenging, albeit potentially the most beneficial, approach to tissue engineering. The ultimate goal of this approach to tissue engineering is to enhance the body's ability to heal and repair itself by introducing a scaffold that the body recognizes and incorporates directly into the reparative process of the tissue. These systems are meant to mimic the native ECM and can serve as a structural framework for both cells and signaling molecules using the body as a bioreactor, exerting normal physiologic, biomechanical, and biochemical signals upon the scaffold. The idea being that the ECM analog will induce cells to more accurately reproduce their normal physiological behavior, thereby improving tissue regeneration and repair [8, 9, 105]. A number of tissue-inducing signaling molecules have been incorporated into ECM analog scaffolds to aid this process. These signaling molecules can include a wide number of cytokines and chemokines to promote cell growth, instruct differentiation, and promote cellular migration [78]. Since they are open to immunological attack, much research has been done on the material composition of these matrix analog systems in order to produce matrices that elicit little to no immune response and most closely mimic both the structure and function of native ECM.

3.2.2 Native ECM

Mammalian tissue is composed of two major components: cells (both parenchymal and mesenchymal) and ECM. A large majority of the tissue volume is composed of the ECM component, which also provides much of a tissue's geometric shape. From a structural standpoint, the ECM is a complex arrangement of proteins and polysaccharides such as collagen, hyaluronic acid, proteoglycans, glycosaminoglycans, and elastin (Table 3.1). These ECM components are constantly being synthesized, secreted, oriented, and modified by the cellular components that they support. Historically, the function of the native ECM was only believed to be a structural framework for tissues. However, it is now understood that the ECM, through interaction with receptors on the surfaces of cells, directly takes part in promoting cell adhesion, migration, growth, differentiation, and apoptosis. The ECM also plays a role in cytokine activity and intracellular signaling. Growth factors and signaling molecules can be stored within the ECM to preserve against their degradation, or they can attach to the surface of the ECM to present themselves more efficiently to the cell receptors [25, 65, 78].

Interactions between the cells and the ECM are complex and dynamic and play critical roles during development, wound healing, and environmental maintenance (Fig. 3.1). During development, the cell–ECM interaction is responsible for pattern formation, morphogenesis, and phenotype acquisition and maintenance. During the wound healing process, clot formation, inflammation, formation of granulation tissue, and remodeling are all mediated by the cell–ECM interaction. Initial attraction and adhesion of the cells to the ECM is induced by multiple, low affinity charge and hydrophobic interactions. During the spreading phase of adhesion, heterodimeric transmembrane proteins known as integrins on the cell surface bind to specific small peptide fragment sequences on the ECM molecules. This allows for the cells to bind to the ECM, through focal adhesions, and promote direct communication between the two. Integrin binding is both specific and reversible and allows the cells to differentiate, secrete and absorb the matrix, and transmit signals [25]. Signals are sent from the ECM across the cell membrane to the soluble molecules in the cytoplasm and through direct connections with the cytoskeleton into the cell nucleus, evoking a cellular response, termed "outside-in" signaling. This direct contact allows for stronger, more specific signaling than through the release of diffusible signaling molecules. The cell–ECM interactions can also be of an "inside-out" nature, when changes within the cell feed back to alter the activity of surface receptors,

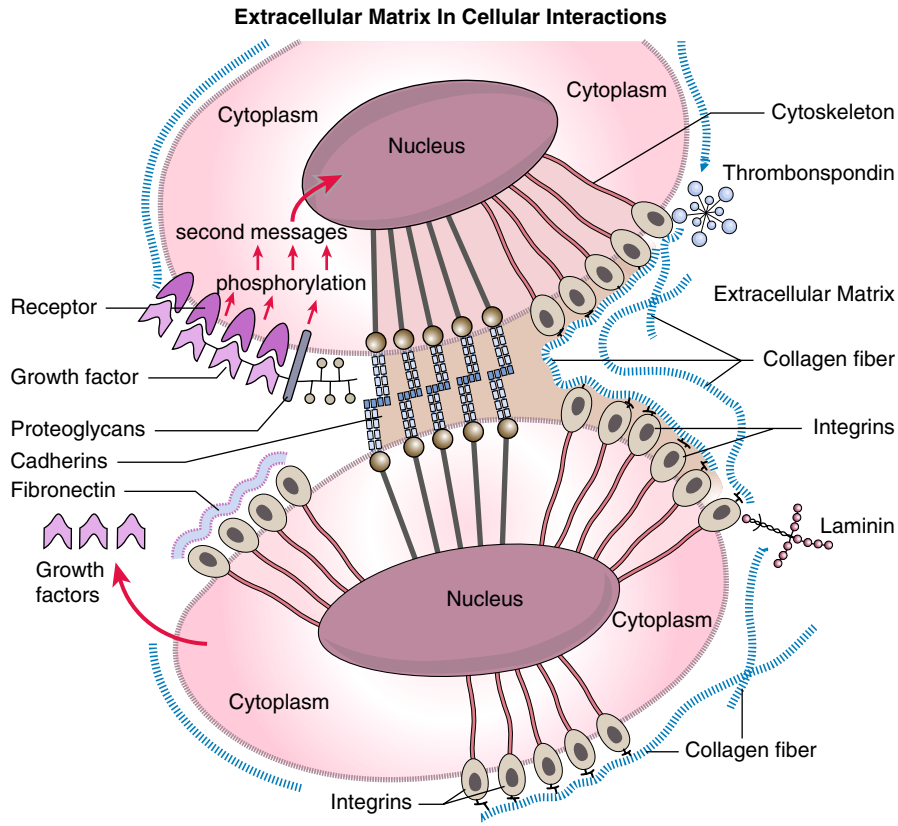

Fig. 3.1 ECM–cell interaction depicting integrin receptors. Reprinted from [78]

ultimately creating changes in the integrin and nonintegrin receptors in focal adhesions [25]. In what is known as dynamic reciprocity, the cellular response to the ECM signaling can often alter the state of the ECM. For example, the cells may release matrix metalloproteases to break down an overly dense ECM to allow for their migration or proliferation [65, 78].

3.2.3 ECM Analog Scaffolds

As complex a structure as the native ECM has been revealed to be, it should be no surprise that the creation of a successful engineered ECM analog has proven to be extremely challenging. Ideally, one would like to mimic both the fibrillar form and the complex function of the native ECM [1, 11, 128]. To attain a successful ECM analog scaffold, there are several design and material criteria that must be met. First and foremost, the scaffolding material should be subjected to the same standards as any other biomaterial implanted in the body, namely, the scaffold should not initiate any adverse tissue or immune reactions. For many applications, scaffolding materials should be biodegradable or bioabsorbable at a rate that will allow for their gradual incorporation into the surrounding tissue without any fibrous encapsulation or residual evidence of their presence [1, 11, 111, 119]. ECM analog scaffolds have been fabricated from an extensive array of materials through a number of different fabrication techniques. A wide number of different polymers, both synthetic and natural in origin, have been used as ECM analogues. The most common matrix materials in use today are polymers such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer, polylactide-co-glycolide (PLGA). However, extensive work has also been done with polycaprolactone (PCL) and polydioxanone (PDO), as well as some polyanhydrides, polyorthoesters, polycarbonates, and polyfumarates [1, 111]. For ECM analogues engineered from natural materials, collagens [66], elastin [90], fibrinogen [113], and silk [4] have been used. ECM substitutes of this variety have the potential for a greater upside than their synthetic

counterparts due to the fact that they are constructed from native ECM materials and may be expected to retain some of their biologic behavior [74, 111]. Inorganic materials such as hydroxyapatite, tricalcium phosphate, ceramics, and glass have also been used [91, 111]. Both the materials and their fabrication techniques will be discussed in detail later in this text.

The architecture of the scaffold is every bit as important as the material from which it is fabricated. As previously stated, an ECM analog should mimic the form of the native ECM. To be ideal, this ECM analog would need to mimic the topographical features and geometry on the macroscale, microscale, and even nanoscale levels, as each influences the cell response to the scaffold [77]. Native ECM is composed of nanoscale fibers that can provide structural integrity to the tissues. Recent advances in fabrication techniques (self-assembly, phase separation, and electrospinning) have made the creation of consistently nanofibrous scaffolds possible. The use of nanofibrous scaffolds creates structures with a very high surface area to volume ratio to support cell growth and infiltration [107, 119, 128]. In addition, the morphological similarities between the nanofibrous structures and the native ECM are believed to improve cellular response and overall biocompatibility [128].

Success of a tissue engineering scaffold in many applications is ultimately dependent upon the ability of the cells to infiltrate the ECM analog, migrate throughout its thickness, proliferate, and restore normal physiologic function [10, 100]. The scaffold's porous structure, a combination of microporous (pore diameters <2 nm), mesoporous (pores with diameter 2–50 nm), or macroporous (pore diameters >50 nm) void spaces, plays a major role in cellular penetration [60, 74]. As yet, there has been no concrete claim to an ideal pore diameter, but it has been documented that pores with a small diameter, yet larger than the diameter of a cell, are favorable [119]. Not only do the pores of an ECM analog scaffold need to be of a sufficient size for tissue growth to occur, but they also need to be open and interconnected. Interconnectivity refers to the extent of which the pores are connected with their neighboring pores, and has a large effect on nutrient and waste diffusion, cell migration, and overall scaffold permeability [52, 61, 111]. The terms porosity and permeability are often incorrectly used interchangeably in the realm of tissue engineering and in the consideration of ECM analog scaffolds. By definition, porosity is the amount of void space contained within a structure, while permeability is a measure of the ease with which a fluid can move through the structure. Matrix permeability ultimately depends on the combination of scaffold porosity, pore size and distribution, pore interconnectivity, and pore orientation and scaffold porosity to determine the hydraulic permeability of an ECM analog scaffold [52, 61].

Although not commonly reported for tissue-engineered scaffolds, permeability and porosity are extremely important to the success of an ECM analogue. Healthy, living tissue *in vivo* relies on the microvasculature to distribute blood and exchange metabolites through a combination of diffusion over short distances and flow-limited exchange. There are currently no tissue-engineered products that contain their own prevascularized capillary bed to provide nutrients to the structure, chaining their initial effectiveness to the limits of passive diffusion [51]. The limitations of diffusion-based nutrient transport restrict the maximum thickness of avascular tissue-engineered constructs to less than 2 mm [34]. Scaffolds with increased porosity and permeability help promote the diffusion of nutrients to cellular constituents, while promoting the diffusion of metabolic waste away from the cells. An increase in nutrient penetration distance will promote cell migration away from the scaffold periphery, and the presence of interconnected macropores will augment their ability to migrate [52, 61]. The degradation behavior of synthetic polymer-based scaffolds is also controlled in part by the permeability of the ECM analog. Low porosity and permeability scaffolds made of poly(α-hydroxy acids) have exhibited increased rates of degradation due to an increase in autocatalytic activity. Essentially, as the polymers breakdown via hydrolysis, the acidic byproducts become trapped within the scaffold and lower the local pH. This reduced pH then accelerates the degradation of the polymer from the inside-out resulting in a rapid loss of mechanical stability [52, 88].

3.3 State of the Art

3.3.1 Synthetic Scaffolds

Synthetic scaffolds, as mentioned above, have been used as ECM analogues and offer many advantages

over natural polymers, as well as limitations. For one, the material properties of the synthetic polymers can be controlled to suit specific functions, and therefore, can be more beneficial as a scaffold for multiple tissue engineering applications. In addition, many synthetic polymers are bioresorbable and have a known degradation rate, mechanical strength, and are readily available, and therefore, degradation time should not vary significantly between hosts [11]. Synthetic scaffolds provide many positive characteristics for their use as scaffolding materials, and almost seem to be the answer for ideal tissue engineering scaffolds, but the key dilemma is that they lack one of the major requirements of an ECM analog. Although the surface and structural characteristics of the synthetic polymers can be controlled, they are synthetic, as their name implies, and therefore, are deficient in the biological component of the native ECM [8, 114]. Another disadvantage is that the degradation products of these polymers can be toxic products, mostly weak acids, which can cause an adverse reaction if they accumulate locally [119].

Biodegradable synthetic polymers have been the primary focus for tissue-engineered scaffolds, with most belonging to the polyester family. Some of the most commonly used polymers will be more specifically outlined in the following paragraphs. A brief overview of the polymers' characteristics is displayed in Table 3.1 [11, 35, 106, 119].

3.3.1.1 Poly(Glycolic Acid)

PGA is a biodegradable, linear, aliphatic polyester that possesses a compact, repeating structural unit [14, 114, 119] (Table 3.2; Fig. 3.2). It is currently used in a variety of medical applications, but was initially developed as a commercially available suture in the 1970s because of its superior biocompatibility and reproducible mechanical properties [8, 27]. PGA is formed from the ring opening polymerization of glycolide and produces high molecular weight materials [70]. Some characteristics of PGA include a high crystallinity (46–55%), a high melting point (185–225°C), a glass transition temperature of 35–40°C, and a low solubility in organic solvents [14, 27, 35, 70, 119]. The glass transition temperature of PGA is very close to physiological temperature (35–40°C), and thus, water infiltration and loss of mechanical strength may occur more easily after implantation. Mechanical properties of PGA sutures include a tensile strength of 106 Kpsi, an elongation of 24%, and a knot retention of 65 Kpsi [8, 12]. Due to its hydrophilic nature, PGA degrades into glycolic acid over a period of 2–4 weeks in vivo. The predictable bioabsorption of this polymer and the fact that its degradation product is metabolized in the body makes it an attractive option for many tissue engineering applications. More specifically, for applications where an initially tough, but fast degrading material is

Table 3.1 Some major ECM components, their function, and location, adapted from [78]

Component	Function	Location
Collagen	Tissue architecture, tensile strength, cell–matrix interaction, matrix–matrix interaction	Widely distributed
Elastin	Tissue architecture, elasticity	Tissues requiring elasticity (lung, blood vessel, skin)
Proteoglycans	Cell–matrix interaction, matrix–matrix interaction, cell proliferation, cell migration	Widely distributed
Hyaluronan	Cell–matrix interaction, matrix–matrix interaction, cell proliferation, cell migration	Widely distributed
Laminin	Basement membrane component, cell migration	Basement membranes
Fibronectin	Tissue architecture, cell–matrix interaction, matrix–matrix interaction, cell proliferation, cell migration	Widely distributed
Fibrinogen	Cell proliferation, cell migration, hemostasis	Blood, wound healing

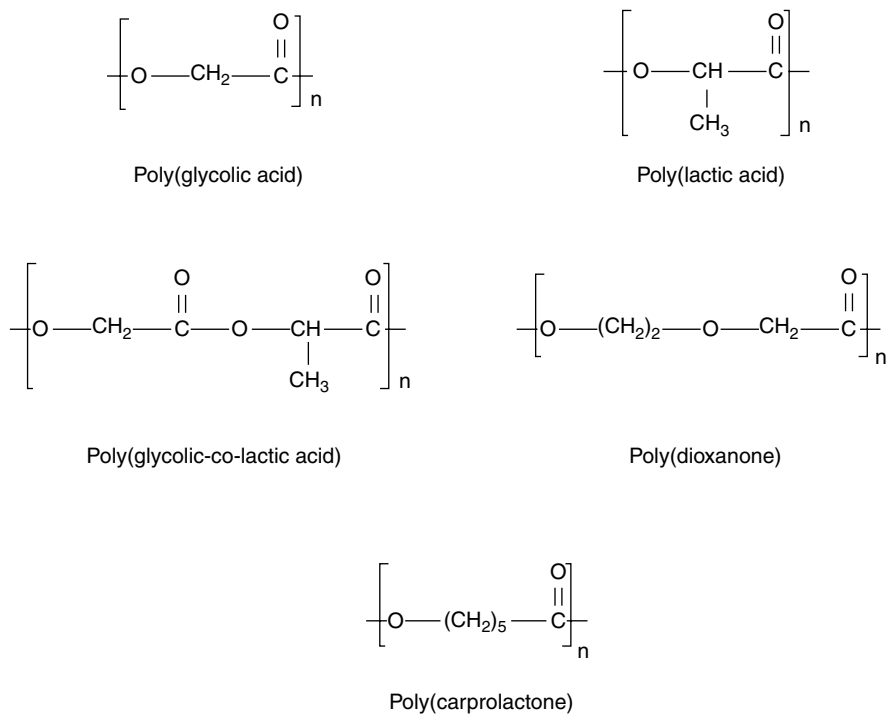

Fig. 3.2 Chemical structures of biodegradable synthetic polymers. [114]

desired, this polymer is a superior choice, as 60% of its strength is lost by hydrolytic degradation during the first 2 weeks.

PGA scaffolds have previously been formed by traditional extrusion methods [71], electrospinning [13], and solvent casting or particulate leaching [35] (fabrication techniques discussed later). Extrusion methods produce fibers with a minimum diameter above 10 μm which is a highly unfavorable size compared to native ECM analogues. Electrospinning methods have produced fibers of 200 nm–1.5 μm, depending upon polymer concentration. During an *in vitro* biocompatibility study, PGA scaffolds that were pretreated with hydrochloric acid demonstrated the ability to increase the rates of proliferation of cardiac fibroblasts compared to the cells on tissue culture plastic. In vivo studies with the same scaffolds resulted in full incorporation of the scaffolds into the hind leg tissue of rats [14]. Freed et al. demonstrated that PGA nonwoven mesh scaffolds seeded with chondrocytes have the ability to aid in cellular attachment and proliferation and the regeneration of cartilaginous matrix [27].

3.3.1.2 Poly(Lactic Acid)

Another biodegradable, aliphatic polyester commonly used in clinical applications is PLA [8] (Table 3.2; Fig. 3.2). Formed from the polymerization of lactide, this polymer is present in two isoforms: D(-) for dexorotary, l(+) for levorotary, and the synthetic blend, DL for racimic [70]. P(l)LA and P(d)LA are semicrystalline solids, and P(dL)LA is amorphous. The difference in the polymer's crystallinity has an effect on its clinical application. P(dL)LA is usually used in drug delivery applications because of its low tensile strength, high elongation, and rapid degradation time, while the semicrystalline PLA is preferred for load-bearing applications such as orthopedic fixations and sutures because of its higher tensile strength and modulus and lower elongation [114, 70]. Because of the extra methyl group in the monomer, PLA is more hydrophobic than PGA. The chemical structure of PLA contains an ester bond, which makes it less likely to undergo hydrolysis. Because of this, it degrades much slower than PGA (typically 30–50 weeks) and has a higher solubility in organic solvents. Since the degradation of PLA yields

L-lactic acid, for most tissue engineering applications, P(l)LA is chosen over P(d)LA, because lactic acid is naturally present and metabolized in the body [8, 35, 114, 119]. Other characteristics of PLA include 37% crystallinity, a melting point of 96–185°C, and a glass transition temperature of 57–65°C [114, 70]. It is important to mention that caution should be taken when sterilizing this polymer via gamma-radiation, because this may cause chain separation, cross-linking, and a decrease in crystallinity.

PLA scaffolds have been processed using traditional fiber extrusion methods, similar to PGA, and have resulted in fiber diameters on the microscale. Electrospun PLA scaffolds produce fibers with diameters ranging from 100 nm to 10μm, depending upon the solvent and concentration. In vitro experiments done by Yang, et al. demonstrate neural stem cell elongation and outgrowth parallel to the direction of P(l)LA fibers and higher cell differentiation for P(l)LA nanofibers vs. microfibers [116]. Human articular chondrocytes cultured on PLA scaffolds formed from a spin-casting method revealed that cellular attachment was increased on P(dL)LA scaffolds vs. P(l)LA scaffolds. In contrast, cellular proliferation was greater on P(l)LA scaffolds as compared to P(dL)LA scaffolds. This is most likely due to the differences in crystallinity of the two polymers, which could cause a difference in the amount of serum proteins that are absorbed [45].

3.3.1.3 Poly(Lactide-Co-Glycolide)

PLGA (Table 3.2; Fig. 3.2) is the copolymer formed by PGA and PLA. Unlike each individual homopolymer, this copolymer is amorphous when either monomer is present below 70 mol% because of the disruption of the regularity of the polymer chain by the other monomer [70]. This characteristic results in a decrease in the degradation rates and mechanical strength [114, 119, 130]. If either homopolymer is present above 70 mol%, the copolymer exhibits some crystallinity. PLGA also exhibits lower crystallinity and melting temperature compared to PGA or PLA. This copolymer degrades in bulk by ester hydrolysis (Table 3.2), and the rate of degradation can be altered by adjusting the ratio of PLA/PGA.

PLGA has been used for many medical and tissue engineering applications such as surgical sutures, cardiac tissue regeneration, and drug delivery, because it is biodegradable and biocompatible [8, 55, 130]. Bone formation on PLGA foams fabricated by solvent casting/particulate leaching (to be discussed later) by mesenchymal stem cell-derived osteoblasts has also been reported [120]. PLGA scaffolds have also been shown to support the cell growth and function of a variety of other cell types including fibroblasts, chondrocytes, and smooth muscle cells (SMCs) [55, 119].

Table 3.2 Properties of degradable synthetic polymers used for tissue engineering scaffolds

Polymer	Degradation method	Degradation time	Primary degradation products	Bulk mechanical stiffness: E-modulus (GPa)	Tissue engineering applications
PGA	Ester hydrolysis	1–12 months	Glycolic acid	5–7	Skin, cartilage, bone, ligament, tendon, vessels, nerve, bladder, liver
PLA	Ester hydrolysis	5–60 months	Lactic acid	2–3	
PLGA	Ester hydrolysis	1–12 months	Lactic acid and glycolic acid	2–7	
PDO	Ester hydrolysis	3 weeks to 6 months		0.002–0.04	Orthopedics, drug delivery, bone, vessels
PCL	Ester hydrolysis	1–3 years	Caproic acid	0.4	Skin, cartilage, bone, ligament, tendon, vessels, nerve
PEG/PEO	Nondegradable	Not applicable	Not applicable	0.1–5	Drug delivery, cartilage

PGA poly(glycolic acid); *PLA* poly(lactic acid); *PLGA* polylactide-co-glycolide; *PCL* polycaprolactone; *PDO* polydioxanone; *PEG* poly(ethylene glycol); *PEO* poly(ethylene oxide)

3.3.1.4 Polydioxanone

PDO is a biodegradable polyester formed from the ring opening polymerization of the monomer paradioxanone [16, 70] (Table 3.2; Fig. 3.2). Originally, this polymer was developed for use as a suture because of its superior degradation rate of 6–12 months, falling in between PGA and PLA. PDO exhibits a high crystallinity of 55% and a glass transition temperature of −10–0°C. Another attractive property is its excellent flexibility because of an ester oxygen group in the backbone structure [8, 16, 70]. In addition, PDO has shown no acute or toxic effects upon implantation. A negative aspect of the polymer when used as a suture is its shape memory. Because of the suture's ability to retain its spooled shape, knot retention becomes very difficult. Although this may prevent PDO from being an ideal suture material, shape memory may be a positive aspect for tissue engineering applications. For example, if used in a vascular graft, it can provide rebound and kink resistance [8, 16, 70]. Also, the degradation rate of PDO sutures can be a negative aspect, because it may be too rapid to allow for a durable closure of wounds and may cause abnormal healing.

Electrospinning of PDO has been performed and has resulted in fiber diameters of 180 nm–1.4 μm, depending upon the solution concentration, and exhibits material properties within the same range as the major structural components of the native vascular ECM (collagen and elastin). As fiber diameter decreases, it has been shown that cell interaction improves and immune response is reduced [8, 16]. PDO scaffolds have also been used in other applications including orthopedics, plastic surgery, drug delivery, cardiovascular, and bone repair [16].

3.3.1.5 Polycaprolactone

PCL is another linear aliphatic polyester that has demonstrated good biocompatibility, favorable mechanical properties, and a slow degradation rate of 1–3 years [8, 35, 106] (Table 3.2; Fig. 3.2). Prepared by the ring opening polymerization of the cyclic monomer ε-caprolactone, PCL is a repeating unit of one ester group and five methylene groups [35, 70, 119]. This semicrystalline polymer is highly soluble and exhibits a low melting point of 58–64°C and a glass transition temperature of −60°C [35, 70, 119]. Degradation of PCL occurs by bulk or surface hydrolysis of the ester linkages, producing a byproduct of caproic acid [119]. To increase its degradation rate, PCL has been easily copolymerized with a variety of polymers including collagen, PGA, PLA, and poly(ethylene oxide) (PEO) [8, 35, 70, 119].

Electrospun scaffolds of PCL that were seeded with mesenchymal stem cell-derived osteoblasts promoted cellular penetration as well as ECM formation [120]. Other studies have shown that PCL scaffolds support human osteoblast and dermal fibroblast cell viability and the proliferation of human biliary epithelial cells [119]. PCL scaffolds have also been used for cartilage [26] and vascular tissue engineering applications [108].

3.3.1.6 Poly(Ethylene Glycol)/ Poly(Ethylene Oxide)

Poly(ethylene glycol) (PEG) is a linear-chained polymer consisting of an ethylene oxide repeating unit (Table 3.2). PEO has the same backbone as PEG, but a higher molecular weight because of its longer chain length [119]. PEG and PEO are both hydrophilic and are synthesized by anionic or cationic polymerization of ethylene oxide [59, 119]. They have the ability to act as swelling polymers, which has led to their use as a hydrogel, and makes them excellent polymers for medical applications such as drug delivery [82]. Another attractive property for their use in tissue engineering is their low toxicity and biocompatibility [59]. A limitation of PEG and PEO is their inability to naturally degrade, but if they are copolymerized with a hydrolytically or enzymatically degradable polymer, they can be made degradable [59, 119, 121]. PEO-based copolymers, such as the triblock copolymer with poly(propylene oxide) (PPO), PEO-PPO-PEO, could be designed to form gels at body temperature by forming a liquid crystalline phase, and have been mainly used in drug delivery applications, where they have been known to enhance drug penetration [59]. Riley et al. cultured chondrocytes on PEG-based hydrogel scaffolds and found that the scaffolds supported the survival of the cells and the deposition of a cartilage-like matrix [84].

3.3.2 Natural Scaffolds

To overcome the disadvantage that synthetic polymers have of lacking the biological component, natural polymers are widely used, either by themselves,

or as a blend with synthetics. Derived from renewable resources such as plants, animals, and microorganisms, natural polymers used in scaffolds offer an advantage because of their excellent biocompatibility and biodegradability [33]. They are biologically similar to the native ECM, which allows the cells to interact with these polymers in a natural manner through receptors and signals, and also aids in the correct functioning of the cells such as attachment, proliferation, and differentiation. Disadvantages of natural polymers include variations in degradation rates, batch-to-batch inconsistency, and poor mechanical strength [8, 17, 119]. Another important disadvantage to consider is the ability of these polymers to induce a negative immune response due to the presence of impurities and endotoxins, depending on their source [33]. A detailed description of some of the most widely used natural polymers in tissue engineering is discussed below.

3.3.2.1 Collagen

Collagen is the most abundant protein found in the body. It functions to provide the overall structure and strength to the tissues and is the principal component of the native ECM. In addition, the collagen structure provides the cells with the appropriate microenvironment for embryologic development, organogenesis, cell growth, and wound repair and also stores and releases important cell mediators such as growth factors [8, 33]. Currently, there are over 20 different kinds of collagen that have been identified, with the basic structure composed of three polypeptide chains, coiled around each other to form a triple helix (Fig. 3.3).

The most common triple helix of collagen is formed by the peptides, proline, hydroxyproline, and glycine in the sequence Pro–Hyp–Gly. The individual triple helices arrange to form collagen fibrils, which account for the structural integrity of the tissue. Collagen fibril formation is an extracellular process, which occurs through the cleavage of terminal procollagen peptides by specific procollagen metalloproteinases. The different types of collagen are found in different areas of the body depending upon the role and makeup of the tissue. Type I collagen is the most common type, found in the dermis, bone, tendon, fasciae, sclera, organ capsules, and fibrous cartilage, and is a major component of mature scar tissue. Type II collagen is mostly found in hyaline and elastic cartilage, the developing cornea, and in the vitreous body of the eye. The wall of the blood vessels and hollow intestinal organs consist mostly of type III collagen, which also copolymerizes with type I. Types V and XI are less abundant and mostly occur copolymerized with type V and type XI [85]. One reason for collagen's wide use in tissue engineering applications is that it can be isolated from many different sources and is relatively nonimmunogenic. The primary sources of collagen are animal tissues (porcine and calf skin, bovine tendon, rat tail), in which it is purified from the tissues using an enzymatic treatment and salt/acid extraction. Deriving this polymer from animals, although an easily accessible source, does pose some problems for its use as a scaffold because of the possibility of the transmission of infectious agents and rejection from the host tissue. Fortunately, many new attempts have been made to derive collagen from safer sources, such as jellyfish [95], and to produce human recombinant collagen [115]. These alternatives provide a more reliable and predictable source of collagen that is free of animal components.

One feature that is necessary for all types of collagen for their use in tissue engineering applications is chemical cross-linking. Although this process can be a

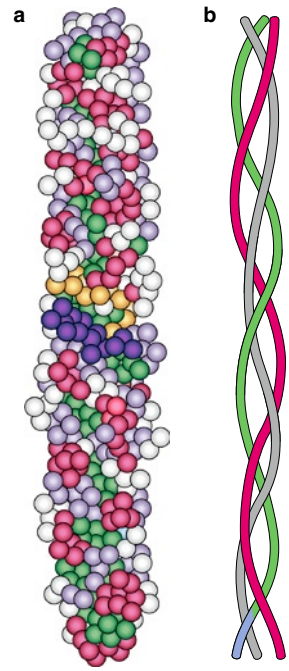

Fig. 3.3 Picture depicts triple-helical collagen structure. (**a**) Model of collagen peptide where Gly is *green*, Pro is *gray*, and Hyp is shown in *magenta*. (**b**) The schematic of the triple helix. [33]

disadvantage for this natural polymer's use in tissue engineering applications because of the addition of toxic solvents, this is necessary to stabilize the polymer in order to control the mechanical properties. Cross-linking can be achieved in various ways, including chemically (glutaraldyhyde, EDC, genipin), physically (freeze drying, UV radiation, heating), and enzymatically [85, 33].

Degradation of collagen is done naturally by matrix metalloproteinases, specifically collagenase and serine proteases [85], which also provide an advantage for its use in tissue engineering. It is used in various medical applications, such as wound dressings and artificial skin, and collagen scaffolds have also been used in a variety of tissue engineering applications, such as vascular grafts [15], tendon/ligament [30, 31], cartilage [99], and breast tissue [41].

Some drawbacks of collagen for tissue engineering applications include the polymer's low mechanical properties, the need for cross-linking (mentioned above), risk of viral infection, increased antigenicity potential, and an extremely fast biodegradation rate [85].

3.3.2.2 Gelatin

Gelatin is obtained by a controlled hydrolysis of collagen and is a natural polymer that is of interest in tissue engineering because of its excellent biocompatibility and biodegradability, and cost efficiency. Generally speaking, there are two types of gelatin: Type A and Type B. Type A is extracted from collagens, and processed by an acidic pretreatment, and Type B is obtained by an alkaline pretreatment, which causes it to have higher carboxylic acid content than Type A. For many years, it has been used as a vascular prosthetic sealant, a dressing for wounds, and a carrier for drug delivery, and recently, it has gained interest for its use as a scaffold for tissue engineering applications [8, 127]. One drawback of gelatin is that it dissolves as colloidal sol at temperatures at or above 37°C, and gels at lower temperatures around room temperature. However, when combined with other synthetic polymers, or cross-linked, this limitation can be reduced.

In a study done by Zhang et al., a mixture of gelatin and PCL solutions were electrospun to produce fibrous scaffolds. These scaffolds proved to have excellent biocompatibility with bone marrow stromal cells and also aided in the process of cellular migration, proliferation, and penetration [126]. Gelatin scaffolds have also been used for nerve [32], hepatic [48], and cartilage [98] tissue engineering applications.

3.3.2.3 Elastin

Elastin is the most linearly elastic biosolid known and is a key structural protein found in the native ECM of connective tissues where elasticity and recoil are critical parameters (Fig. 3.4). Elastin consists of several repetitive amino acid sequences, including VPGVG, APGVGV, VPGFGVGAG, and VPGG [33]. Mature elastin is formed from tropoelastin, a 70-kDa protein consisting of alternating hydrophobic regions (responsible for the elasticity) and cross-linking domains. Additionally, it ends with a hydrophilic carboxy-terminal sequence containing its only two cysteine residues [23]. A highly insoluble protein, elastin constitutes the walls of arteries and veins, ligaments, lung parenchyma, skin, and intestines.

There are some drawbacks of elastin that have limited its use as a biomaterial. Upon implantation, elastin preparations have a strong tendency to calcify. This may be due to the microfibrillar components, such as calcium-binding fibrillin, within the elastic fiber that are difficult to remove, although there have been studies that disprove this theory. Another limitation of elastin is the complexity of its purification process [21]. Similar to collagen, pure elastin scaffolds need to

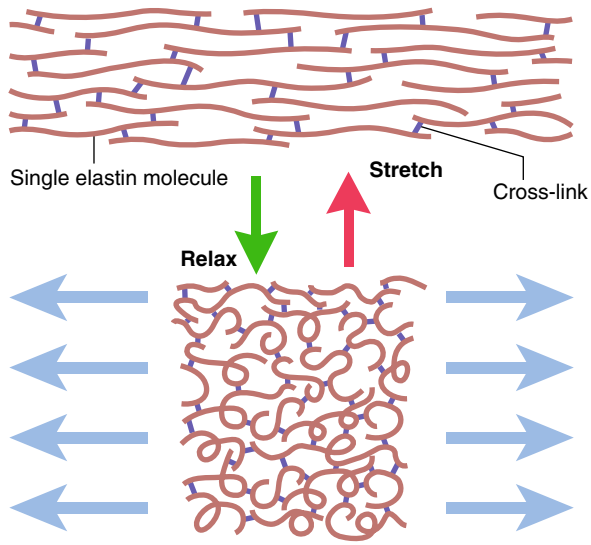

Fig. 3.4 Elastin structure reprinted from [33], © Elseiver

be cross-linked to improve their mechanical integrity, which also presents a limitation [67].

Elastin scaffolds have mostly been fabricated by electrospinning [90], but others have used insoluble elastin in gels [81] and elastin-like polypeptide block copolymers [62]. In previous studies, electrospun elastin scaffolds have been shown to regulate the proliferation, migration, and differentiation of SMCs as well as reduce the vascular proliferative response to arterial injury in vivo [15, 21]. Several other studies have been performed using elastin scaffolds for cardiovascular tissue engineering purposes [22, 39, 93].

3.3.2.4 Fibrinogen

Fibrinogen is a naturally occurring plasma protein (340 kDa) that plays an important role in blood clotting, fibrinolysis, cellular and matrix interactions, inflammation, wound healing, and neoplasia [68, 69, 75]. Fibrinogen molecules are elongated 45-nm structures consisting of two outer D domains, each connected by a coiled-coil segment to a central E domain (Fig. 3.5).

These molecules consist of six polypeptide chains: two Aα, two Bβ, and two γ, and when fibrinogen reacts with thrombin, two peptides are cleaved to produce fibrin monomers. In the presence of calcium and factor XIII, fibrous clots and other fibrous structures are formed by the fibrin monomers. These structures play a role in the wound healing process, serving as a provisional matrix on which tissues rebuild and repair themselves. For this reason, fibrinogen is an attractive protein for its use in tissue engineering applications.

Another advantage of fibrinogen is that it has two integrin binding sites, RGDF and RGDS, in which many cellular interactions occur through binding to these sites. Cells that have receptors for these binding

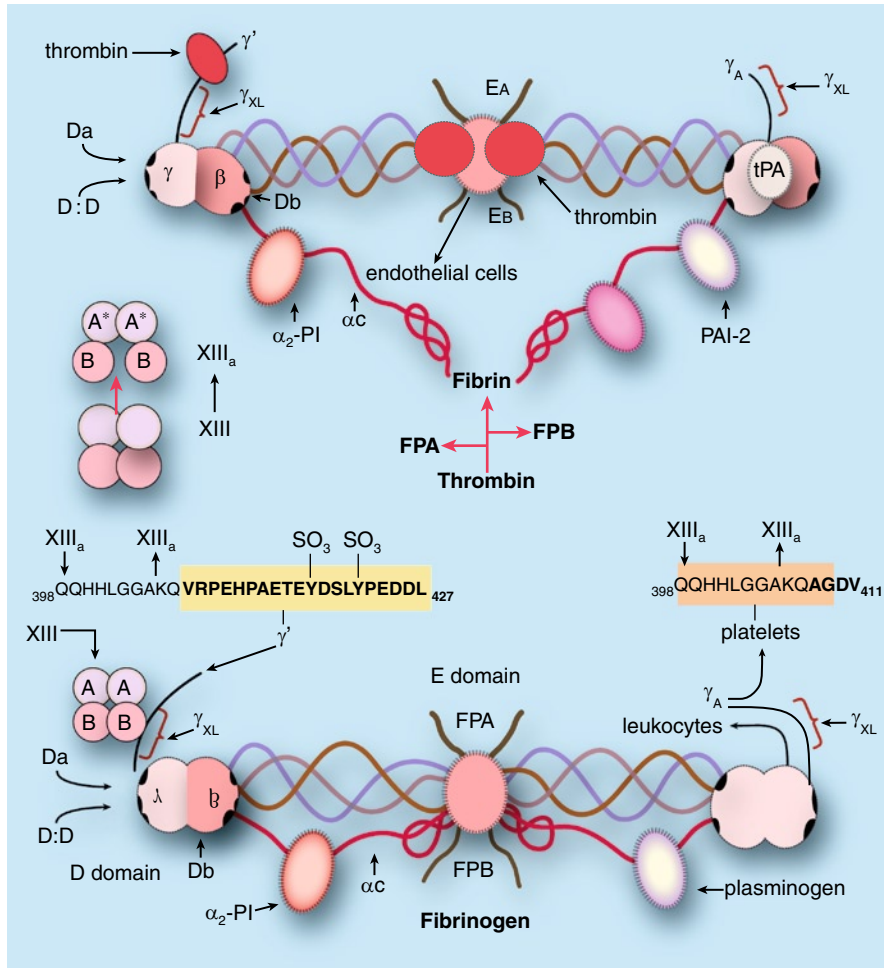

Fig. 3.5 Major structural features of fibrinogen. [75]

sites include platelets, endothelial cells (ECs), melanoma cells, fibroblasts, monocytes, and neutrophils [75].

Fibrin-based scaffolds have been developed previously in the form of fibrin gels and wet extrusion fibronectin-fibrinogen cables. Although these gels were easily degradable, nonimmunogenic, and promoted cell migration, they lack the structural integrity needed for practical use in a tissue. The limitation of the wet extruded cables is their resulting large fiber size; 200–250 μm in diameter is many orders of magnitude larger than the native fibers of the ECM, resulting in an unfavorable environment for the cells to function correctly. Electrospun fibrinogen has also been accomplished and has shown excellent cellular interaction as well as mechanical properties similar to those of the native tissue [68, 69].

3.3.2.5 Silk

Silk is an extremely common fibrous protein that has been used as a medical grade suture for centuries. More recently, silk has become a material of interest in the creation of tissue engineering scaffolds due to its unique blend of material characteristics. Silk exhibits excellent biocompatibility with a foreign body response comparable to other degradable sutures, hemocompatibility, and oxygen and water permeability. Although it is classified as a nondegradable suture, it has been shown that silk will break down through proteolytic degradation and will be slowly absorbed *in vivo* as biocompatible amino acids. The rate of degradation varies based upon the implantation site and the size of the implanted fibers but has been reported to lose the majority of its tensile strength between 6 weeks and 1 year after implantation. Silk also possesses remarkable mechanical properties not seen in other naturally occurring proteins [3, 4, 110]. It has been reported that natural silk fibers have tensile strength and yield at fracture values comparable to synthetic fibers such as Kevlar [128].

Silk is produced in nature by a wide variety of insects and spiders, with the silk from *Bombyx mori* silkworm cocoons being the most commonly harvested. Natural silk is composed of two distinct proteins: a glue-like sericin protein, which serves to hold the fibers together, and a fibroin filament component which acts as the mechanical backbone. As sericin has proven to elicit an adverse immune response inside the body, it is the degummed silk fibroin (SF) that is used in medical applications such as braided suture. This 325 kDa protein consists of repetitive hydrophobic blocks, which form crystalline β-sheets through hydrogen bonding, and amorphous hydrophilic regions. The β-sheets provide the structure with its tensile strength, while the amorphous regions provide elasticity and toughness [4, 110, 128]. SF fibers have been used as both knitted constructs [63, 92, 102] and as electrospun scaffolds of reconstituted SF [2, 47, 49, 50, 53, 72, 97, 109, 122]. It has been well documented that the reconstituted fibers of SF are almost completely amorphous in their structure and must be annealed in a methanol or ethanol solution to create β-sheet crystallization [2, 29, 47, 50, 53, 72, 109, 132]. Despite this annealing process, reconstituted SF scaffolds have proven to be highly conducive to cell seeding, with literature reporting successful proliferation of fibroblasts [2, 4, 72, 92], osteoblast-like cells [4], keratinocytes [72], and bone marrow stem cells [3, 4, 50, 63, 102, 110].

3.3.2.6 Acellular Matrix and Submucosa

Another natural-based material that has been used as a successful scaffold for tissue engineering applications is decellularized ECM. As mentioned before, nearly all the tissues are comprised of ECM that consists of structural proteins, polysaccharides, cytokines, and growth factors. While this process of decellularization aims at completely removing all the cellular and nuclear material from the tissues, the bulk composition, mechanical properties, and biological activity are still intact [6]. Using this type of scaffold eliminates some of the drawbacks and limitations of techniques that will be mentioned later, such as insufficient mechanical strength and possible undesirable inflammatory responses, but still has other disadvantages, including rapid degradation rates and calcification production. Native ECM structures are advantageous for their use as tissue-engineered scaffolds because they induce a positive host response that promotes cell infiltration, rapid scaffold degradation, formation of host-derived neomatrix, and tissue remodeling with a minimum amount of scarring [5].

Briefly, decellularization is the process by which cells are removed from an ECM through a series of mechanical, chemical, or enzymatic steps, leaving an

acellular matrix that can serve as a tissue engineering scaffold. The main components of the native ECM that are left after the decellularization process are collagen and elastin [11]. ECMs that have been successfully processed this way include the urinary bladder, the dermis of the skin, small intestine, pericardium, basement membrane and stroma of the decellularized liver, and decellularized Achilles tendon [5].

The submucosa is the layer of tissue beneath a mucous membrane or the layer of connective tissue beneath the tunica mucosa. Similar to the decellularized ECM described above, subintestinal submucosa (SIS) is an attractive material for tissue engineering scaffolds because of the structural and biological factors present in the tissue [7, 20, 58, 96]. *In vivo* studies done with a bladder submucosa confirm that this tissue can contribute to bladder tissue regeneration. Normal cellular organization and phenotype were demonstrated, along with the presence of nerve fibers [117]. Two drawbacks of both decellularized ECM and SIS are that they exhibit a rapid rate of degradation *in vivo*, and may elicit calcification. This former problem is especially prevalent in the submucosal tissue, while the native ECM tissue also has problems with scaffold shrinkage *in vivo*.

Another method to process the ECM and submucosa include adding chemical cross-linking agents to modify the mechanical properties, but doing so may cause a fibrous response [117].

3.3.3 Fabrication Techniques

There are several fabrication techniques that are used to try and meet the design requirements listed above to produce an ideal tissue-engineered scaffold for specific applications. These techniques include electrospinning, phase separation [11, 46, 125], self-assembly [46, 36, 123], solvent casting/particulate leaching [11], melt molding [11], and CAD techniques such as rapid prototyping and SFF [42, 44, 64]. Each technique produces scaffolds that exhibit many different characteristics such as pore size, fiber diameter, mechanical strength, and types of polymers that can be used; each parameter is unique depending upon the tissue engineering application that the scaffolds are being made for. Some of these techniques will be discussed in further detail below.

3.3.3.1 Electrospinning

Electrospinning is a textile manufacturing process that is widely used as a fabrication technique for tissue-engineered scaffolds. It was first described as electrostatic spraying, which has been around for more than 100 years, and can produce polymeric scaffolds with fiber diameters ranging from nano- to micrometer range. The electrospinning process consists of a polymer solution, or melt, that is placed in a syringe or pipette. The tip of the pipette or needle that is attached to the syringe is charged with a voltage, and when the electric field produces a force that overcomes the surface tension of the solution, a jet of polymer is drawn from the syringe and attracted to a grounded collecting plate placed some distance away from the needle. The jet forms a Taylor Cone immediately after leaving the needle, and as it travels toward the grounded target, the solution gradually evaporates, leaving small fibers to collect on the target. The charge from the fibers dissipates into the surrounding environment, and a nonwoven fiber mat consisting of tiny fibers, ranging from 50 nm to 10 μm, is formed on the target [8, 11, 24, 64] (Fig. 3.6). Processing parameters including solution properties (viscosity, elasticity, conductivity, and surface tension), processing conditions (voltage, needle diameter, distance from the needle to the grounded target), and the environment (temperature, humidity, and static electricity) can be varied to modify the fibers for individual applications. For example, fiber diameter increases with increasing polymer concentration and increasing voltage [8, 46].

This fabrication technique has many attractive features to produce scaffolds for tissue engineering applications. For one, the mechanical properties of the scaffolds can be adjusted by changing the orientation of the fibers (parallel alignment or random arrangement), and the types of polymer(s) used (natural vs. synthetic). In addition to altering the orientation of the fibers, variations in the scaffolds themselves can be obtained for different applications. For example, multilayering electrospinning results in a hierarchically ordered structure composed of different types of polymers, while multicomponent electrospinning, where multiple polymers are simultaneously electrospun together, forms a mixed fiber mesh [54]. Also, the way the fibers are collected on the grounded target can influence the fiber orientation as well as scaffold fabrication. Collection schemes currently used include

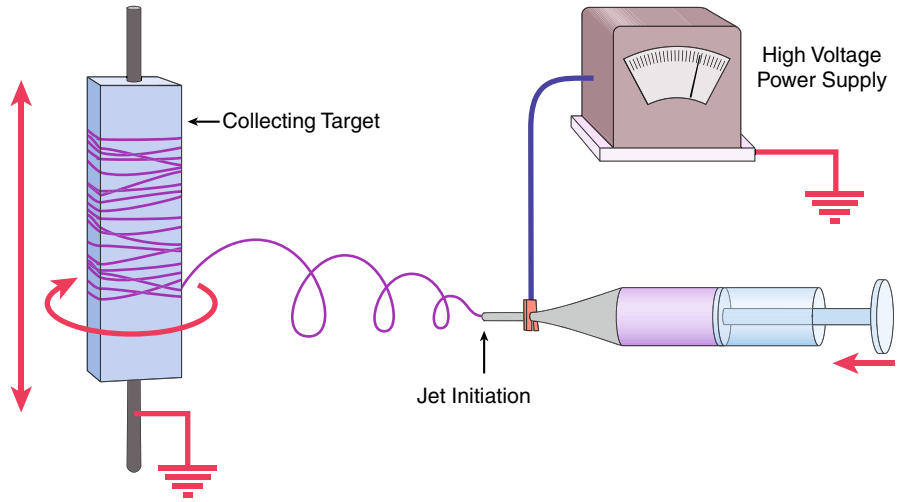

Fig. 3.6 Electrospinning setup [16]

a single ground, rotating single ground, dual bar, dual ring, single horizontal ring, electrospinning *in vitro* onto cells, or electrospinning cells with polymer (Fig. 3.7) [44].

To date, many different polymers, both synthetic and natural, have been successfully electrospun. These polymers include synthetics such as PLA, PGA, PCL, and PDO, naturals including collagen, elastin, silk, and fibrinogen, and blends of naturals and synthetics [8, 11, 15, 16, 50, 68, 130] (Fig. 3.8). With these different polymer combinations, scaffolds can be produced with strengths and stiffness that mimic the native ECM. Many *in vivo* and *in vitros* studies have shown that electrospun scaffolds support cell infiltration and have excellent biocompatibility as well [11]. Cardiac myocytes have been shown to interact with electrospun PLGA-based scaffolds and these constructs provided both flexibility and guidance for cell growth [128]. Boland et al. demonstrated the ability of the electrospun collagen and elastin blended scaffolds to be used in vascular tissue engineering applications. These scaffolds, when cultured with ECs, SMCs, and fibroblasts, exhibited a three-layered structure, similar to a native artery, with a confluent EC lining on the intima, a complete cellular infiltration of SMCs throughout the media, and a dense population of fibroblasts and SMCs in the adventitia [15]. Mesenchymal stem cells (MSCs) have been cultured on electrospun scaffolds of PLGA and PCL. After 7 days, the MSCs showed a fivefold population increase on the PLGA scaffolds. On the PCL scaffolds, the cells migrated through 1 mm, depositing collagen along the way, and subsequently differentiated into osteoblasts, producing calcifications [94]. In addition, electrospinning of different polymers has been performed to develop scaffolds for wound dressings [73], cartilage [84, 91, 98, 99, 101], bone [4, 28, 120], ligament [87, 86], nerve tissue [32, 80], and breast tissue engineering [41].

There are many benefits of using this technique, as well as some limitations. The simplicity of this process allows it to be used in a scaled-up setting for mass production purposes. Also, the fibers produced are similar in arrangement and size to those of the native ECM. As stated previously, the adjustment of many parameters to this technique allows for additional control and modification of scaffold characteristics. Drawbacks of this process include the use of toxic solvents (HFP) and cross-linking agents, which could adversely affect the cellular response if not fully extracted. Also, a lack of mechanical strength has been reported, especially when electrospinning natural polymers, although if they are blended with synthetic polymers, this limitation can be resolved [8, 11].

3.3.3.2 Phase Separation

Phase separation is a technique that produces three-dimensional, highly porous scaffolds with the aim of incorporating bioactive molecules. As a process that produces fibers in the submicron range, it can be accomplished in several ways, including nonsolvent-induced phase separation, chemically induced phase separation, and thermally induced phase separation

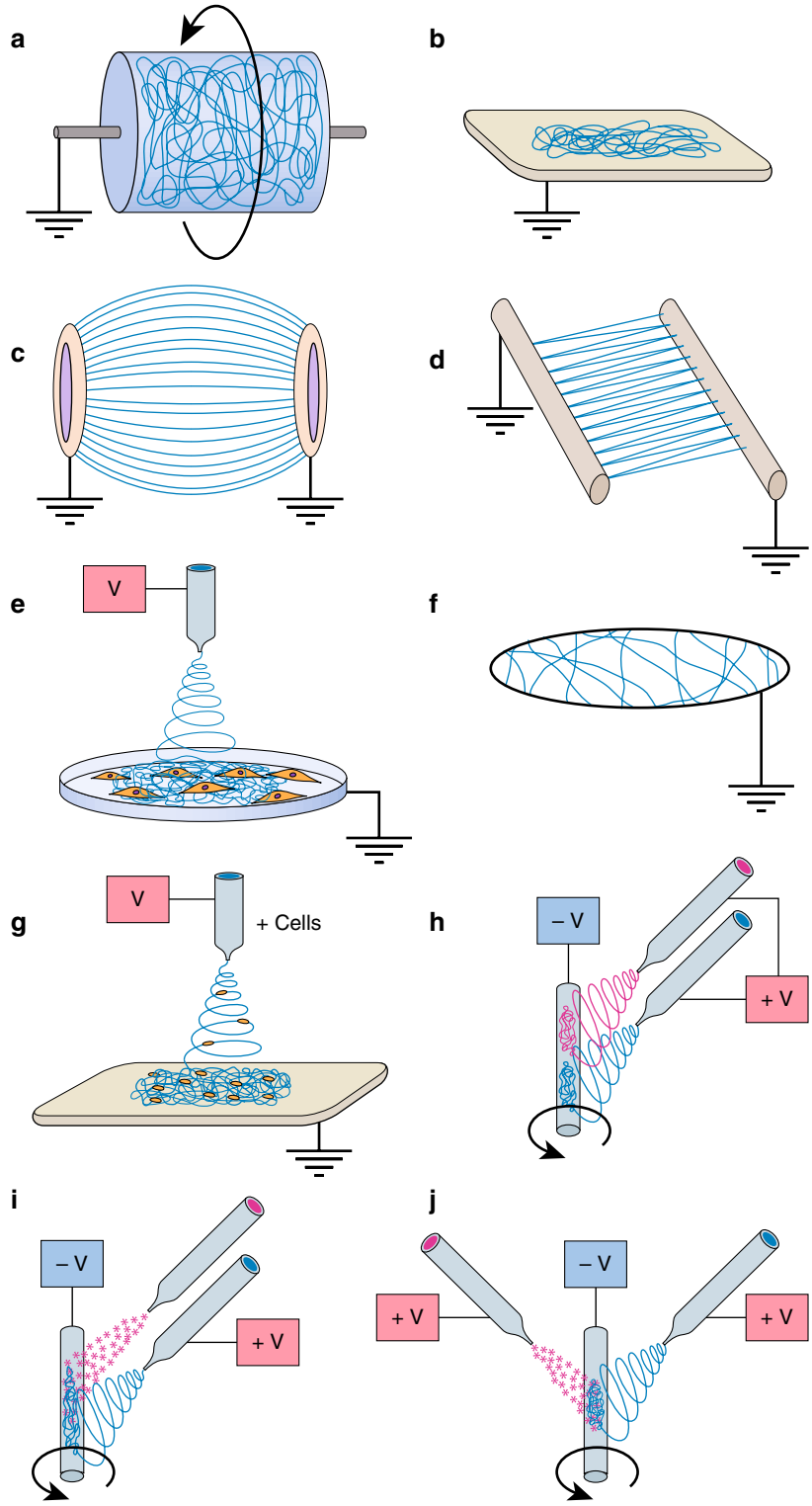

Fig. 3.7 Different electrospinning collecting schemes. (**a**) Rotating single ground, (**b**) single ground, (**c**) dual ring, (**d**) dual bar, (**e**) electrospinning in vitro onto cells, (**f**) single horizontal ring (**g**) electrospinning cells with polymer, (**h**) dual spinneret electrospinning (with second pink fibers), (**i**) electrospinning/electrospraying with parallel and (**j**) perpendicular spinnerets. [44]

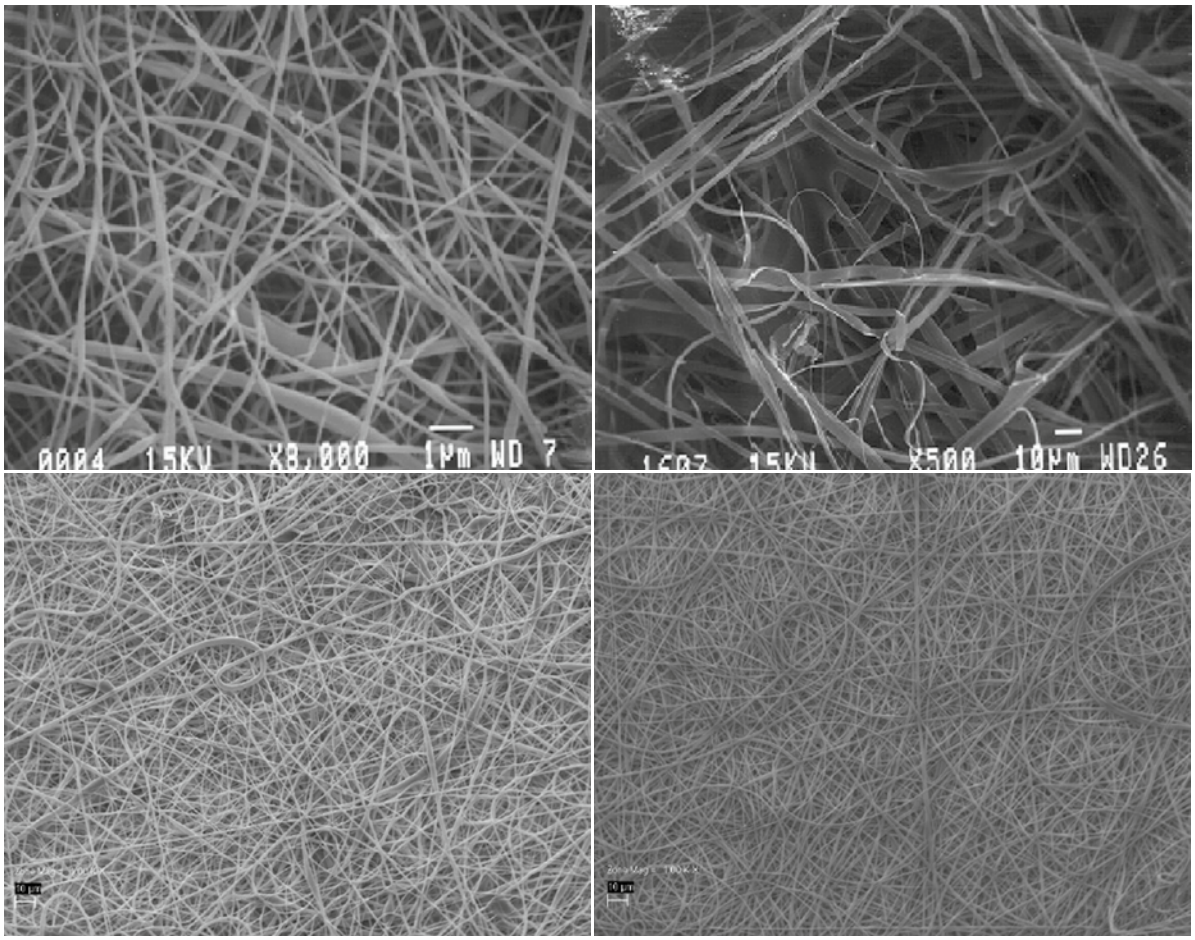

Fig. 3.8 SEM images of electrospun collagen type I (calf skin) (*left*, magnification 8,000×, left-middle, magnification 500×). Reprinted from [95], © Frontiers in Bioscience. Electrospun PDO (80 mg/mL) (right-middle, magnification 1,000×). Electrospun PLGA (80 mg/mL) (*right*, magnification 1,000×)

(TIPS) [8, 11, 24, 124]. In the TIPS process, the temperature of a polymer solution is cooled to induce phase separation, that is, to form a polymer solution into a polymer-rich component and another into a polymer-poor/solvent-rich component. After the solvent has been removed by processes such as extraction, evaporation, or sublimation, the polymer in the polymer-rich phase solidifies into the skeleton, and the spaces originally occupied by the solvent in the polymer-lean phase become the pores of the polymer foam. The pore morphology of the membrane can be altered depending on the polymer, solvent, concentration of the polymer solution, and the phase separation temperature. An additional advantage to this process is that scaffolds can be molded into a variety of shapes and sizes [19, 44, 46, 125].

There are two methods of TIPS; liquid–liquid phase separation, which has been illustrated to produce foams with the potential for drug delivery, and solid–liquid phase separation, which has been developed to enhance the mechanical properties of the scaffolds [11]. Further detail is given below for both methods.

Liquid–Liquid Phase Separation

When the crystallization temperature of the solvent is much lower than the phase separation temperature of an amorphous polymer solution, the thermally induced liquid–liquid phase separation takes place by cooling the polymer solution below an upper critical solution temperature. Figure 3.9 shows a binary phase diagram

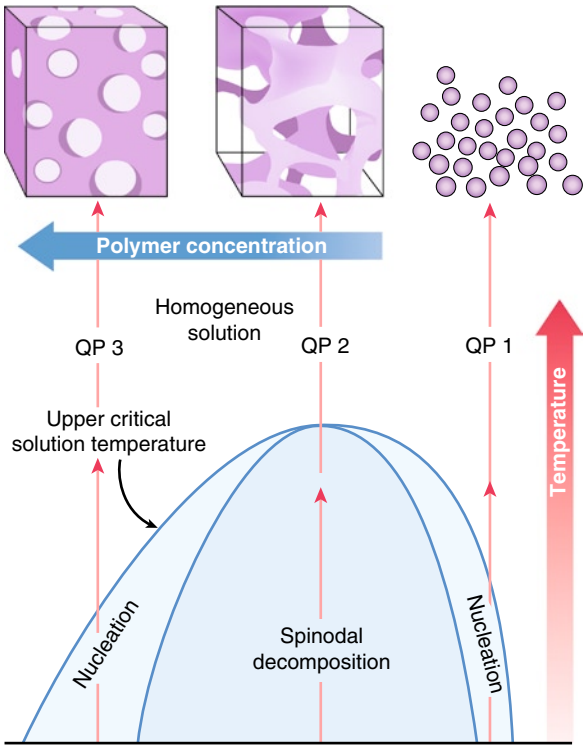

Fig. 3.9 Binary phase diagram with TIPS properties representing the three distinct morphological types. [44]

of the amorphous polymer solution system to determine the solvent/polymer relationship. The phase boundaries are shown as a function of temperature and composition and such regions provide three different morphologies.

In a polymer solution of very low concentration, when the temperature is lowered, the structure formation consists of a polymer-rich phase dispersed in the matrix of the polymer-lean phase (QP1). In this case, the powder-like polymer solid is obtained after the solvent has been removed and the result is a material without structural integrity.

When the composition-temperature point is in the metastable region of a solution with very high polymer concentration (QP3), droplets of the polymer-lean phase are formed in the matrix of the polymer-rich phase and results in foams with a closed-pore structure. This results in a noncell invasive material.

When the composition-temperature point is in the unstable region (QP2), a bicontinuous structure, where both the polymer-rich phase and polymer-lean phases are completely interconnected, will form because of

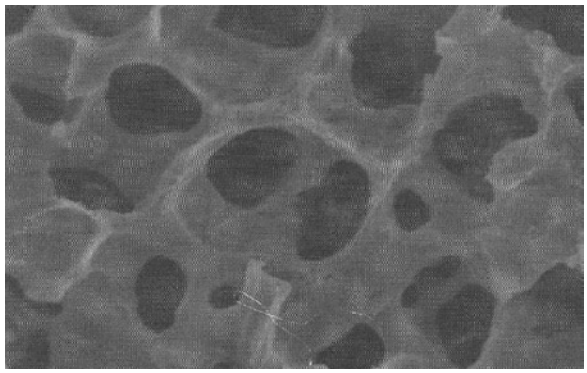

Fig. 3.10 SEM micrograph of a PLGA scaffold prepared with thermally induced liquid–liquid phase separation. Reprinted from [96]

the spinodal decomposition. Foams with a continuous pore network structure are obtained and are of interest to tissue engineering applications because they provide both mechanical integrity and interconnecting porosity for cell invasion.

Structural morphology of liquid–liquid phase separated scaffolds is influenced by quenching rates, crystallinity and vitrification of the polymer phase, and crystallization of the solvent. Scaffolds formed by this method include PLGA, PLLA, and PDLA in tetrahydrofuran (THF) or dioxanone/water and provide a relatively uniform pore distribution with porosities of up to 87% and diameters of 50–100 μm [11, 44, 125]. An example of this scaffold is illustrated in Fig. 3.10.

In addition to the advantages given above, the foremost advantage of the liquid–liquid method is the potential use of the scaffold as a delivery vehicle for bioactive molecules.

Solid–Liquid Phase Separation

Solid–liquid phase separation process occurs when the freezing point of the solvent is higher than the liquid–liquid phase separation temperature of the solution. When the temperature of the solution decreases, the solvent crystallizes and the polymer is expelled from the solvent crystallization front [44, 125]. The scaffold morphology reflects the solvent crystal structure and can be altered with the solvent used, the polymer concentration, the crystallization temperature, and the temperature gradient applied to the polymer solution. Many scaffolds have been formed using solid–liquid

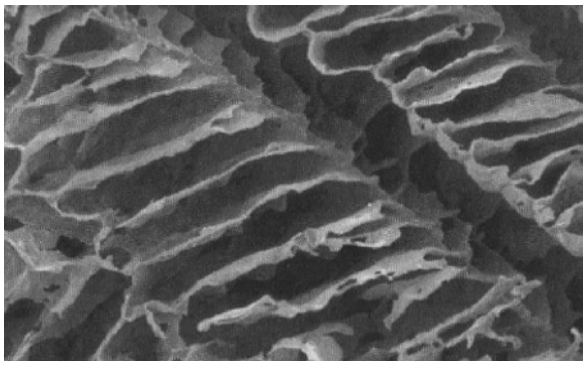

Fig. 3.11 SEM micrograph of a PLLA scaffold prepared with a thermally induced solid–liquid phase separation process. Reprinted from [96]

phase separation, including the most common PLLA, PLGA, PDLA, and hydroxyapatite powder and mixtures of this in dioxanone/water (Fig. 3.11). This technique creates scaffolds with pores having diameters as large as 600μm and a porosity of up to 95% [11]. One advantage of this method, as stated before, is increased mechanical strength, which can be of interest in bone tissue engineering applications.

Overall, using phase separation for the fabrication of tissue-engineered scaffolds provides advantages including simplicity compared to self-assembly (discussed below), and a low requirement of equipment. In addition, it is easy to achieve batch-to-batch consistency and controllable scaffold mechanical properties and architecture.

Both the methods use organic solvents that may have detrimental effects on the cells. This technique is also limited to only a select number of polymers [8, 46]. Also, for upper micrometer ranges, this process needs to be combined with salt leaching [125].

3.3.3.3 Self-Assembly

Self-assembly is the independent organization of individual components into functional structures and patterns with preprogrammed noncovalent bonds. Self-assembly processes are common throughout nature and technology, for example, nucleic acid synthesis, protein synthesis, energy transduction, weather systems, and crystals are all associated with this process [36, 37, 46, 112]. From an ECM analog perspective, this technique can be used to modify the chemical, physical, and biological properties of a scaffold to influence cell adhesion, differentiation, migration, and orientation [123]. This technique to create nanofibers has been accomplished for four kinds of polymer configurations: diblock copolymers, triblock copolymers, triblocks from peptide-amphiphile (PA), and dendrimers. The most common configuration for the production of nanoscale fibers is PA.

PA is a triblock polymer consisting of five key structural features: a long alkyl tail providing the hydrophobic driving force, a peptide region with four consecutive cysteine residues to create disulfide bonds for polymerization, the head group region (the C-terminal end) containing three glycine residues to provide flexibility, a phosphorylated serine residue that helps direct mineralization due to its interaction with calcium ions, and RGD, a cell adhesion ligand [46, 94]. Modifications to these regions have been made in a better attempt to explore the ways in which the molecule can be modified for use in other applications [36, 37, 123]. Specifically, Hartgerink et al. designed the standard PA configuration explained above for bone tissue engineering applications, and others have used this technique in nerve tissue engineering and cartilage repair [36, 37, 111].

This process creates nanofibers 5–8 nm in diameter and pores of around 5–200 nm, with an extremely high water content of 99.5%, or 1–5 mg/mL (Fig. 3.12). Because of these characteristics, these scaffolds have the potential for use specifically in three-dimensional cell culture. The porous structure mimics that of the native ECM and can allow for the molecules (growth factors and nutrients) to diffuse in and out slowly [123]. Other ways to induce self-assembly include acid-induced (addition of HCl in a sealed chamber), divalent ion-induced (adding Ca^2 to cause gelation), and simply dissolving PA in water at pH 8 and allowing it to dry on a surface [37].

In contrast to the advantages of this technique listed above, drawbacks include the complexity of the laboratory procedure, a limited availability of polymer configurations, and restriction as a large-scale tissue engineering option. Most three-dimensional scaffolds formed from this technique are of the hydrogel type, which presents many limitations, including mechanical strength and the rate of degradation [112]. However, to overcome this limitation, artificial amphiphilic protein scaffolds have been synthesized with over 200 amino acids [36].

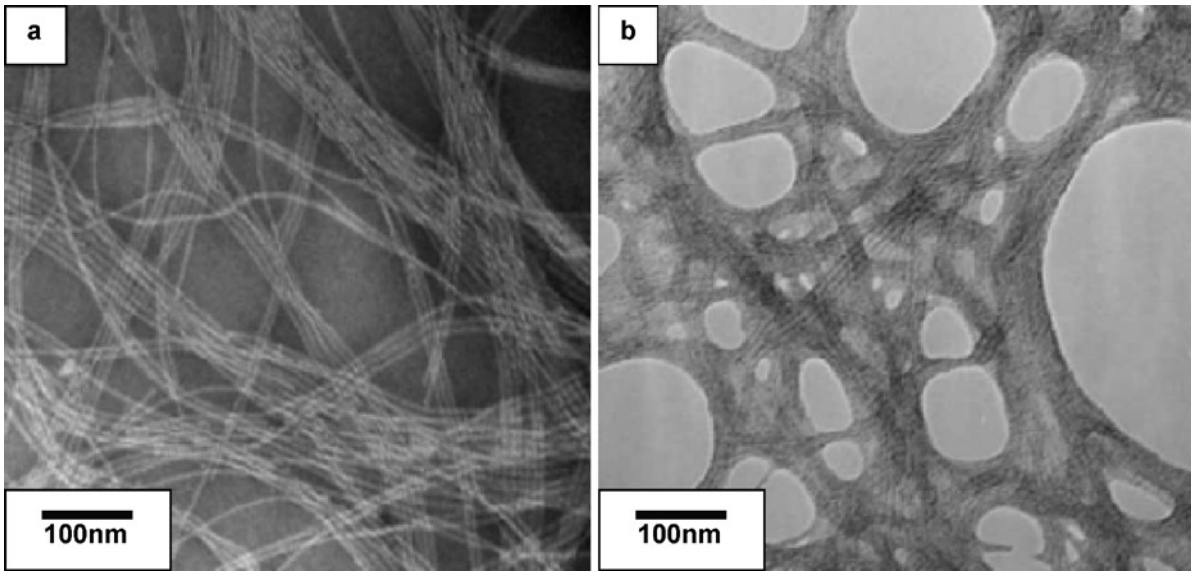

Fig. 3.12 TEM images of a PA molecule (**a**) self-assembled by drying onto a grid, and (**b**) self-assembled by mixing with $CaCl_2$. Reprinted from [36], © Elsevier

3.3.3.4 Leaching Techniques

Leaching techniques to produce porous scaffolds for tissue engineering applications are most commonly associated with solvent casting and particulate leaching and are based on the principle that porogens (NaCl, paraffin spheres, sugar crystals, or gelatin) are dispersed throughout a polymer solution and later dissolved by immersion, creating a highly porous, interconnected scaffold [11, 111]. Polymers such as PLLA, PLGA, and PEG have been used to produce pliable, highly porous scaffolds, with porosities of 90% and 500 μm diameter pores [11]. Riddle et al. demonstrated that scaffolds formed using smaller particulate sizes (in the range of 75–106 μm) result in scaffolds that allow for greater tissue infiltration *in vivo* because of their enhanced pore connectivity [83].

An advantage to this technique is that the pore size, porosity, molecular weight, and surface-to-volume ratio can be precisely controlled and readily manufactured [64, 83]. Limitations of this technique include the requirement of potentially toxic solvents, and rigorous processing conditions, which could potentially prevent the insertion of bioactive molecules [11].

3.3.3.5 Computer-Aided Design Techniques

CAD techniques include three-dimensional printing (3-DP), rapid prototyping, and SFF. 3-DP, which was developed at Massachusetts Institute of Technology, creates a solid-freeform object by ink-jet printing a binder into areas of sequential layers of powder (Fig. 3.13). A thin layer of powder is spread over the surface of a powder bed to create each layer. Each layer is precisely constructed by a CAD representation of the component. The powder bed is set on a piston which has the ability to descend when the powder is spread and each layer is printed (or the bed remains stationary and the ink jets and spreader are raised after the printing of each layer). This layer-by-layer process continues until the part is completed. When the ink droplet impresses on each layer of the powder, the binder solution joins single powder particles, and causes the adjacent powder aggregates to merge. Each layer will form a solid powder-based band, which will finally compile to form the full, solid 3D structure. The powder that is not bound supports the unconnected portions of the component while the structure is built, but is removed after the printing is completed by air jet flow [42, 43, 44, 111].

Similarly, rapid prototyping and SFF are techniques in which scaffolds are produced from a model taken

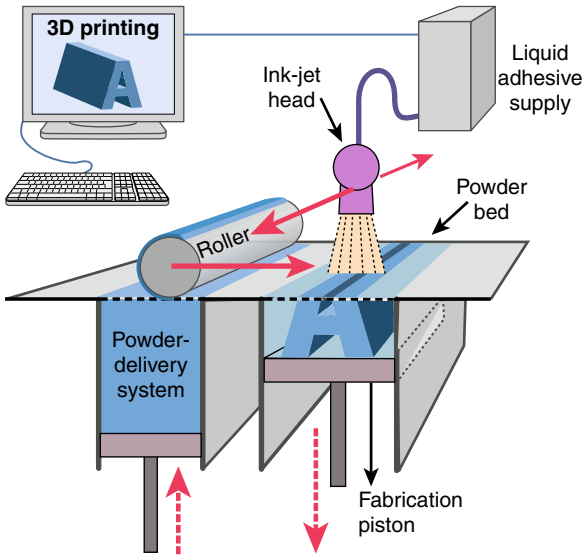

Fig. 3.13 Schematic of 3-DP setup. [44]

directly from a CAD data set. The specific structure is built up, layer-by-layer, from a selected material, and the process is lead by a computer program [111].

For these techniques, parameters such as porosity, interconnectivity, pore size, and geometric stability can be precisely controlled and present an advantage for tissue engineering applications. Furthermore, processing techniques where cells are printed on the surfaces have been accomplished, which implies that biological substances could be incorporated into the scaffold layers.

One limitation of this fabrication technique is timing. If a solvent that has a rapid drying rate is used, the printed component tends to warp. However, this factor can be eliminated if a solvent is chosen with a low vapor pressure. For example, PCL prepared with chloroform have undetectable amounts of warping, while PCL made with methylene chloride display significant amounts of warping. Combining solvents has also been shown to resolve the warping problem. Another drawback of this technique is that open pores must be present within the structure in order for the unbound powder to be removed to allow for a porous structure for tissue engineering applications. Also, the particle size of the powder used affects the thickness of each layer that is produced. The thickness of each layer ranges from 100–400 μm depending on the printer. Limitations also exist in the choice of materials available and the resolution obtained because of the small size of the scaffolds and imprecise machine tools used [44, 111].

3.4 Clinical Application

The use of ECM as a commercially available product has grown dramatically in recent years, with the majority of products consisting of decelullarized xenogenic ECM materials. SIS-based products such as Restore™, CuffPatch™, Surgisis®, Oasis®, and Durasis® are packaged as sheets and used for tissue reinforcement and wound repair. TEI Biosciences produces a number of products for specialized applications derived of fetal bovine skin. These include TissueMend® for rotator cuff repair, Durepair® for repair of cranial or spinal dura, Xenoform™ for gastrointestinal and urologic repair, SurgiMend™ for soft tissue membranes, and PriMatrix™ for general wound management. Horse and bovine pericardium-based products have been created and used for a number of different repair and reinforcement applications by Pegassus Biologicals (OrthADAPT™, and DurADAPT™) and Synovis Surgical (Veritas®, Dura-Guard®, Vascu-Guard®, and Peri-Guard®), respectively.

Allogenic decellularized ECM-based products have also made it to the market for a number of different applications. Alloderm is a human skin product that has been used in the abdominal wall, breast, and grafting applications. Graft Jacket® is a skin-based product used for foot ulcers, while Axis™ Dermis has been used for pelvic organ prolapse. Another allogenic product on the market is Suspend™, composed of decellularized human fascia lata and used as a urethral sling.

Currently, with the writing of this chapter, there are four cellular-based ECM products on the market. Smith & Nephew produces both Dermagraft™ and Transcyte™. Dermagraft™ is a full thickness diabetic ulcer graft composed of fibroblasts on a bioabsorbable ECM scaffold, while Transcyte™ is a graft for mid to intermediate partial thickness burns made from the ECM produced by human fibroblasts. Organogenesis, Inc. markets Apligraf®, composed of human fibroblasts on a matrix of collagen and secreted ECM, used to treat venous and diabetic foot ulcers. Finally, a product made from bovine collagen seeded with human fibroblasts, OrCel™ is manufactured by Ortec International to treat burn wounds [6].

To date, there are no engineered ECM analog products in clinical use. While there has been a large amount of funding and research committed to the development of such a product, engineered ECM products are currently limited strictly to the laboratory setting. There are a number of different limitations still to be overcome before such a product can reach the marketplace, and they will be discussed in a subsequent section.

3.5 Limitations/Critical View

As the engineering of ECM analog scaffolds is still in its infancy, there remain a large number of limitations, many of which are associated with the search to create an ideal scaffold. As stated in the previous section, the use of decellularized ECM has made it possible to create marketable products. However, these products are limited in their scope to patches and skin grafts. A SIS-based material would not be sufficient to support the level of biological complexity needed to engineer a whole tissue or organ. Moreover, the use of these mostly xenogenic materials brings with them the inherent risk of zoonotic disease transmission [6]. An engineered matrix will need to be created, be it natural or synthetic in origin, which can serve as an effective delivery vehicle for the cells or act as a catalyst for cellular remodeling. This will require a prevascularized construct capable of efficient mass transfer of nutrients over long distances. Currently, engineered ECM structures are limited in their overall thickness to the distance of passive diffusion to carry the nutrients to the cells [79]. In addition to the material limitations in matrix creation, there are fabrication limitations as well. Many of the fabrication techniques used to create synthetic ECM analogues in the laboratory currently require the use of toxic organic solvents, or would be nearly impossible to scale up, severely limiting their effectiveness in creating a mass producible product. As yet, no one has been able to mass produce an ideal scaffolding material that will exhibit the necessary mechanical strength and degradation time *in vivo*, while remaining bioactive.

Of course, scaffolds cellularized *in vitros* bring with them their own set of risks and limitations; chief among these are the problems associated with immunogenicity. Nearly, every differentiated cell line in the body will elicit an immune response. Even adult stem cells, nonimmunogenic in their undifferentiated state, will cause an immune reaction when they are fully differentiated. Depending on the intended usage time in the body, the degree of immunogenicity may or may not be acceptable. Additionally, allogenic cells have been shown to exhibit phenotypic and functional differences based on sex, age, and location in the body. They may not behave in the same way in a recipient as they did in their donor. To skirt the issues associated with allogenic cells, autologous cells are an option. However, the use of autologous cells no longer makes the product an off-the-shelf option, as it takes time to extract, culture, and seed a product prior to its usage. Ideally, a tissue-engineered product would need to be readily available as a cost and time-effective off-the-shelf option to warrant commercial success. The use of embryonic stem cells are an option to bypass these technical limitations, but bring with them their own set of moral and legal complications [76, 103].

As with the use of the embryonic stem cells, there are challenges and limitations outside the realm of basic science and engineering that must be addressed to ensure the success of ECM analog materials. The FDA, which must provide the approval for all the medical products prior to their clinical usage, categorizes products as either a device, biologic, drug, or blood based. In the past, this gross characterization severely limited as to which devices would readily gain approval to cell therapies (biologic) or acellular products (device). Combinations of the two, which have the potential for greater effectiveness, were often miscategorized. This inadvertently forced companies in need of getting a product on the market to produce simplified products, rather than complex combinations of cells, scaffolds, and growth factors, in hopes of gaining rapid approval. The creation of the Office of Combination Products as a coordinating body in 2002 was designed to assuage some of the difficulties associated with passing a complex combination ECM analog; however, this office does not have any authoritative power [76].

3.6 Expert Opinion

The basis of our being is a nanofabric, the ECM, which creates a complex microenvironment of structural elements, cells, and ground substance that act as the structural framework for each tissue and organ.

As tissue engineers, our ultimate end goal is to replicate, for repair and/or replacement, the damaged and diseased tissues and organs. In order to create such structures one must replicate the basis for each tissue or organ, the ECM, via some fabrication method. Unfortunately for the tissue engineer, each tissue and organ presents a unique challenge by possessing an ECM with an exclusive composition and organization. In order to be successful in tissue engineering, we must be able to replicate the ECM to provide the proper microenvironment for tissue regeneration and maintenance. Without proper cellular microenvironments, the best one can achieve will be the pathological tissue. Thus, as the field continues to move forward a major obstacle remains: Can we engineer an ECM analog for individual tissues or organs to allow for functional, yet marketable, tissue-engineered products to improve the quality of life? This is a daunting challenge and one that must be overcome to produce successful tissue-engineered products. It is this author's belief that through the use and refinement of the current nanoscale fabrication techniques, or a combination of these fabrication techniques, an acceptable ECM analog will be produced which will allow for the successful creation of tissue-engineered products for a number of different applications.

3.7 Five-Year Perspective

The past two decades have seen tissue engineering grow from a fledgling field, to a field filled with the promise of new commercially available ECM analogues entering the market. Tissue engineering has expanded from its infancy of simple cell injection treatments to complex bioactive scaffolds created from a marriage of cells and nanotechnology-based biomaterials. As tissue engineering has grown, our knowledge base in the fields of cell and molecular biology, polymers science, immunology, and tissue mechanics has grown symbiotically, spawning further collaboration and more multidisciplinary approaches, and creating the possibility for continued exponential growth. However, we have reached a point where the public eye needs to see tissue engineering as a viable, product producing field capable of improving their quality of life. The benchtop successes we have seen in the laboratory need to translate to the marketplace to ensure success and enhance the probability of achieving long-term goals, namely the creation of fully engineered tissues and organs.

Our understanding of cell behavior during development and the reparative process has increased substantially in recent years since the boom of stem cell science, and should continue to grow at a torrid pace. The discovery and continued rise in popularity of MSCs in tissues such as processed lipoaspirate (PLA), obtained through cosmetic liposuction procedures, will make the usage of adult stem cells more widespread and their incorporation into ECM analogues more prevalent. These cells have proven to be plentiful and are more readily extracted than MSCs from bone marrow, while exhibiting equal if not superior proliferation and differentiation ability. The nonimmunogenic nature of the adult stem cells will make it possible to create a cellularized off-the-shelf ECM analog product containing allogenic cells, rather than extracting autologous cells and seeding them on an acellular scaffold. This type of product would be very effective as a short-term aid to repair, but would run into immunogenicity issues once the stem cells became fully differentiated.

Another area of growth in the near future would be in the creation of vascularized ECM analogues. Currently, there are no prevascularized products on the market, which handcuffs the maximum thickness of an ECM construct to the limits of nutrient diffusion. This requires all the engineered scaffolding to be no more than a couple of millimeters thick, and hinders the creation of tissue constructs of substantial volume. To achieve the end goal of creating a completely engineered tissue or organ, it is necessary to create a structure that has conduits to supply nutrients to its entirety as opposed to limiting ourselves to the thicknesses allowable by diffusion. Current work in the field of computer-aided tissue engineering, such as 3-DP and rapid prototyping, has the ability to create intricately designed scaffolds with ever increasing precision. The creation of a scaffold, designed and constructed with computer accuracy, containing a highly organized network of cellularized conduits throughout to promote capillary formation, would enhance the ability of the nutrients to travel longer distances. Current research into the promotion of angiogenesis through the use of a number of different growth factors incorporated into ECM constructs, coupled with the use of ECs, stem cells, and macrophages to create

vascularized structures has proven to be promising and may prove to be the answer.

Finally, further refinement of the biomaterials and their fabrication techniques will drive tissue engineering down a path toward an increasingly ideal ECM analog construct. The ability of the polymer scientists to create novel polymers, with bulk properties (mechanical strength, thermal properties, degradation, etc.) tailored to specific tissue applications, will have the potential to enhance the overall effectiveness of engineered scaffolds. These polymers can also be combined with any number of growth factors and signaling molecules to improve their bioactivity and promote positive cellular interactions. Further collaborations between the polymer scientists and other members of the tissue engineering community will only benefit the creation of successfully engineered ECM.

3.8 Conclusion/Summary

Tissue engineering has made a profound impact on the quality of life for many, and with the field growing at a rapid rate, that number will continue to increase as more exciting and path breaking discoveries are made. Polymeric scaffolds, both synthetic and natural, used as ECM analogues have the potential to replace, and further regenerate new tissue by providing a suitable environment for the cells to be able to communicate through signals and function properly through attachment, differentiation, migration, and proliferation. Multiple fabrication techniques used to create ECM analogues design the structural characteristics needed to mimic the native ECM. These ideal ECM analogues will open the doors to many new clinical options for applications including bone, cartilage, cardiovascular, nerve, skin, ligament, tendon, breast, and liver. Although there are limitations that still stand in the way of having an ideal scaffold, the challenge of designing a successful ECM analog is slowly being conquered as professionals in the field continue to make significant improvements on a daily basis.

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Badylak SF, et al. Small intestinal submucosa as a large diameter vascular graft in the dog. J Surg Res. 1989;47(1):74-80.

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Zhang R, Ma PX. Synthetic nano-fibrillar extracellular matrices with predesigned macroporous architectures. J Biomed Mater Res. 2000;52(2):430-8.

The first publication on thermal phase separation created ECM analog scaffolds with a predesigned macroporous structure designed to enhance cellular penetration. The macroporous structure was created by the leaching of sugar and salt porogens.

Suggested Readings with Abstracts

Badylak S, Gilbert T, Myers-Irvin J. The extracellular matrix as a biologic scaffold for tissue engineering. In: van Blitterswijk J, et al., editors. Tissue engineering. London: Academic; 2008. p. 121-43.

This chapter contains information on the composition and organization of the native ECM, along with commercially available scaffolds composed of ECM.

Farach-Carson MC, Wagner RC, Kiick KL. Extracellular matrix: structure, function, and applications to tissue engineering. In: Fisher JP, Mikos AG, Bronzino JD, editors. Tissue engineering. Boca Raton: CRC; 2007. p. 3-1–22.

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Gomes M, et al. Natural polymers in tissue engineering applications. In: van Blitterswijk C, editor. Tissue engineering. San Diego: Elsevier; 2008. p. 145-92.

An overview of the origin, structure, and applications of natural polymers used in tissue engineering is given in this chapter. Advantages and disadvantages of natural polymers are discussed as well.

Hutmacher D, et al. Scaffold design and fabrication. In: van Blitterswijk C, editor. Tissue engineering. San Diego: Elsevier; 2008. p. 403-54.

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This book reviews the field of tissue engineering as a whole and is divided into parts labeled quantitative cell and tissue biology, cell and tissue characterization, engineering methods and design, and clinical implementation. The book discusses the challenges of the field and the requirement of the understanding of many subject areas including tissue dynamics, stem cell biology, cell communications, biomaterials, physicochemical-rate processes, and bioengineering design.

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Pluripotent Stem Cells: Sources and Characterization

4

Sean P. Palecek

4.1 Introduction

4.1.1 The Promise of Pluripotent Stem Cells in Tissue Engineering

Recent advances in deriving, isolating, and manipulating pluripotent human stem cells, including human embryonic stem (hES) cells and iPS cells, have reinvigorated the search for a novel source of safe, effective cells in tissue engineering and regenerative medicine applications. Pluripotent stem cells possess the unique combination of theoretically limitless self-renewal and pluripotency and the ability to differentiate to progenitors and terminal cells in each of the three embryonic germ layers, in a nontransformed cell. Thus, these pluripotent cells have the potential to supply researchers and clinicians with virtually limitless amounts of any cell type in the human body.

To date, human pluripotent stem cells have served primarily as an in vitro model system to study human developmental processes. These cells permit access to some of the earliest human developmental programs, which are difficult or impossible to study in vivo for technical or ethical reasons. For example, pluripotent stem cells may provide an in vitro system to understand the mechanisms of human germ cell development. Pluripotent stem cells also offer the potential to serve as in vitro disease models. Human iPS cells have been derived from patients suffering from a familial form of amytrophic lateral sclerosis (ALS) and inherited spinal muscular atrophy, and differentiated to motor neurons, the cell type destroyed in these two diseases [20,22]. Disease-specific iPS cells have also been generated from patients with adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne (DMD) and Becker muscular dystrophy (BMD), Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21, and the carrier state of Lesch-Nyhan syndrome [52].

Human pluripotent stem cells may serve as a source of human cells for in vitro cellular diagnostic and toxicology applications. Many differentiated human cells are not easily obtained, expanded, or maintained in culture (e.g., neural and cardiac tissues), and the ability to generate these cells from a progenitor would facilitate toxicology and high-throughput screening applications. For a review of the use of hES cells in drug screening, see [10]. Longer-term applications of human pluripotent stem cells include serving as a source of cells for tissue engineering and regenerative medicine applications.

4.1.2 Challenges Facing Implementing Human Pluripotent Stem Cells in Engineered Tissue

While the promise of human pluripotent stem cells is substantial, several substantial hurdles must be overcome prior to the successful implementation of pluripotent stem cell-derived tissues in the clinic. First, robust methods must be developed to guide differentiation of

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pluripotent stem cells to fully-functional differentiated cell types found in a therapeutically relevant tissue. The progenitor or differentiated cells must also self-assemble or be directed to the appropriate three-dimensional location in an engineered tissue where they can interact with one another and other cells and tissues at the implant site. In addition to generating and assembling the cells, significant questions remain about the molecular and functional characteristics of pluripotent stem cell-derived cells as compared to primary cells. The genetic stability of pluripotent stem cell lines must be assessed and maintained through extended culture and differentiation conditions. Undifferentiated cells must be removed prior to use in vivo to eliminate the risk of teratoma formation. Also, the immunological determinants and engraftment potential of allogeneic pluripotent stem cell-derived cells are not clear. Ethical issues regarding pluripotent stem cells, especially derivation of hES cell lines, must be resolved. Even with these substantial barriers to translation, the promise provided by pluripotent stem cells suggests they may be incorporated into engineered tissues in the coming years. This chapter describes advances in deriving, characterizing, and culturing human pluripotent stem cells with the goal of engineering medically relevant tissues.

4.2 Aim of the Discipline

The goal of the pluripotent stem cell field, as it relates to tissue engineering, is to provide a source of cells that will facilitate construction of fully-functional tissue replacements. To achieve this goal, substantial research into the basic nature of pluripotency is required. We need a better understanding of how to generate pluripotent cells that are suitable for transplantation. Cell safety, including a very low probability of transformation and teratoma formation, is an issue that must be addressed. Likewise, the source of cells should be immune-compatible with the patient. Effective methods of differentiation and incorporation into tissue constructs are also required. Many of these requirements are general problems that face the entire field of tissue engineering. This chapter will focus on the issues specific to pluripotent stem cells, including how we obtain, culture, and characterize these cells.

4.3 State of the Art

The hES cell and iPS cell lines currently available have permitted basic research into mechanisms of pluripotency and differentiation. This understanding has enabled development of improved methods to generate pluripotent stem cell lines. To facilitate further research and development of pluripotent stem cell-derived therapeutics, new pluripotent stem cell lines will be necessary. Recent advances in hES cell line derivation protocols have demonstrated that embryo destruction is not necessary, reducing ethical concerns regarding creation of new lines. Furthermore, demonstration that adult somatic cells may be reprogrammed to iPS cells may enable creation of genetically designed or patient-specific pluripotent stem cell lines suitable for research or clinical use.

4.3.1 Human Embryonic Stem Cell Derivation

Human embryonic stem cells were first derived by James Thomson in 1998 by harvest of the inner cell mass from blastocyst-stage embryos and subsequent culture on mouse embryonic fibroblast (MEF) feeder cells [74]. Blastocyst formation begins at day 5 post-fertilization and the blastocyst contains approximately 70–100 cells. The gold standard for hES cell line derivation remains harvest and culture of the entire inner cell mass of the blastocyst, although several studies have demonstrated that hES cell lines can also be derived from the earlier stage morula (day 3–4 post-fertilization; 16 cell stage) [70,92]. The rate of successful derivations is lower with morula-stage embryos than with blastocyst-stage embryos, and it is not yet clear whether significant differences in self-renewal or differentiation propensity exist between these lines. Embryo storage and quality prior to derivation attempts are important factors in the derivation success rates. Low morphological grade embryos that have arrested prior to the blastocyst stage do not efficiently yield hES cell lines, but 8.5% of poor quality embryos that reached the blastocyst stage were shown to yield hES cell lines [40].

4.3.1.1 Derivation of Human Embryonic Stem Cells Without Embryo Destruction

Human ES cell line derivation does not necessarily require embryo destruction. Multiple cell lines have been derived using single cell biopsies from morula-stage embryos [13]. The biopsied embryos were able to subsequently develop to the blastocyst stage in vitro. Although this method has not yet been used to derive an hES cell line for an embryo that implanted and formed an organism, the technique of harvesting the donor cell is similar to that used in preimplantation genetic diagnosis (PGD). This protocol is labor-intensive and thus is not likely to become a widespread method for generating new hES cell lines, but may provide an alternative derivation method that reduces the ethical concerns associated with embryo destruction.

Blastocysts can be generated from unfertilized oocytes by parthenogenesis, a process that uses a chemical or electrical stimulus to induce asexual development. These blastocysts are suitable for derivation of hES cell lines; parthenogenesis has been used to derive human HLA homozygous hES cell lines from HLA heterozygous donors, demonstrating that parthenogenesis may be suitable for generating and banking HLA-matched hES cell lines for regenerative therapies [58,59].

Somatic cell nuclear transfer (SCNT) has been used to derive ES cell lines from cloned embryos in numerous species, including primates [8]. Human blastocyst-stage embryos have been created by transferring the nucleus of an hES cell into an unfertilized human oocyte [69]. Transfer of adult fibroblast nuclei into human oocytes has also been used to generate cloned human blastocysts [27]. Human ES cell lines have not yet been derived from cloned blastocysts. While attempts have been made to use cloned blastocysts to generate hES cell lines, concerns regarding the ethics of such efforts will likely limit the use of this protocol for generating new lines even if success is demonstrated.

Fusion of existing hES cell lines with adult somatic cells, including human foreskin fibroblasts and hES cell-derived myeloid progenitors, has been used to generate novel pluripotent cell lines that express hES cell markers and exhibit DNA methylation patterns similar to those found in hES cells [15,71,90]. While this method may reduce immunological concerns regarding cell transplantation, the tetraploid nature of the resulting cells likely renders them unsuitable for clinical applications.

4.3.2 *Human Induced Pluripotent Stem Cell Derivation*

The ability to reprogram adult somatic cells to a pluripotent state offers tremendous potential for generating a source of patient-specific pluripotent stem cells. Human iPS cells do not pose the same ethical concerns regarding embryo destruction as hES cells, and cells differentiated from iPS cells will HLA match the donor.

In 2007, the Thomson and Yamanaka labs demonstrated reprogramming of embryonic and adult human fibroblasts by expression of four transcription factors. Takahashi et al. retrovirally transduced *OCT4*, *SOX2*, *KLF4*, and *C-MYC* into primary adult human dermal fibroblasts to induce pluripotency [72]. Yu et al. used lentiviral transduction of *OCT4*, *SOX2*, *NANOG*, and *LIN28* to reprogram IMR90 fetal fibroblasts and human foreskin fibroblasts to a pluripotent state [91]. These iPS cell lines express the hES cell markers SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, alkaline phosphatase, NANOG, OCT3/4, and SOX2. The iPS cell lines also possess other similarities to hES cell lines, including high telomerase activity, histone methylation patterns, embryoid body (EB) formation, directed differentiation to cells in each of the three germ layers, and teratoma formation in mice. When global gene expression patterns, determined by microarray analysis, were compared between iPS cell and hES cell lines, only minor differences were observed; in fact, these differences were no greater than the normal line-to-line variation observed between hES cell lines.

4.3.2.1 Pluripotency Reprogramming Factors

The first successful efforts to derive human iPS lines, each used four transcription factors. Takahashi et al. based their selection of *OCT4*, *SOX2*, *KLF4*, and *C-MYC* on factors that reprogrammed mouse somatic cells to murine iPS cells [46,50,73,81]. Yu et al. selected

OCT4, *SOX2*, *NANOG*, and *LIN28* based on the analysis of genes preferentially expressed in hES cells [91]. Each of these methods requires expression of *OCT4*, a homeodomain transcription factor in the POU family, and *SOX2*, an HMG box-containing transcription factor, to obtain pluripotent cells. *OCT4* expression is required for inner cell mass development and maintenance of a pluripotent state. *SOX2* is expressed in neural stem cells, extraembryonic ectoderm, and trophoblast progenitors in addition to ES cells.

Transduction with *NANOG* improved reprogramming efficiency, but was not necessary since *NANOG* expression is induced by other reprogramming factors [91]. *NANOG* is expressed in the inner cell mass, and loss of expression is generally correlated with differentiation. The *C-MYC* gene is also not required for reprogramming [48,54], removing some concerns regarding the effects of *C-MYC* expression on transformation of iPS and iPS-derived cells. However, reprogramming efficiency also declined when *C-MYC* induction was omitted [48]. The mechanistic role of *C-MYC* in reprogramming is not yet known. Table 4.1 lists factors that have been implicated in inducing or facilitating pluripotency during somatic cell reprogramming.

Table 4.1 Genetic factors involved in reprogramming somatic cells to a pluripotent state

Gene	Role
OCT4/POU5F1	Homeodomain transcription factor of POU family; required for the maintenance of cells in pluripotent state; expression appears to be required for reprogramming
SOX2	Transcription factor involved in pluripotent stem cell self-renewal. All successful human cell reprogramming reports to date have introduced Sox2
KLF4	Transcription factor involved in cell survival, proliferation, and differentiation. Expression is not required for reprogramming, but it enhances efficiency
C-MYC	Protooncogene encoding a transcription factor that regulates cell survival and proliferation. Expression is not required for reprogramming, but it enhances efficiency
NANOG	Transcription factor that regulates self-renewal and pluripotency
LIN28	RNA-binding protein. Blocks Let7 processing of micro-RNAs. Expression improves reprogramming efficiency
SV40LT	Simian virus 40 large T antigen. Involved in cell immortalization. Expression improves reprogramming efficiency
hTERT	Human telomerase reverse transcriptase. Maintains telomere lengths. Involved in cell immortalization. Expression improves reprogramming efficiency

4.3.2.2 Inducing Pluripotency Through Nonviral Methods

Viral integration can efficiently deliver transgenes to a target cell, but poses safety concerns while using the reprogrammed iPS cells in downstream applications. During iPS cell generation, the virally-transduced reprogramming transgenes are silenced and expression of the endogenous factors is initiated and maintained, leading to a stable, pluripotent cell [54,72,91]. However, transgene reactivation events and insertional mutagenesis of genes at integration sites pose substantial concerns.

The integrated reprogramming transgenes can be removed by Cre/LoxP recombination; this method was successfully used to derive human iPS cell lines from patients with Parkinson's disease [66]. All reprogramming factors can be introduced on a single polycistronic vector, which can be excised following reprogramming by Cre/LoxP [11]. Insertional mutagenesis, however, remains a concern with these methods. The piggyback (PB) transposon/transposase system can be used to reprogram human fibroblasts to pluripotent cells using inducible *OCT4*, *SOX2*, *KLF4*, and *C-MYC* [37,82]. Following reprogramming, the PB sequences may be removed by reexpression of the transposase.

Expressing reprogramming genes in the target cell using plasmids was shown to generate iPS cell lines that completely lacked vector and transgene sequences [89]. The low stable transfection efficiency of the plasmids reduced reprogramming efficiency, but expression of multiple reprogramming factors, including *OCT4*, *SOX2*, *NANOG*, *LIN28*, *C-MYC*, *KLF4*, and *SV40LT*, improved efficiency and led to stable iPS cell lines.

Selection of reprogrammed cells may also improve the efficiency of identifying iPS cell clones, even if reprogramming rates are low. Lentiviral vectors have been developed to aid isolation of iPS lines by

expressing EGFP and puromycin resistance genes under the control of Early Transposon promoters and *OCT4* and *SOX2* enhancers [33]. Nonviral selection systems are needed to complement nonviral reprogramming methods.

Small molecule inhibitors may supplement, or even potentially replace, expression of transgenes in reprogramming somatic cells to a pluripotent state. Valproic acid, a histone acetylase inhibitor, facilitates reprogramming of primary human fibroblasts with concomitant expression of just two genetic factors, *OCT4* and *SOX2* [34]. The requirement of *SOX2* expression can also be replaced with chemical inhibitors in reprogramming MEFs to iPS cells [63].

Direct protein transduction is another method to introduce reprogramming factors into cells. Zhou et al. reprogrammed murine embryonic fibroblasts to a pluripotent state using valproic acid and recombinant OCT4, SOX2, KLF4, and C-MYC proteins fused to polyarginine protein transduction domains [94]. The reprogramming efficiency was low – an OCT4-GFP reporter cell line was used to identify reprogrammed clones. While this method has not yet been demonstrated in human cell reprogramming, a similar approach is likely to be effective.

4.3.2.3 Somatic Cell Sources for Human-Induced Pluripotent Stem Cells

Since the first derivations of human iPS cells, several groups have shown that different types of somatic cells have the potential to be reprogrammed to a pluripotent state. Adult dermal fibroblasts, epidermal keratinocytes, and CD34+ blood cells have been used to generate human iPS cell lines [1,42,54,72,91]. Any of these cell types may be suitable for large-scale construction of iPS cell lines and banks since harvest of these cells from adults can be accomplished via noninvasive or minimally invasive medical procedures. It is not yet known whether the somatic cell type influences the efficiency of reprogramming. Assen et al. reported a higher reprogramming efficiency of human keratinocytes than human fibroblasts using *OCT4*, *SOX2*, *KLF4*, and *C-MYC* expression. The iPS cells derived from keratinocytes also contained fewer viral integrations than the fibroblast-derived iPS cells, suggesting a more efficient reprogramming process that generates iPS cell lines with less genomic damage [1].

While iPS cells appear similar to hES cells based on marker expression, gene expression profiling, and epigenetic analysis, the somatic cell used as a source may affect the differentiation potential of the iPS clone. As pluripotent cells, iPS lines should retain the ability to differentiate to any cell type in the adult, but the efficiency of differentiation may vary based on the somatic cell source, donor age, or reprogramming method.

4.3.3 Characterizing Pluripotent Human Stem Cells

One of the easiest ways to identify human pluripotent stem cells, including hES cells and iPS cells, is by their tightly-packed colony morphology (Fig. 4.1). The cells

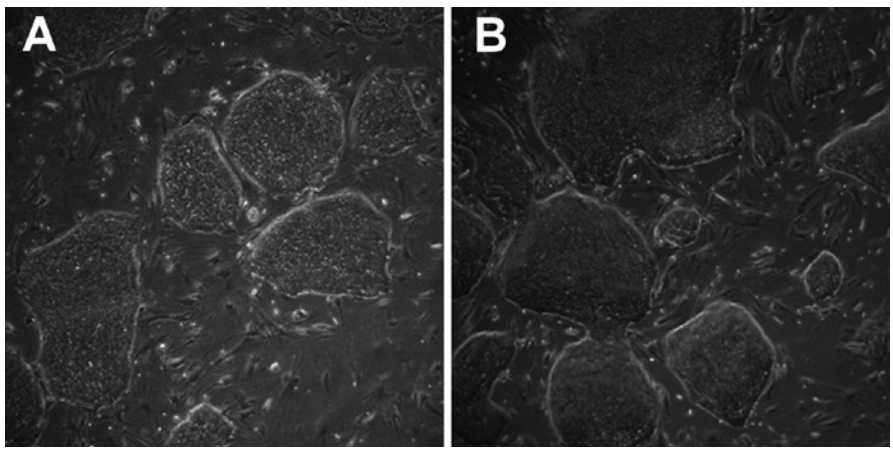

Fig. 4.1 Morphology of (**a**) hES cells and (**b**) iPS cells cultured on MEF feeder layers. The hES cells are line H9 and the iPS cells were generated from IMR90 fibroblasts by transduction with *OCT4*, *SOX2*, *NANOG*, and *LIN28* [91]. The undifferentiated cells grow in small, compact colonies with well-defined boundaries

are very small and have a high nucleus-to-cytoplasm ratio. The colonies generally have well-defined edges, and the cells may grow in a monolayer or pile up on the colony. Morphology is not sufficient to verify the two critical characteristics, pluripotency and extremely high self-renewal, of hES cells and iPS cells. Demonstration of these criteria with absolute certainty is infeasible, but several standard assays to suggest these properties are commonly employed.

4.3.3.1 Demonstrating Self-Renewal Potential

Pluripotent stem cells do not appear to undergo senescence, and therefore, can be maintained in culture for hundreds of doublings [9]. Quantifying telomere activity or monitoring telomere lengths provide evidence of high self-renewal capacity; hES cells have high telomerase activity and maintain telomere length as the cells divide [60,74]. During reprogramming to iPS cells, telomere elongation occurs as a result of increased telomerase activity, and telomeres in iPS cells exhibit similar methylation patterns and expression profiles as telomeres in hES cells [44]. Pluripotent stem cells maintain a lack of senescence in the absence of cell transformation, and thus should not exhibit hallmarks of transformation in conjunction with increased telomerase activity.

While telomere length remains relatively constant during long-term hES cell culture, chromosomal abnormalities are commonly acquired [5,21]. Relatively little is known about how these abnormalities arise or how they can be repressed. Presumably, current culture methods induce a selective pressure that favors these abnormalities, which may enhance proliferation rate or reduce spontaneous differentiation. Mechanical dissociation of colonies during passaging has been suggested to decrease the rate of chromosomal abnormalities as compared to chemical dissociation of colonies [47], but mechanical dissociation is much more labor-intensive. Monitoring karyotype of cells in culture (e.g., G band analysis) is an important aspect of pluripotent stem cell characterization, especially for cells to be used in clinical applications.

4.3.3.2 Demonstrating Pluripotency

The "gold standard" for demonstrating pluripotency in murine ESCs is injection of a labeled putative pluripotent cell into a blastocyst, then monitoring the label during embryonic development. The daughter cells of a pluripotent cell will appear in tissues representing each of the three germ layers. However, this analysis is not ethical to perform in humans, so alternative measures of pluripotency have been established.

4.3.3.3 Differentiation Potential

The ability of hES cells and iPS cells to generate differentiated cells in the ectoderm, endoderm, and mesoderm lineages can be established in vivo via teratoma formation in mice, or in vitro through EB formation or directed differentiation protocols. To form teratomas, a critical mass of undifferentiated cells is injected into immune-compromised mice in subdermal, intramuscular, intrakidney, or intratesticular loci. The presence of differentiated cells in ectoderm, endoderm, and mesoderm lineages is typically assessed by histology and immunocytochemistry [2,29]. Teratoma formation is considered to be the most rigorous assessment of pluripotency since certain nonembryonic cell types have been shown to differentiate to each of the three germ layers in vitro [39], but do form teratomas in mice [17,39]. Both hES cells and human iPS cell lines have been shown to be competent for teratoma formation [72,74,91].

In vitro pluripotency assessment most often involves EB formation, in which undifferentiated cells are cultured in suspension in the presence of serum. After several days to several weeks in suspension, the EBs are plated and maintained in serum-containing medium. Immunocytochemistry for lineage-specific markers is used to verify differentiation to ectoderm, endoderm, and mesoderm. As an alternative to EB formation, directed differentiation to cell types in each of the three main germ layers may be employed. For example, differentiation to neuronal lineages indicates ectoderm formation potential, to cardiac myocytes demonstrates the ability to form mesoderm, and to pancreatic islets shows potential to form endoderm. EB formation is a simpler assessment of pluripotency than directed differentiation, although directed differentiation absolutely demonstrates the ability to generate specific cell types of interest.

4.3.3.4 Marker Expression

Human ES cells and iPS cells express transcription factors and cell surface markers that suggest pluripotency

Table 4.2 Markers of undifferentiated hES cells and iPS cells

Protein	Localization
OCT4	Nucleus
NANOG	Nucleus
SOX2	Nucleus
SSEA3	Cell surface
SSEA4	Cell surface
TRA-1-60	Cell surface
TRA1-81	Cell surface
GCTM2	Cell surface
GCTM343	Cell surface
Alkaline phosphatase	Cell surface
Thy1	Cell surface
CD9	Cell surface

(Table 4.2). Expression of these markers can be quantified at the protein level on a population basis by Western blotting or on a cellular basis by flow cytometry. Immunocytochemistry provides a more qualitative assessment of expression of these markers. Gene expression can be measured by quantitative reverse-transcription polymerase chain reaction (RT-PCR) or by microarray studies. Expression of these markers is often used to measure the extent of differentiation, either desired or spontaneous, in a cell population, or to assess heterogeneity in the population (e.g., flow cytometry).

The transcription factors, OCT4, SOX2, and NANOG, are the primary regulators of the pluripotency program in pluripotent stem cells. Expression of these three factors is often used to support pluripotency, although expression of any one of these factors is not sufficient to demonstrate that a cell is in a pluripotent state. Figure 4.2 shows localization of OCT4 to the nuclei of hES cells and iPS cells. These factors induce activation of pluripotency promoters and/or repress expression of genes involved in cell differentiation.

Cell surface antigens commonly used as hES cell and iPS cell markers include SSEA3, SSEA4, TRA-1-60, TRA-1-81, GCTM2, GCTM343, Thy1, CD9, alkaline phosphatase, and HLA class 1 antigens. Multiple hES cell lines express these antigens [2], as do several iPS cell lines [72,91], although expression in iPS lines has not yet been well characterized. Expression of surface antigens is not as direct of a measure of pluripotency as expression of transcription factors involved in pluripotency, but surface antigens are more easily quantified by flow cytometry and permit sorting of live cells.

In 2007, the International Stem Cell Initiative (ISCI) published a comparison of global gene expression in 59 hES cell lines and between undifferentiated and differentiated hES cells. This effort suggested the following six genes, each of which exhibited specific expression in undifferentiated cells in every line, as a core set of markers that can define undifferentiated hES cells: *NANOG*, *TDGF*, *POU5F1* (*OCT4*), *GABRB3*, *GDF3*, and *DNMT3B* [2].

Global gene expression analysis (e.g., microarrays) is becoming a common method to compare similarities and differences between pluripotent stem cell lines. For example, differences in hES cell vs. iPS cell gene expression profiles are no greater than differences between distinct hES cell lines [72,91], suggesting strong genetic and functional similarity between these cell types.

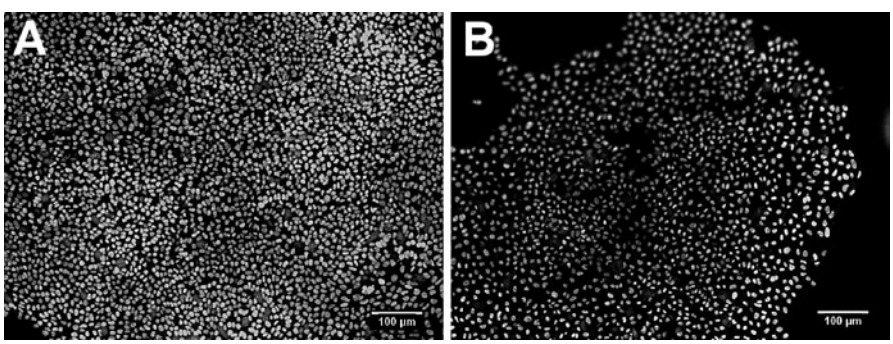

Fig. 4.2 OCT4 expression and localization in hES cells (**a**) and iPS cells (**b**) as determined by immunofluorescent staining of fixed cells. The hES cells are line H1 and the iPS cells were generated from IMR90 fibroblasts by transduction with *OCT4*, *SOX2*, *NANOG*, and *LIN28* [91]

4.3.3.5 Epigenetics

The use of epigenetic analysis to characterize and compare pluripotent stem cell lines is becoming more common. The 2007 ISCI study identified monoallelic expression of four paternally-expressed genes (*IPW*, *SNRPN*, *KCNQ1OT1*, and *PEG3*) in all hES lines [2]. The absence of X inactivation in female hES cell lines, followed by X activation upon differentiation, has been reported; however, this is not a general property of all hES cells [19,32].

Human iPS cell lines have similar epigenetic profiles as hES cell lines. Cytosine guanine dinucleotides (CpG) in the promoter regions of genes involved in pluripotency (e.g., *OCT4*, *NANOG*) are highly unmethylated in both hES cells and iPS cells [72]. Furthermore, histone H3 lysine 4 is characteristically methylated, while lysine 27 is demethylated at the promoter regions of pluripotency genes in both hES cells and iPS cells [51,72,91]. An analysis of 66,000 CpG sites found a greater level of cytosine methylation in pluripotent stem cells than in fibroblasts and a slightly higher amount of methylation in iPS cells than hES cells [18].

4.3.4 Pluripotent Stem Cell Culture

A key element of implementing human pluripotent stem cells in engineered tissues involves development of scalable, defined, and robust methods to expand and maintain the cells in vitro. The initial hES cell lines were cultured in undefined conditions, which contributed to heterogeneity in cell proliferation and differentiation states and would limit the ability to utilize these cells in clinical settings. Several defined culture systems exist and scalable culture systems are on the horizon. Culture systems developed and implemented for hES cells are also currently being used for iPS cells; to date, no differences in self-renewal or proliferation requirements are known between these cell types.

4.3.4.1 Feeder Cultures

The original hES lines were maintained in an undifferentiated state by coculture with primary MEF feeders [74]. An immortalized MEF line was developed to facilitate hES cell culture [53].

Concerns about xenogenic culture systems motivated the development of human feeder cells for hES culture. A wide variety of fibroblast feeders have proven to be effective, including cells from skin, muscle, placenta, and the uterus [67]. Fibroblasts generated directly from hES cells have been isolated and expanded to serve as feeder cells for hES culture [68], offering the advantage of an autogenic source of feeder cells. While many different cell types can serve as feeders, not all perform identically. For example, MEF cells secrete more activin A and less basic fibroblast growth factor (bFGF) than human foreskin fibroblasts [23]. Different lines or preparations of a particular feeder cell type can also be more effective than others, reducing reproducibility of culture.

4.3.4.2 Defined Culture Media

Direct contact with feeder cells is not necessary for the maintenance of hES cells. The cells can be expanded on Matrigel in MEF-conditioned medium [85]. Analyses of media conditioned by several types of fibroblasts have provided insight into the roles these feeder cells play in maintaining hES cells in a pluripotent state. One proteomic study identified 175 proteins enriched in medium conditioned by human fetal, human neonatal, or MEFs [56]. These factors include known pluripotency regulators such as Activin A, insulin-like growth factor-1 (IGF1), and transforming growth factor β1 (TGF-β1). Another proteomic analysis compared molecules secreted by MEFs that can support hES cell self-renewal with MEFs that cannot support hES cell expansion and identified a mixture of six proteins (pigment epithelium-derived factor, plasminogen activator inhibitor, insulin-like growth factor binding proteins 2 and 7, monocyte chemoattractant protein 1, and interleukin 6) that can replace the need for MEF-conditioned medium when culturing hES cells on fibronectin [12].

Basic fibroblast growth factor (bFGF, FGF2) is one of the primary factors added to culture media, both conditioned and defined, to enhance self-renewal and repress spontaneous differentiation [4,86,87]. At high concentrations of bFGF (~100 ng/mL or 6 nM), hES cells can be maintained in the pluripotent state in unconditioned medium [41]. This concentration is significantly higher than the K_d of bFGF binding to its receptor (<1 nM), possibly as a result of the low

stability of bFGF in culture or may instead permit signaling through lower affinity bFGF interactions [41].

Insulin-like growth factors (IGFs) have also been implicated in hES cell self-renewal. Treatment of feeder cells with bFGF stimulates secretion of IGF, which then enhances hES cell self-renewal [7]. Inhibition of the IGF1 receptor induces hES cell differentiation, while addition of an IGF1 analog in conjunction with an ERBB2 ligand, bFGF, and Activin A supports long-term expansion of undifferentiated hES cells [78].

The TGF-β/Activin/Nodal signaling pathway also plays a crucial role in regulating hES cell self-renewal by activating the SMAD2/3 pathway [6,36,61]. Combining Activin A with bFGF enhances self-renewal in the absence of feeder cells or feeder-conditioned medium [76]. Activin A induces expression of pluripotency regulators, including Oct4 and Nanog, as well as growth and differentiation factors, such as bFGF, FGF8, Nodal, and Wnt3 [77,84].

Inhibition of differentiation pathways, including those induced by bone morphogenetic proteins (BMPs), can also contribute to maintaining pluripotent cells in a self-renewing state. For example, Noggin, an antagonist of BMP4, reduces spontaneous differentiation in hES cells [79,87].

While growth and differentiation factors, proteins that bind cell surface receptors and initiate signal transduction, are crucial determinants of pluripotency, lipid molecules also regulate hES cell self-renewal. Sphingosine-1-phosphate (S1P), when added in combination with platelet-derived growth factor (PDGF), can suppress spontaneous differentiation of hES cells cocultured with MEF feeders [55]. Sphingolipid metabolites, including S1P and creamed, enhance hES cell proliferation, repress hES cell apoptosis, and may selectively promote apoptosis of certain differentiated cells [35,62,83]. Because of their hydrophobic nature, these lipids require a carrier such as albumin to remain soluble in culture media. Lipid-rich bovine serum albumin (BSA) was shown to stimulate hES cell self-renewal while lipid-poor BSA could not [28].

Pluripotent stem cells are passaged as small clumps, dissociated from larger colonies either mechanically or enzymatically. Single cells exhibit very low rates of survival, complicating passaging, genetic manipulation, and culture purification. However, clonal isolation of hES cells and iPS cells can be substantially enhanced by inhibiting Rho-associated kinase (ROCK) [14,80]. Similarly, clonal recovery can be enhanced by culturing cells under low oxygen conditions [26] or by adding neurotrophins and pleiotrophin, which inhibit apoptosis [57,65].

Clinical applications of pluripotent stem cells will require application of good manufacturing practices (GMP) during derivation, expansion, and differentiation [75]. Existing hES cell lines that have been derived and cultured in the presence of animal-derived feeder cells or animal serum have incorporated animal components, including the sialic acid Neu5Gc [45]. These lines appear to lose Neu5Gc, and potentially other animal derivatives, following extended culture in humanized conditions [31]. Thus, existing lines may be useful in clinical settings if they are demonstrated to be free of pathogens and infectious agents.

Allogeneic human fibroblasts, including hES cell-derived fibroblasts, provide a humanized option for feeder culture, although these culture systems remain undefined [67]. Human feeder cells have been used to derive novel, xeno-free hES lines, which can be maintained in conditioned medium containing human serum [24,64].

A completely defined culture medium (mTeSR) was shown to promote long-term self-renewal and hES cells and iPS cells [43,91]. This medium contains a combination of proteins and small molecules, including bFGF, TGF-β1, LiCl, GABA, and pipecolic acid. Pluripotent stem cells can be cultured in mTeSR on Matrigel, or alternatively on a humanized extracellular matrix containing human collagen IV, vitronectin, laminin, and fibronectin [43]. This system is also suitable for derivation of xeno-free hES cells [43]. Another defined, serum and feeder-free medium, STEMPRO, contains IGF1, bFGF, Activin A, HRG1-β, among other components [78].

4.4 Clinical Application

An established clinical application of human pluripotent stem cells does not yet exist. Geron has received US Food and Drug Administration (FDA) clearance for the first human clinical therapy using pluripotent stem cells. Human ES cell-derived oligodendrocyte progenitor cells (GRNOPC1) have improved motor skills in animal acute spinal injury models [38]. The safety and functionality of these cells in treating acute human spinal cord injuries will be investigated.

Even though the results of Geron's study are not known, the approval represents an important step for the fields of pluripotent stem cells and cell-based regenerative medicine. GMP production of hES cell-derived cells and product testing methods have been developed to enable this project. Presumably, these advances may be applied to other ES cell-derived therapies.

Before pluripotent stem cells are used in tissue engineering, the stem cells will provide a useful source of differentiated cells for drug screening and toxicology applications. Hepatic and cardiac toxicity are main characteristics leading to failure of drug candidates and are often not detected until very late in the drug development process. Animal models are not always accurate predictors of toxicity in humans, while human cell lines typically do not have fully-differentiated phenotypes. Methods to generate hepatocytes and cardiac myocytes from pluripotent stem cells have been developed [3,88,93] and may provide the large amounts of these cells required by the pharmaceutical and biotechnology fields.

4.5 Expert Opinion

4.5.1 Cell Source

The optimum source of pluripotent stem cells for tissue engineering applications remains unknown. ES cells have been much more extensively studied and may have a safety advantage since genetic modifications are not necessary. However, iPS cells are much more amenable to autologous transplantation since patient-derived cells could be reprogrammed and differentiated to somatic cells. Recent advances in nonviral reprogramming may ameliorate concerns regarding genetic modifications of reprogramming. Also, iPS cells pose fewer ethical apprehensions than hES cells, even though hES cell line derivation may not necessitate embryo destruction.

More studies of the similarities and differences between hES cell and iPS lines must be performed. With the limited information available today, no substantial distinctions appear evident. However, line-to-line variation has been observed in cell culture and differentiation experiments.

4.5.2 Cell Characterization

The human pluripotent stem cell field currently lacks a consensus set of markers to define, and perhaps sort, pluripotent cells. Ideally, a minimal list that provides a very high probability of pluripotency would become standardized in the field. The set of markers identified and proposed by ISCI is a good step toward identifying a pluripotent population. It is not clear whether or how heterogeneity in pluripotency marker expression affects differentiation to various somatic cell types. Methods to identify optimal clones for particular therapies may provide insight into how to best generate pluripotent cell lines for tissue engineering applications. Initially, such methods will likely rely on functional assays that utilize EB formation or directed differentiation. Correlating functional responses to molecular markers would facilitate clone selection.

4.5.3 Pluripotent Stem Cell Culture

The pluripotent stem cell field is moving toward defined culture conditions, although substantial research is still performed using feeder cells or feeder-conditioned medium. Defined culture conditions may reduce culture heterogeneity in research experiments and translational studies. Culture in xeno-free medium may also be an important component of preventing contamination of cells used to generate therapeutics. A completely defined, humanized culture system is not yet cost-effective for most research labs or development efforts, primarily because of a lack of effective extracellular matrices that support long-term pluripotent stem cell expansion in the current defined media. As defined culture systems improve, they should replace feeder culture as standard methods.

4.6 Five-Year Perspective

4.6.1 The Future of Pluripotent Stem Cell Derivation, Characterization, and Culture

Given that the human pluripotent stem cell field is barely a decade old, and human iPS cells have existed

for less than 2 years, predictions about the future of the field are likely to be inaccurate. The current trajectory, however, suggests the possibility of exciting developments. Based on recent advances in mouse cell reprogramming, we can expect to see methods of cell reprogramming that do not require genetic modification. Reprogramming factors may be RNA, proteins, or small molecules delivered to the cell. We should also see improvements in reprogramming efficiency as the roles of the individual factors become clearer. The field will identify how different sources of somatic cells respond to reprogramming. New sources of pluripotent stem cells may also be identified.

Improvements in cell characterization are also expected. In many ways, these will be motivated by moving pluripotent stem cells to clinical trials. Clear definitions of starting and final cell populations will be required for GMP protocols. Defined, humanized culture systems should also become more prevalent and cost-effective.

4.6.2 Applications of Pluripotent Stem Cells in Engineered Tissues

There are several criteria that may determine whether pluripotent stem cells are a feasible cell source for tissue engineering applications. First, appropriate demand for the particular tissue must exist. Second, primary sources of cells should be difficult to obtain or expand, necessitating an alternative cell source. Effective pharmacological or biological treatments for the disorder or disease should not be available. Protocols to differentiate and purify fully-functional cell types from the pluripotent stem cell source must be robust and efficient. Also, the engineered constructs must be able to be integrated into the body in a way that they survive and function appropriately.

In addition to neural system repair, which generally meets these criteria [16], several other tissues are candidates for pluripotent stem cell engineering applications. Pancreatic islets for the treatment of type I diabetes comprise one of the more promising clinical applications of pluripotent stem cells. A drastic shortage of islets exists to meet the current demand, and constructs can easily integrate with the blood supply, but obtaining cells that are able to appropriately regulate glucose levels has been challenging [25]. Cardiac myocytes are another example of cells that are in high demand. Obtaining fully functional cardiac cells from pluripotent stem cells and integrating them into the heart will, however, be difficult [49]. Pluripotent stem cells may also provide a safer, more reliable alternative for engineering tissues for which primary cells are available, including skin and vascular grafts [30].

4.7 Limitations

There are many technical issues that impede the translation of pluripotent stem cell research to clinical therapies. Safety and immunogenicity issues have already been discussed in this chapter. Pluripotent stem cells have the ability to cause teratomas in vivo, so methods to ensure products contain no undifferentiated cells will be critical. Expense and ease of implementation will also limit applications of engineered tissues containing pluripotent stem cell derivatives. Creating these products will be extremely time- and labor-intensive.

It is important to keep in mind that pluripotent stem cells offer real and substantial advantages over primary differentiated cells or adult progenitor cells, but that many technological hurdles must be overcome before pluripotent stem cells are incorporated into engineered tissues for use in patients. As in the adult stem cell and tissue engineering fields, we can expect setbacks in early clinical studies. However, each study will provide valuable information about pluripotent cells and their use in tissue engineering so that the promise of these cells will eventually be realized.

4.8 Conclusion

The first decade of human pluripotent stem cell research has generated substantial advances in deriving embryonic and iPS cells, culturing pluripotent stem cells, and characterizing the mechanisms of self-renewal and pluripotency regulation in these cells. Pluripotent stem cells provide a promising supply of cells for regenerative medicine and tissue engineering, but significant technological barriers remain before this potential can be realized in a clinical application. Methods to generate patient-specific pluripotent stem cells must be made safer and more efficient. In particular, nonviral

reprogramming of adult cells to iPS cells via plasmid, proteins, or small molecules appears to be particularly promising. While tremendous strides have been made toward developing defined, humanized culture systems, the components of these systems are expensive. Finally, robust systems to expand, culture, and monitor pluripotent cell populations must be engineered to generate sufficient number of cells with the desired characteristics for clinical trials or therapies.

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Adult Stem Cells: Sources and Characterization

5

Hitoshi Okochi

5.1 Introduction

In regenerative medicine, when damage is too severe for the body to heal or replace the injured organ or tissue, it should be replaced by transplantation. Owing to the progress of immunosuppressive agents, organs such as the kidney and heart are being transplanted all over the world. However, the supply of donors is far less than demands for tissues and organs. Two goals of cell-based therapy are to utilize stem cells, to solve the problems of donor deficiency and, further, to avoid rejection reactions after transplantation by using the recipient's own cells.

Research on adult stem cells is progressing rapidly. Unlike human embryonic stem cells (ES cells), adult stem cells have no ethical problems. However, there are technical issues. The number of the stem cells in each tissue is generally so small that they require culture and expansion in vitro for the clinical use, because there is a critical difference between ES cells and adult stem cells in terms of cell growth. The default standard of the cell cycle is active in ES cells, but it is quiescent in most types of adult stem cells. Some of the adult stem/progenitor cells found in bone marrow, skin, and gastrointestinal tract undergo a rapid turnover to replenish any cell loss, but others remain quiescent and rarely divide under normal conditions. Any tissue regeneration mediated via adult stem/progenitor cells is usually accompanied by environmental changes in the niche and orchestrated by several growth factor and cytokine-initiated cascades. The inherent difficulties involved in expanding adult stem cells create a major obstacle for clinical application. One specific problem is the fact that culturing adult stem cells requires specific conditions and the current conditions are not yet satisfactory.

Until recently, it was thought that differentiated cells were irreversible to an immature state because their genetic programs had been modified by epigenetic changes such as the methylation of DNA and the acethylation of histone proteins. However, the generation of a cloned sheep, Dolly, clearly demonstrated that the nucleus of a differentiated cell can be reprogrammed by somatic nuclear transfer into an unfertilized egg [6]. Furthermore, cells have been reprogrammed by molecular biological techniques. Dr. Shinya Yamanaka et al. first reported that retroviral-mediated introduction of four transcription factors was able to dedifferentiate mouse fibroblasts into ES-like pluripotent cells [46]; they named these cells iPS cells (induced pluripotent stem cells). Soon afterwards, they succeeded in getting similar results with human cells [45]. Although these human iPS cells have almost the same potential as ES cells, they do not involve the ethical dilemma of destroying human embryos. This new technology that can generate patient-specific iPS cells to treat various types of disease has been accepted with enthusiasm, and it has the potential to become so powerful that it will have great impacts on both stem cell research and regenerative medicine.

In this chapter, the basic characteristics of adult stem cells are described. Among the various types of stem cells, the focus will be on mesenchymal stem cell (MSC) and iPS cells because of their close relationships to clinical applications.

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5.2 Aim of the Discipline

In regenerative medicine, cell-replacement therapy is considered promising, because stem cells are key players in tissue repair. Exogenous application of specific growth factors and cytokines is also effective in stimulating the resident adult stem cells in damaged areas. The expectation after transplantation of stem cells is that they will participate in tissue regeneration not only by integrating themselves but also by secreting various growth factors and cytokines that restore the endogenous tissue regeneration program. Therefore, a thorough understanding of adult stem cells is essential for selection of the proper cell source for specific regenerative problems. Other goals include defining the functional and molecular markers that will enhance the reproducibility of results and shortening the length of preclinical studies. It is also important to determine the relative risks of various cell culture techniques.

Elucidating the mechanisms of micro-RNA (miRNA) and the epigenetic states of adult stem cells will help us maintain cells in an immature state and control the microenvironment supported by the niche.

5.3 State of the Art

5.3.1 Micro-RNA (miRNA)

miRNA is a class of small noncoding RNA segments consisting of 20–22 nucleotides. The discovery of RNA-silencing phenomena that are mediated by miRNAs has unveiled the ability of RNA to impact an unanticipated variety of biological processes through posttranscriptional modulation of gene expression. They are involved in many cellular functions and play important roles in the determination of cell fates [44]. Recently, miRNAs have emerged as areas of intense interest for the maintenance of stemness [42] and have been recognized as differentiation signatures in many stem cell types [43]. For example, MSCs have discrete miRNA expression profiles that may indicate such intrinsic stem cell properties as quiescence, self-renewal, differentiation, and pluripotency; such signatures can be used as reliable molecular markers specific to MSCs [48].

5.3.2 Aging

It is generally accepted that the number of stem cells in a human decreases with increasing age. When grouped by decade, dramatic decreases in MSCs per nucleated bone marrow cell have been observed, with a tenfold decrease from birth to teens and another tenfold decrease from teens to the elderly. These decreases paralleled observed fracture healing rates: very rapid in the young and very slow in older patients [7]. On the other hand, several studies have argued that the stem cell number may not decline with increasing age, but their functional capacity has deteriorated in aging individuals [4]. As an example, dramatic functional and phenotypic changes occur in hematopoietic stem cells (HSC) with respect to age. HSC may play a role in immunosenescence through cell-fate decisions leading to an overproduction of myeloid cells and an underproduction of lymphocytes [8]. In addition, an age-related loss of DNA damage repair pathways may pose a significant threat to stem cell survival and longevity. Normal stem cells appear to have strict control of gene expression and DNA replication, but stem cells with loss of DNA repair may have altered patterns of proliferation, quiescence, and differentiation [26]. One recent paper reported the age-related loss of local platelet-derived growth factor signals that promote the induction of cardiac myocytes from Oct3/4+ bone marrow stem cells [15].

5.3.3 iPS Cells (Induced Pluripotent Stem Cells)

iPS cells are somatic cells that have been reprogrammed to revert to an ES cell-like pluripotent state. The introduction of four transcription factors – Oct3/4 (octamer-binding transcription factor 3/4), Sox2 (SRY-related high-mobility–group -box protein 2), Klf4 (Kruppel-like factor-4), and c-Myc turned mouse fibroblasts into ES-like pluripotent cells [46]. More recently, human iPS cells have been developed from adult fibroblasts by the same group [45]. At the same time, another group reported that they had succeeded in obtaining iPS cells from human neonatal foreskin fibroblasts by using a different set of genes (Oct3/4, Sox2, Nanog, Lin28) [51]. Hanna et al. demonstrated a new therapeutic strategy in a mice model in which

genetic disease could be treated by modifying patient-specific iPS cells [20]. First, they established iPS cells from a mouse model of sickle cell anemia and replaced the defective gene using homologous recombination. These cells were then differentiated into hematopoietic cells and transplanted to treat the sickle cell mouse.

Many doctors have thought that the use of a retrovirus for establishing human iPS cells created significant regulatory hurdles for therapeutic applications because several patients have suffered from leukemia after receiving gene therapy involving the retrovirus for the X-linked severe combined immunodeficiency (X-linked SCID) [39]. Moreover, because c-Myc is known as an oncogene, its usage also raised concerns about promoting tumor formation. However, c-Myc is not essential for producing human iPS cells; it can be avoided, although the efficiency of derivation decreases [33]. iPS cells have recently been established not only from skin fibroblasts but also from adult mouse liver and stomach cells [2]. Efficient and rapid generation of iPS cells from human keratinocytes is also reported [1]. Encouragingly, iPS cells have been generated from patients with ALS (amyotrophic lateral sclerosis) that could differentiate into motor neurons [14]. It has also been shown that the reprogramming of neural progenitor cells into iPS cells does not require exogenous Sox2 expression [16].

It is conceivable that immature cells are easier to reprogram than mature cells. However, it was recently reported that terminally differentiated mature B cells can be reprogrammed [19]. In addition, Huangfu et al. reported new methods for the induction of pluripotent stem cells from primary human fibroblasts using only Oct4 and Sox2 [21]. Additionally, Yamanaka's group has reported that they were able to generate iPS cells without viral vectors, indicating that integration of the exogenous gene into the chromosomes was unnecessary [36]. This method improves future prospects because we do not have to consider the risks of retrovirus, which improves the chances for clinical application.

Patient-derived iPS cells are expected to contribute both to treating the patient and to drug screening for the disease. Table 5.1 summarizes the remarkable progress in iPS that is expected to continue to produce fruitful results.

Table 5.1 Current methods for establishing induced pluripotent stem (iPS) cells

Reference	Transcription factors	Species	Cell type	Vector
Takahashi and Yamanaka [46]	**Oct3/4, Sox2, Klf4, c-Myc**	Mouse	Fibroblasts	Virus
Takahashi et al. [45]	Oct3/4, Sox2, Klf4, c-Myc	**Human**	**Adult** skin fibroblasts	Virus
Yu et al. [51]	Oct3/4, Sox2, **Nanog, Lin28**	**Human**	Newborn foreskin fibroblasts	Virus
Hanna et al. [20]	Oct3/4, Sox2, Klf4, c-Myc	Mouse	Fibroblasts from humanized knock-in model of **sickle cell anemia**	Virus
Nakagawa et al. [33]	**Oct3/4, Sox2, Klf4**	**Human**	Fibroblasts	Virus
Eminli et al. [16]	**Oct3/4, Klf4, c-Myc**	Mouse	**Neural progenitor cells** from neonatal brain	Virus
Huangfu et al. [21]	**Oct3/4, Sox2**	**Human**	Fibroblasts	Virus
Aoi et al. [2]	Oct3/4, Sox2, Klf4, c-Myc	Mouse	**Hepatocytes and gastric epithelial cells**	Virus
Aasen et al. [1]	Oct3/4, Sox2, Klf4, c-Myc	**Human**	**Keratinocytes**	Virus
Dimos et al. [13]	Oct3/4, Sox2, Klf4, c-Myc	**Human**	Fibroblasts from **amyotrophic lateral sclerosis (ALS) patient**	Virus
Hanna et al. [19]	Oct3/4, Sox2, Klf4, c-Myc, **C/EBPα**	Mouse	**B lymphocytes**	Virus
Okita et al. [36]	Oct3/4, Sox2, Klf4, c-Myc	Mouse	Embryonic fibroblasts	**Plasmid**

New findings are emphasized in bold

5.4 Stem Cells and Clinical Applications

Every tissue has its own stem cells. Even in the heart and brain, long thought to lack stem cells, they surely exist. Among tissue stem cells, the first ones transplanted were bone marrow cells about 50 years ago. In the 1970s, the culture methods for keratinocytes were developed, and cultured keratinocyte sheets were grafted onto severely burned patients. The FDA approved the first tissue-engineered skin for diabetic ulcers in 1998 [50]. Although HSCs are well characterized and widely used in bone marrow transplantation, MSCs from bone marrow have also been reported to be multipotent and play an important role in supporting the maintenance of HSCs. In addition, autologous transplantation of bone marrow cells was reported to be effective in inducing therapeutic angiogenesis in patients with limb ischemia [47]. These results indicate that bone marrow-derived stem cells, adipose-derived stem cell (ADSC), and muscle-derived stem cells (MDSCs) may all contribute to the repair of an injured cardiovascular system via multiple molecular mechanisms [32].

5.4.1 HSC (Hematopoietic Stem Cells)

HSCs play critical roles in normal bone marrow; they continually renew all of the new mature and differentiated hematopoietic cells in the peripheral circulation, including the leucocyte, erythrocyte, and thrombocyte lineages. The most immature and quiescent multipotent HSCs are characterized by their expression of biomarkers including CD34− or CD34+/CD38−/low/Thy1+/CD90+/ C-kit−/low/Lin−/CD133+/ vascular endothelial growth factor receptor 2 (VEGFR2+). They are colocalized with the osteoblasts in a specialized niche within the bone marrow designated as the endosteum [32].

Bone marrow banking systems have been established worldwide and contribute largely as donor cell supplies for bone marrow transplantation.

5.4.2 Umbilical Cord Blood (UCB)

Human umbilical cord blood (UCB) contains the same kinds of hematopoietic stem and progenitor cells as bone marrow. It also contains a plastic adherent fibroblastoid population expressing characteristics similar to those of bone marrow-derived MSC [5]. UCB has already been used extensively for HSC therapy. Allogenic UCB transplantations have effectively treated leukemia, hematological diseases, and inherited metabolic diseases; UCB banking systems have also been established worldwide.

5.4.3 MSC (Mesenchymal Stem Cell, Multipotent Stromal Cell)

Recently, MSCs have been reported to reside in the connective tissue of many organs. A large number of publications have revealed that they have great plasticity. According to the International Society for Cellular Therapy (ISCT), an MSC is defined by the following criteria [13]:

1. Its property of adherence to plastic
2. Its phenotype: CD14− or CD11b−, CD19− or CD79α−, CD34−, CD45−, HLA-DR−, CD73+, CD90+, CD105+
3. Its capacity to differentiate into any of three lineages: chondrocyte, osteoblast, or adipocyte

Although the MSCs are defined by their capacity to differentiate toward these three cell lineages, they display an even broader differentiation potential. Thus, MSCs are also characterized by their potential to differentiate into myocytes, tendinocytes, ligamentocytes, cardiomyocytes, neuronal cells, and other cell types [27].

The functions of bioactive molecules secreted by MSC are categorized as follows:

(a) They inhibit apoptosis and limit the field of damage or injury
(b) They inhibit fibrosis or scarring at sites of injury
(c) They stimulate angiogenesis and bring in a new blood supply
(d) They stimulate the mitosis of tissue-specific and tissue-intrinsic progenitors, such as cardiac or neural stem cells

Infused MSCs also secrete immunomodulatory agents that turn off T cell surveillance and chronic inflammatory processes. Therefore, allogeneic MSCs can be therapeutically effective [7].

5.4.3.1 Adipose-Derived Stem Cell (ADSC)

The stromal immature cell population, alternatively called the stromal vascular fraction or preadipocytes, can be easily isolated from human lipoaspirates and proven to be multipotent [53]. These cells are now named ADSC or adipose-derived MSC. ADSCs are equally able to differentiate into cells and tissues of mesodermal origin, such as adipocytes, cartilage, bone, and skeletal muscle, as are bone marrow-derived human MSCs [40]. They express CD29, CD44, CD71, CD90, CD105/SH2, and SH3 [52]. Neuronal cells were differentiated from ADSC [12], and differentiation toward hepatocytes was also reported [3]. Subcutaneous adipose tissue is one of the most attractive sources of adult stem cells, because they are highly accessible and the procedure to obtain them is minimally invasive (Fig. 5.1). Both isolation and culture techniques are easy to perform. However, the molecular key events and transcription factors that initially allocate the ADSC to a nonmesodermal lineage remain almost completely unknown. Decoding these molecular mechanisms is of great interest for more effective development of novel cell therapies [40].

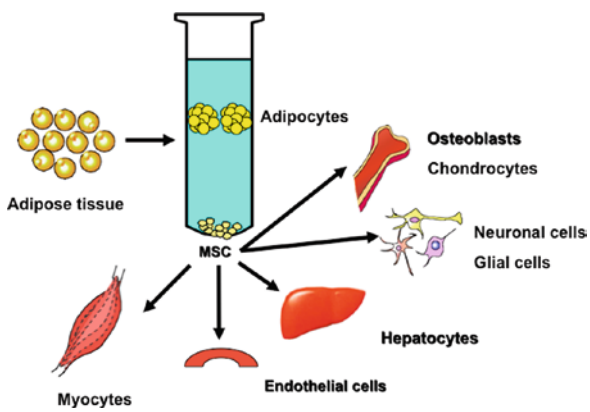

Fig. 5.1 Isolation method and multipotency of adipose-derived stem cell (ADSC). ADSC or adipose-derived mesenchymal stem cell (MSC) is easily concentrated by centrifugation after collagenase treatment because they are heavier than mature adipocytes. ADSCs are able to differentiate into cells and tissues of mesodermal origin, such as endothelial cells, cartilage, bone, and skeletal muscle. They also differentiate into neuronal cells and hepatocytes

5.4.4 Stem Cells in the Epidermis and Hair Follicle

In 2001, stem cells in the bulge area of hair follicles were shown to become epidermal keratinocytes, sebaceous glands, and follicular epithelial cells [37]. Normally, they are destined to become follicular epithelial cells in a steady state. However, they behave differently in cases of emergency such as wounds. At wound sites, stem cells in the bulge have been shown to migrate toward the damaged epidermis to cover the wound but to reside only transiently [23]. One of the key molecules in determining the stem cells' fate is beta-catenin, which drives them to differentiate into follicular epithelial cells [22]. Using K14deltaNbeta-cateninER transgenic mice, short-term, low-level beta-catenin activation stimulates de novo hair follicle formation from sebaceous glands and interfollicular epidermis [41]. New follicles induced in the interfollicular epidermis derive from that cellular compartment, not through bulge stem cell migration. Another report demonstrated that transgenic mice expressing an activated beta-catenin are predisposed to developing skin tumors resembling pilomatricomas. In fact, 75% of this type of human tumor possesses mutations affecting the amino-terminal segment of beta-catenin [9]. Furthermore, the involvement of increased beta-catenin signaling has been reported in malignant human squamous cell carcinomas [31]. Beta-catenin signaling was shown to be essential in sustaining the cancer stem cell phenotype; ablation of the beta-catenin gene resulted in the loss of cancer stem cells and complete tumor regression [31].

On the other hand, after large full-thickness skin wounds in mice, de novo hair follicles developed in the middle of the scar tissue [24]. This is a surprising report, because it is generally accepted that new hair formation never happens in full-thickness skin wounds in adults due to the lack of responsible cells. Ito's data suggested that cells can repeat the developmental process once again in an adult in cases of emergency. If so, it may be possible to improve regeneration capacity if we mimic the embryonic environment.

5.4.5 Muscle-Derived Stem Cells

Adult skeletal muscles contain two distinct stem/progenitor cells, MDSCs and the satellite cell population.

Muscle-committed satellite cells expressing markers, such as M-cadherin, myogenic factor 5 (MYF5), paired box gene 7 (PAX7) transcription factors, and neural cell adhesion molecule-1, are quiescent progenitor cells located at the periphery of skeletal myofibers under homeostatic conditions [38].

5.4.6 Cardiac Stem/Progenitor Cells

The heart muscle is comprised of cardiac involuntary striated muscle cells, also called cardiomyocytes. Cell renewal in the adult myocardium may be accomplished via the activation of cardiac stem/progenitor cells found within specialized niches localized at the apex and atria of the heart [32]. Under both physiological and pathological conditions, these cardiac stem cells are able to give rise to the three major cell types constituting the myocardium, cardiomyocytes, smooth muscle, and vascular endothelial cells.

5.4.7 Neural Stem Cells

The adult central nervous system has been long considered to be nonrenewable tissue. However recent evidence has clearly shown that neurogenesis does occur in the adult brain. Neural stem cells were found within two specific regions designated as the lateral subventricular zone of the lateral ventricle in the forebrain and the dentate gyrus subgranular zone in hippocampus [32]. Neural stem cells in the subventricular zone can give rise to three neural cell lineages such as neurons, astrocytes, and oligodendrocytes. The local microenvironment of neural stem cells seems to influence their behavior. The changes in the niche components including neighboring endothelial cells are suggested to play an important role during regeneration [30].

5.5 Expert Opinions

5.5.1 Label-Retaining Cells

Stem cells are characterized by their self-renewal and multipotency. For self-renewal, stem cells are required to divide asymmetrically to generate one stem cell and one daughter cell called a transit amplifying cell or progenitor cell, which can divide several times and become a terminally differentiated cell. However, stem cells should also be able to divide symmetrically to increase the number of stem cell in cases of accidental loss. Therefore, stem cells divide symmetrically or asymmetrically dependent on the situation, although the precise mechanisms for this discrimination have not been elucidated. Each stem cell probably has its own hierarchy, as clearly demonstrated by the HSCs.

Because adult stem cells do not have any universal specific markers and the number of stem cells is very low, they are not easy to detect and isolate. However, we can label stem cells by adding a nucleotide analog, BrdU (Bromo-deoxy-uridine), for specific durations in vivo or in vitro. BrdU is incorporated into the DNA during cell division. Because DNA replication is semiconservative, the concentration of BrdU per cell steadily decreases after repeated cell divisions. However, if the cell does not divide for a long-time period after incorporating BrdU, the concentration of BrdU remains high. We can detect these label-retaining cells and identify them as slow cycling ones. Stem cells are expected to be slow cycling. In the skin, for example, label-retaining cells are reported to reside in the bulge area of hair follicles in mice [10]. This method is useful in animal experiments; however, we cannot ethically perform the same experiments in humans.

5.5.2 SP Cells (Side Population Cells)

Adult stem cells have been isolated by fluorescence-activated cell sorting (FACS) with a combination of specific antibodies directed against stem cell markers, because no unique specific marker for stem cells has yet been identified. Another new technique focuses on these cells' ability to efflux Hoechst 33342 dye, which binds to DNA. It is considered essential for stem cells to have a strong capability to efflux unwanted toxic materials in order to maintain themselves. When Hoechst 33342 dye was applied to bone marrow cells, the main population cells emitted fluorescence after UV radiation, but other cells did not. The latter cells were named SP cells; they exhibited potent HSC activity [17]. SP cells highly express ATP-binding cassette (ABC) transporters

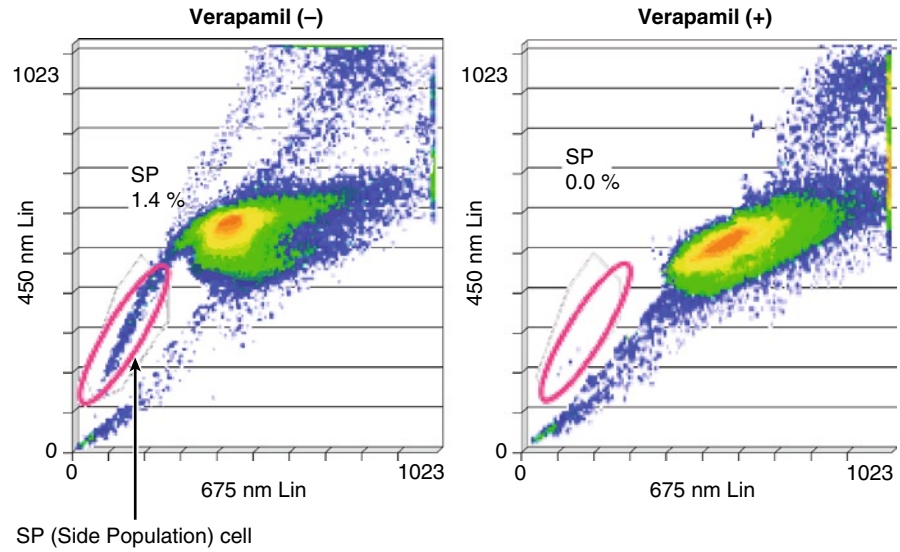

Fig. 5.2 Side population (SP) cells in the skin. When Hoechst 33342 dye was applied to skin-derived keratinocytes, the main population cells emitted fluorescence after UV radiation, but other cells did not. The latter cells were SP cells (circled in *red* in the *left*); they exhibited several stem cell markers for keratinocytes [49]. SP cells highly express ATP-binding cassette (ABC) transporters such as ABCG2, which are closely related to the exclusion of dye. After the administration of verapamil, a calcium channel blocker, the SP phenotype was no longer detected (*right*)

such as ABCG2, which are closely related to the exclusion of dye. After the administration of verapamil, a calcium channel blocker, the SP phenotype was no longer detected (Fig. 5.2). The multipotential capacity of SP cells isolated from variable tissues is supported by an increasing number of studies. A comparison of SP cells with differentiated main population cells within a tissue revealed that SP cells are in an active transcriptional and translational state and underexpress genes reflecting tissue-specific functions. [29].

5.5.3 The MSC Niche: The Pericyte

Caplan proposed that MSCs can function as vascular pericytes [7]. A pericyte is a cell that is released from its position on a vascular tube in cases of focal injury and functions as an immunomodulatory and trophic MSC. This immune modulation turns off T cell surveillance of the injured tissue and thus blocks autoimmune reactions. Crisan et al. reported that blood vessel walls harbor a reserve of progenitor cells that may be integral to the origin of such elusive MSCs and other related adult stem cells [11].

5.6 Five Year Perspective

5.6.1 Immunomodulation

Adult bone marrow-derived and tissue-resident MSCs are only slightly immunogenic and display immunomodulatory and antiinflammatory effects in the host in vivo [28]. These therapeutic properties of MSCs support their possible clinical application to preventing the tissue/organ allograft rejection and severe acute graft-versus-host diseases. A recent clinical study in Europe showed that the overall response to MSC administration was about 70% in patients with steroid-refractory severe acute GVHD [28]. It may also be possible to treat such autoimmune disorders as inflammatory bowel disease and inflammation of the heart muscle walls associated with autoimmune myocarditis for which immunomodulation and tissue repair are required.

While in vitro data consistently demonstrate the immunosuppressive capability of MSC, current studies in animals and humans suggest that MSCs are less effective in vivo in producing systemic immunosuppression. Further mechanistic studies and randomized controlled trials using standardized cell populations are needed to define the optimal conditions for the use of MSC as immunotherapy [25].

5.6.2 Anticancer Therapy

Recent evidence suggests that MSCs selectively home in on tumors and contribute to the formation of tumor-associated stroma. This effect could be utilized by genetically modifying MSC to produce high levels of anticancer agents that blunt tumor growth kinetics and inhibit the growth of tumors in situ [18].

5.6.3 iPS Cells

The iPS-cell technology can be used for the production of cell lines from patients with various diseases. For genetic diseases, iPS cells provide a new approach to pathogenesis based on a particular genetic trait at the cellular level [35]. The cost of generating iPS cells cannot be ignored. Therefore, the establishment of cell banks of iPS cells should be considered to treat as many patients as possible [34].

5.6.4 Engineered Skin

Engineered skin is currently available. Ectopic production of proteins in skin grafts can provide an alternative approach to treating human endocrine or hematological disorders [50].

5.7 Limitations/Critical View

5.7.1 The Risk of Transformation

In vitro culture conditions are very artificial and pose many potential risks for clinical applications. We must not underestimate the effects of culturing cells. During culture, cells are exposed to various "culture shocks" such as oxidative stress, mechanical stress, enzymatic digestion etc. These stressors may cause epigenetic changes in the cultured cells, leading to abnormalities. In fact, the accumulation of genetic and/or epigenetic alterations arising in stem/progenitor cells during aging and severe injuries, including chronic inflammatory atrophy, could trigger their malignant transformation into cancer stem/progenitor cells [32].

Although it is very important to validate the current condition of culture cells and to minimize the potential risks, we also have to consider the cost performance. For example, if we add animal-derived materials to the medium, we have to rule out contamination by infectious organisms. Nowadays, many recombinant proteins and serum-free media are commercially available; however, they are relatively expensive. Regulatory requirements elongate the research timeline and increase the cost of such therapies. Therefore, patient-specific tailor-made therapy is theoretically possible, but applications may be limited fiscally. Another problem is that, using current modalities, it takes more than several weeks or months to provide a large number of the patient's own cells. Thus adult-stem-cell-based therapy is not currently suitable for the acute diseases such as myocardial infarction or stroke, although it may be useful for degenerative diseases such as Parkinson's and Alzheimer's.

Because MSCs are immunosuppressive, there is a high probability of allogenic usefulness. In this case, an MSC bank will be as important a stem cell bank as an umbilical cord bank.

5.8 Conclusion/Summary

Among the various types of adult stem cells, bone marrow-derived and adipose-derived MSCs are promising for future stem-cell-based therapy because of their easy accessibility. Patient-derived iPS cells will be useful in future regenerative medicine and also be powerful tools for the elucidation of disease mechanisms and drug screening.

However, we must be cautious and consider the safety issues of cell culture techniques, because culture has own potential risks and may induce unwanted epigenetic changes in the cell.

5.9 Literature with Abstracts

Mimeault M, Batra SK. Recent progress on tissue-resident adult stem cell biology and their therapeutic implications. Stem Cell Rev. 2008;4:27–49.

Recent progress in the field of the stem cell research has given new hope for treating and even curing diverse degenerative disorders and incurable diseases in humans. In particular, the identification of rare populations of adult stem cells in the most tissues/organs in human has emerged as an attractive technique for obtaining multipotent stem/progenitor cells for cell replacement-based therapies and tissue engineering in regenerative medicine. These tissue-resident adult stem/progenitor cells offer the possibility of stimulating their in vivo differentiation or using their ex vivo expanded progenies for cell replacement-based therapies with multiple applications in humans. Among the diseases that could be treated by the stem-cell-based therapies are hematopoietic and immune disorders, multiple degenerative disorders such as Parkinson's and Alzheimer's diseases, type 1 or 2 diabetes mellitus, eye, liver, lung, skin and cardiovascular disorders, and aggressive and metastatic cancers. In addition, genetically modified adult stem/progenitor cells could also be used as delivery systems for expressing therapeutic molecules in specific damaged areas of different tissues. Recent advances in cancer stem/progenitor cell research also offer the possibility of targeting the undifferentiated and malignant cells that provide critical functions in cancer initiation, progression, and disease relapse in order to treat patients diagnosed with advanced and metastatic cancers, which remain incurable with the current therapy modalities.

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Isolation and Growth of Stem Cells

6

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6.1 Introduction

Multiple tissues can serve as a source of adult or somatic stem cells. Some of these tissues are available at only one point in the lifecycle, such as the umbilical cord, Wharton's jelly, and placenta. In contrast, others are available throughout life and these include adipose tissue, bone marrow, and skeletal muscle. This chapter focuses on the latter three tissues due to their availability and utility for autologous and allogeneic transplantation. While none of these tissues yields a "perfect" stem cell, they do exhibit some of the following ideal properties:

Abundant, accessible, and replenishable tissue source
Multiple lineage differentiation potential
Nontumorigenic
Suitable for both autologous and allogeneic transplant
Capable of retaining the desired properties after long-term storage
Available in quantities of billions of cells
Low immunogenicity
Limited donor to donor variability
Simple and reproducible isolation procedure

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6.2 Aim of the Discipline

The aim of this chapter is to review the following:

1. Isolation methods for adult/somatic stem cells from adipose, bone marrow, and skeletal muscle tissues.
2. Culture and expansion procedures.
3. Quality assurance and quality control issues relating to the manufacture of clinical-grade stem cells.
4. Regulatory assurances necessary for clinical trial applications of adult stem cells.
5. Current and pending clinical applications.
6. Success and limitations of the technology.

6.3 State of the Art

Review of the methods for the isolation and expansion of adipose-derived stem cells (ASCs), bone marrow-derived mesenchymal stem cells (BMSCs), and skeletal muscle-derived satellite cells (SMSCs).

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Investigators have isolated stem cells from multiple tissues. Academic and biotech centers have exploited umbilical cord and placenta as rich sources of hematopoietic and mesenchymal stem cells [17, 37]; however, these tissues are available only once during an individual's life cycle. In contrast, adipose tissue, bone marrow, and skeletal muscle are available throughout one's lifetime and can be harvested without major morbidity to the donor [6, 14, 17, 38]. Accordingly, we have focused on these sites as stem cell sources. The most extensive literature and experience relates to the use of bone marrow as a stem cell source. Bone marrow aspirates were the original source of hematopoietic stem cells for the transplant rescue of cancer patients undergoing chemo-or radiation therapy. Friedenstein's pioneering studies in the 1960s and 1970s led to the recognition of BMSCs [13]. Originally, the BMSCs were selected by density gradient centrifugation and subsequent adherence to plastic and were characterized based on their multilineage differentiation potential (adipogenic, chondrogenic, osteogenic). Similar cell populations have been found in adipose tissue (ASCs) and skeletal muscle (skeletal muscle stem cells or SMSCs) [14, 38]. Following digestion with collagenase, trypsin, and/or hyaluronidase, individual nucleated cells are concentrated from these soft tissues by differential centrifugation. The frequency of the progenitor cells is often calculated using a limit dilution based colony forming unit-fibroblast (CFU-F) assay. This assay determines the number of plastic adherent, colony forming cells per unit volume of tissue. The number of CFU-F in marrow decreases with advancing age based on human and experimental animal models. While it is estimated that there are between $1{:}10^4$–$1{:}10^6$ CFU-F per mL of human marrow, the frequency of CFU-F in adipose tissue and skeletal muscle is ~2–3 orders of magnitude greater [4, 29]. Despite this difference, the ASCs, BMSCs, and SMSCs all share common immunophenotypic features. Consequently, the cells can be positively selected with immunobeads or by flow cytometry using antibodies directed against the following surface antigens: CD29, CD44, CD73, CD90, CD105, or CD166. The antibody selected cells can be used directly or after culture expansion on tissue culture plasticware, uncoated or coated with collagen, fibronectin, or gelatin. Protocols differ with respect to the density of cell seeding. Several investigators recommend seeding the adherent cells at low density (50 per cm^2) while the majority use a higher density ($>10^3$ per cm^2) [11, 42]. Since some evidence suggests that cells begin to lose their multipotent properties when maintained at confluence, low seeding density and subconfluent passaging are recommended [42].

The majority of studies have used fetal bovine serum (FBS) supplementation at concentrations of 2–20% to maintain or expand the adherent cells (Table). Bovine and animal proteins present potential hazards such as the transmission of bovine spongiform encephalopathy or the stimulation of an immune response to xenoproteins in a transplant recipient. Consequently, the research community has increased its interest in developing animal serum-free growth media. The following cytokine growth factors are routinely employed to reduce the need for FBS: basic fibroblast growth factor (bFGF) which promotes the proliferation of the cells without loss of stem cell characteristics, epidermal growth factor (EGF) which promotes proliferation, insulin, and/or transforming growth factor β1 (TGF β1). A substantial body of work (Table) has begun to explore the use of serum-free substitutes as well as human serum, plasma, or platelet lysates to support adherent stem cell expansion in vitro. While the majority of these studies have examined the growth of BMSCs, some studies have focused on ASCs as well as cells from other tissue sources (synovium). The literature consistently concludes that autologous serum supports the expansion of stem cells that retain their characteristic differentiation potential and immunophenotype as well as or better than FBS. Since there is concern that the use of autologous cell products and serum will face commercial and manufacturing restrictions, further attention has been placed on the use of allogeneic or heterologous serum as well as platelet-rich plasma lysates. While some early studies found that allogeneic serum failed to support BMSC proliferation, the majority of recent publications report successful expansion of multipotent cells retaining their characteristic multipotent differentiation, immunophenotype, immunosuppressive, and migratory/mobility properties in vitro. Some evidence suggests that it may be necessary to use AB serum donors and to heat inactivate the complement factors in

allogeneic serum. Based on rodent studies, even if BMSCs are expanded initially in FBS, a brief culture period in the presence of allogeneic serum reduces the cell's surface presentation of xenoantigens and improves their engraftment in vivo. Additional studies have demonstrated that human ASCs can be expanded successfully in concentrations as low as 0.1% human serum when supplemented with cytokines (bFGF, EGF). Thrombin-activated platelet-rich lysates and similar platelet products also support adherent cell expansion. Their action can be correlated with the level of platelet-released cytokines, including bFGF, EGF, and platelet-derived growth factor (PDGF). The development of animal protein-free culture medium is critically important to the advancement of clinical-grade stem cells. Only those proprietary "serum free" growth medium with regulatory agency approval should be used, since the details of their contents remain a trade secret.

	ASCs	BMSCs	SMSCs
Tissue harvest	Subcutaneous adipose tissue obtained by lipoaspirate	Bone marrow aspirates	Skeletal muscle biopsy
Tissue digestion	Collagenase (type I) ± hyaluronidase	None	Trypsin
Centrifugation	Differential centrifugation to separate stromal vascular fraction (SVF) from mature adipocytes	Density gradient centrifugation (Ficoll Hypaque) to separate nucleated cells from erythrocytes	Differential centrifugation and concentration
Purification	Red cell lysis Plastic adherence Immunobead isolation Flow cytometry		
Expansion protocol	In vitro culture Perfusion bioreactors Seeding density (low vs. high) Harvest density (low vs. high)		
Expansion medium composition	DMEM/Ham's F12	DMEM high glucose	DMEM low glucose
Fetal bovine serum (FBS)	10%	10%	9% (heat inactivated)
Supplementary growth factors	Epidermal growth factor (EGF) bFGF $TGF\beta_1$	EGF	EGF Insulin Dexamethasone Fetuin
Antibiotic	Penicillin Streptomycin Amphotericin	Penicillin Streptomycin Amphotericin	Gentamycin Fungizone
Cell doubling times	2–4 days [29]	1–4 days	1–3 days [7]

6.3.1 Use of Human Serum, Platelet Lysates, and Serum-Free Supplements

Reference	Cell type	Autologous serum	Allogeneic serum	Platelet lysates	Serum free	Outcomes
[34]	Synovial MSCs and BMSC	Autologous serum 5–20%				Increased synovial MSC and decreased BMSC proliferation and CFU-F in human serum compared to FBS
[30]	BMSC	Autologous serum 10%				~1.5-fold increased proliferation relative to 10% FBS
[46]	BMSC	Autologous human serum (1–10%)				10% autologous serum with 1.8-fold increased yield and enhanced osteogenesis relative to 10% FBS, but reduced adipogenesis, comparable immunophenotype
[47]	BMSC	Autologous human plasma (10%)				~2-fold increase in CFU-F numbers and osteogenesis with human plasma with reduced adipogenesis and apoptosis relative to 10% FBS
[51]	BMSC	Autologous serum (10%)				Comparable CFU-F formation and osteogenesis with autologous human serum relative to FCS
[36]	ASC	Autologous human serum (0.5%)				Maintains rapid proliferative rate with the retention of differentiation (adipo-, chondro-, osteo-) and immunophenotype comparable to 10% FBS
[43]	BMSC	Autologous serum (10%)	Allogeneic serum (10%)			Autologous serum increased proliferation by 1.6-fold relative to 10% FBS, while allogeneic serum caused cell death, accelerated adipo- and enhanced chondro-differentiation with FBS relative to autologous serum
[15]	Human and rat BMSC	Autologous human serum heat inactivated (20 or 10% supplemented with EGF 10 ng/mL, bFGF 10 ng/mL)	Heterologous human serum heat inactivated (20 or 10% supplemented with EGF 10 ng/mL, bFGF 10 ng/mL)			Autologous or heterologous human serum supports proliferation rates similar to equal concentrations (10 or 20%) of FBS, increased with cytokine supplements, autologous human serum proliferated cells with similar differentiation potential (adipo-, osteo-) relative to FBS, heterologous serum improves engraftment and viability of rat BMSCs in a fibrin scaffold in vivo relative to FBS

Table (continued)

Reference	Cell type	Autologous serum	Allogeneic serum	Platelet lysates	Serum free	Outcomes
[20]	BMSC	Heat-inactivated autologous serum with or without bFGF	Allogeneic serum			1.4-fold increase in proliferation with autologous serum and further increase with bFGF relative to FBS; variable response with allogeneic serum; autologous and allogeneic serum increase BMSC motility
[1]	BMSC	Autologous serum 1–15%	Type AB serum 1–15%			1.5-fold increase in CFU-F relative to 15% FBS
[25]	BMSC		10% heat-inactivated AB serum			>2-fold increase in proliferation relative to FBS, increased HLA-DR in early passages; comparable immunophenotypic, immunosuppressive, and differentiation capacity
[44]	BMSC, cord blood-derived MSC		Umbilical cord blood serum			~2-fold increased proliferation relative to 10% FBS, similar differentiation potential, 96 vs. 99% $CD73^{+}CD105^{+}CD45^{-}$ immunophenotype
[39]	ASC, BMSC		Umbilical cord blood serum 10%			Two to threefold increased proliferation relative to 10% FBS, equivalent differentiation potential (adipo, osteo, neuronal) and CFU-F numbers, decreased immunophenotype for ASMA, CD54, CD105, CD106
[23]	BMSC		Human serum			Reduced proliferation and osteogenesis with human serum relative to FBS
[50]	BMSC and cord blood $CD34^{+}$ cells		Human AB serum			No significant difference in $CD34^{+}$ expansion support by BMSC in human AB serum or FBS
[35]	BMSC		Human serum			Enhanced adipogenic and osteogenic differentiation
[22]	ASC		Allogeneic/heterologous human plasma and platelet-poor plasma without or with EGF or bFGF (1 nM)			Platelet-poor plasma with enhanced proliferation and adipogenic differentiation relative to plasma; further increases with EGF or bFGF supplementation
[19]	BMSC, ASC		Allogeneic human serum (5–20%)			Human serum (10%) maintains proliferative and differentiation potential comparable to 5% FBS as well as the ability to blood cell engraftment in NOD-SCID mice

(*continued*)

Table (continued)

Reference	Cell type	Autologous serum	Allogeneic serum	Platelet lysates	Serum free	Outcomes
[28]	BMSC, ASC, trabecular bone, dental pulp cells		Human serum (15%)	Platelet lysate (PL) (5–10%)		PL increased the number of cell generations while retaining the immunophenotype relative to 20% FBS or 15% human serum
[21]	Human ASC		Pooled human AB serum	Thrombin-activated platelet-rich plasma		Both with ~2.5–3-fold increased proliferation relative to 10% FBS, comparable differentiation potential and immunophenotype except for increased CD14/CD45 positive with human serum and PRP
[16]	BMSC			10% Pooled thrombin platelet releasate		Threefold increase in proliferation relative to FBS, improved hepatogenesis; no morphological, osteogenic differentiation, immunophenotype differences
[41]	BMSC			2.5–10% Pooled platelet lysate		Mean yield of 7.8×10^8 cells within 11–16 days per 15 mL bone marrow aspirate
[40]	BMSC			10% Buffy coat of four type O with plasma from one type AB (to avoid ABO exposure)		>4-fold increase in proliferation and increased CFU-F colony size and density relative to FBS, comparable immunophenotype
[33]	BMSC			2.5–10% fresh frozen plasma with 10^8 platelets		>2-fold increase in proliferation relative to 10% FBS, comparable immunophenotype, immunosuppressive properties, and differentiation potential
[3]	BMSC			1–5% pooled PL		Twofold increase in CFU-F and 50% increase in proliferation at 5% PL relative to 10% FBS, comparable immunophenotype, immune regulatory, and differentiation potential (adipo, osteo)
[24]	BMSC			5% pooled platelet lysate		2.8-fold increase in CFU-F and twofold increase in the proliferation of 5% PL relative to 10% FBS, improved adipogenesis with FBS relative to PL, but comparable chondro-, osteo- differentiation, immunophenotype, and immunosuppressive function

Table (continued)

Reference	Cell type	Autologous serum	Allogeneic serum	Platelet lysates	Serum free	Outcomes
[5]	Trabecular bone-derived cells					Both untreated and heat-inactivated human serum supported a lower proliferation and yield of MSC relative to FCS
[48]	BMSC			Pooled platelet-rich plasma (3%)		Twofold increase in proliferation for 3% PL relative to 2% FBS, comparable adipo-, chondro-, osteo-differentiation in vitro and in vivo (osteo)
[10]	BMSC			Platelet-rich plasma lysates		Enhanced CFU-F formation, proliferative rate relative to FCS with the retention of differentiation potential (adipo, chondro, osteo) and immunosuppressive properties
[18]	BMSC			Thrombin-activated platelet-rich plasma		
[2]	BMSC, placental MSC				Serum-free, bFGF (5 ng/mL), on gelatin coated surface	Four to fivefold increased proliferation on gelatin + bFGF relative to 10% FBS, but reduced proliferation with bFGF alone, more immature phenotype based on Oct4, nanog, SSEA4 on gelatin/bFGF relative to 10% FBS
[27]	BMSC				Ultroser G (2%) serum substitute	Three to fivefold increase in proliferation, ~8-fold increase in CFU-F, and 1.5–2-fold increased percentage of $CD73^+$ $CD105^+$ for ultroser G relative to 15% FBS, comparable differentiation (adipo, osteo) and hematopoietic support functionality
[45]	ASC				2.5% FBS, EGF (10 ng/mL), bFGF (1 ng/mL), insulin (8.7 μg/mL)	~4-fold increased proliferation rate relative to 10% FBS, improved adipogenic differentiation following expansion

6.4 Manufacturing Issues

6.4.1 Point of Care Generation vs. Culture-Expanded Cells: Pros and Cons

The question of where the stem cells should be generated remains open to debate. There are clinical and commercial reasons to argue in favor of stem cell isolation and generation at the point of care. If physicians and surgeons can isolate autologous primary stem and regenerative cells from bone marrow or adipose tissue within an operating room or other clinical setting, they reduce the degree of regulatory oversight. These procedures are considered "minimally manipulative" of the primary cells and can be performed without the requirement of FDA review or approval. For example, orthopedic surgeons routinely aspirate bone marrow to mix with osteoinductive scaffolds during procedures. More recently, relatively simple devices have been marketed to allow the enrichment of the bone marrow with MSCs that may improve the tissue [26]. Likewise, plastic surgeons have begun harvesting stromal vascular fraction (SVF) cells from adipose tissue in the operating room [12, 32, 49, 52]. These SVF cells can then be used to coat vascular grafts or to enhance intact fat tissue transplantation for soft tissue cosmesis and breast reconstruction. Theoretically, these practices can be conducted without the risk of infection or harm to the patient. In the absence of extensive clinical experience, some questions remain. Can the implantation of adipose-derived SVF cells cause calcification masking the subsequent detection of breast tumors by mammography? Does the heterogeneity of the SVF cells or bone marrow aspirates interfere with or promote their efficacy in tissue repair? Can a sufficient number of stem cells be isolated in this manner? Are the procedures simple enough to be reproducible from surgeon to surgeon? What if a tissue source of stem cells is unavailable or inadequate due to the factors of age or health status, or otherwise compromised? How can we proactively monitor the isolated cells for evidence of contamination, functionality, and identification for efficacy and safety purposes? Because of these questions, it is necessary to continue the development of culture-expanded adherent stem cells for regenerative medicine.

6.4.2 Lot Quality Assurance/ Quality Control

An array of tests must be applied to any stem cell product that has been manipulated ex vivo [41]. Several address safety. First and foremost, there must be documentation that the product is free of infectious agents. Cells should be evaluated for evidence of endotoxin, mycoplasma, bacteria (aerobic and anaerobic), and viral (CMV, EBV, HIV, Hepatitis) contaminants. Second, the genetic stability and the oncogenic potential of the cells must be evaluated. The karyotype in multiple cells within each lot should be examined. Additionally, studies should determine the adherent-free growth of the cells in agar suspensions in vitro and the migration and tumor forming ability of the cells when implanted in immunodeficient mice in vivo.

The cell features relating to efficacy require equal scrutiny. The individual lots should be quantified for purity and homogeneity. At present, flow cytometric detection of the surface antigens associated with stromal cells (CD73, CD90, CD105) is commonly used. As more global methods are refined, transcriptomic or proteomic cell signatures may be adopted as the "gold standard." Other metrics may include the evaluation of (1) conditioned medium to determine the cytokine profile of the cells; (2) in vitro assessment of the cell differentiation potential along lineage pathways of interest; and (3) in vivo assessment of engraftment and functionality of the cells in relevant animal models. In vivo assays currently employed include the osteogenic capacity of human stem cells combined with biomaterial scaffolds and implanted subcutaneously or the ability of human stem cells to promote revascularization in ischemic injury models, both using immunodeficient mice.

The costs associated with safety and efficacy, quality assurance and quality control will be prohibitive if single lots of cells are generated for autologous transplantation; however, the expense will be reduced if individual lots can be used to generate allogeneic products for multiple recipients.

The reagents used in stem cell manufacture must be obtained from a documented and trackable source or supplier. The potency and chemical definition of each reagent must be provided by the supplier or generated at the site of production according to an established protocol. For example, a collagenase used for tissue

digestion must be certified based on its level of enzyme activity, endotoxin content, bacterial contamination, and stability. The shelf life and storage procedure for each reagent, both before and after preparation in solution, need to be validated and verified. Steps must be taken to confirm the effectiveness of any filtration, purification, and sterilization filtration methods. Quality control and assurance will require a trackable inventory control system for all the materials and reagents used in the manufacturing procedure. Standard operating procedures will need to be established and performed at frequent intervals to insure the safety of the manufacturing process.

6.4.3 Staff Protocols and Equipment and Reagent Certification

Personnel must receive training in safety and manufacturing methods according to defined protocols in a certified manner. While some of this can be performed "online," it is likely that hands-on training under direct supervision will be required. Staff personnel manufacturing stem cells for clinical use will need to meet cGMP standards of personal hygiene. Entry into the cell culture facility will involve passage through a changing room where individuals will change into clean scrubs and shoe covers or don a coverall, wash their hands and exposed skin for a set period of time with antiseptic detergent, and wear a hair and face cover. The cell culture room itself must be monitored for airborne particle count levels. The particle levels must be recorded on a regular basis using certified equipment. Certification testing for performance specifications should be performed and documented on all laboratory equipment at least annually. Cells and tissues should be processed according to defined manufacturing protocols. These interactive documents will include places for technicians and quality assurance monitors to sign their initials confirming that specific steps were carried out as directed or, where necessary, deviations occurred.

While biological safety cabinets alone offer sufficient containment for tissue manipulation and cell isolation in laboratory settings, it is advantageous to have a completely closed system for clinical purposes. Theoretically, a machine will reduce the risk of operator errors. A number of companies have begun to manufacture devices for this purpose (Table 6.4.4). Several of these are designed for the processing, isolation, and/or culture of adherent or nonadherent cells from bone marrow aspirates. Additionally, devices are now marketed for the isolation of SVF cells from adipose tissue. Using them, surgeons can isolate regenerative cells at the point of care. By locating these devices within a hospital operating room as opposed to the blood bank or clinical pathology laboratory, procedures can also be completed more rapidly. Ultimately, these "user friendly" machines will allow any trained technical support personnel to isolate a reproducible and consistent cell product, often with minimal manipulation.

6.4.4 Cell Processing Devices

Company	Instrument (tissue type)	Website
Aastrom biosciences	Tissue repair cell (TRC) technology (bone marrow)	http://www.aastrom.com/
Cytori therapeutics	Cellution® 800/900 (adipose)	http://www.cytoritx.com/us/index.html
DePuy spine (Johnson & Johnson)	Cellect™ (bone marrow)	http://www.depuyspine.com
Thermogenesis	MarrowXpress (bone marrow)	http://www.thermogenesis.com/
Tissue genesis	TGI 1000™ cell isolation system (adipose)	http://www.tissuegenesis.com/

6.5 Clinical Applications and On-Going Human Trials

A review of the Food and Drug Administration Clinical Trials website (clinicaltrials.gov) in August, 2008, identified >50 registered clinical trials involving either BMSCs or ASCs (Tables). Study sites included Belgium, Brazil, China, Denmark, Finland, France,

6.5.1 Regenerative Medical Clinicaltrials.Gov Searching Under "Mesenchymal Stem Cells"

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor (clinical trials identifier #)	Study size	Clinical phase	Primary outcome measure
Moderate to severe Crohn's disease	Allogeneic BMSC (Prochymal™)	2 or 8×10^6 cells/kg body weight	Intravenous	Osiris therapeutics (Baltimore, MD) NCT00294112	10 subjects	Phase II	Crohn's disease activity index @ 28 days
Moderate to severe Crohn's disease	Allogeneic BMSC (Prochymal™)	Placebo, low or high dose	Intravenous	Osiris therapeutics (Baltimore, MD) NCT00543374	200 subjects	Phase III	Duration or reinduction of clinical benefit for 6 months
Multiple sclerosis	Autologous BMSC	2×10^6 cells/kg body weight	Intravenous	University of Cambridge, U.K. NCT00395200	20 subjects	Phase I/IIA	Adverse events
Graft vs. host disease (grades II–IV)	Allogeneic BMSC (Prochymal™)	2 or 8×10^6 cells/kg body weight		MD Anderson (Houston TX) and Osiris Therapeutics (Baltimore, MD) NCT00476762	50 subjects	Phase II	Evaluation of the long-term safety of Prochymal™
Moderate to severe Crohn's disease	Allogeneic BMSC (Prochymal™)	Placebo, low (600×10^6) or high ($1{,}200\times10^6$) dose cells/kg body weight	Intravenous	Osiris therapeutics (Baltimore, MD) NCT00482092	270 subjects	Phase III	Disease remission at 28 days
Chronic obstructive pulmonary disease	Allogeneic BMSC (Prochymal™)		Intravenous	Osiris therapeutics (Baltimore, MD) NCT00683722	60 subjects	Phase II	Pulmonary function test, quality of life, exercise capability at 1 year
Type I diabetes mellitus	Allogeneic BMSC (Prochymal™)		Intravenous	Juvenile diabetes research foundation and Osiris therapeutics (Baltimore, MD) NCT00690066	60 subjects	Phase II	C peptide AUC response
Gastrointestinal graft vs. host disease (grades II–IV)	Allogeneic BMSC (Prochymal™)	2 or 8×10^6 cells/kg body weight		Osiris therapeutics (Baltimore, MD) NCT00136903	75 subjects	Phase II	Resolution or two grade reduction at 42 days
Acute graft vs. host disease patients who failed to respond to steroid treatment	Allogeneic BMSC (Prochymal™)	2 or 8×10^6 cells/kg body weight		Osiris therapeutics (Baltimore, MD) NCT00366145	240 subjects	Phase III	Complete response of ≥28 days
Heart attack and undergoing coronary artery bypass graft	Autologous BMSC	Placebo, low (2×10^7), and high (2×10^8) dose	Intramyocardial injection	NHLBI and Johns Hopkins University NCT00587990	45 subjects	Phase I and II	Incidence of serious adverse events over 6 months

Table (continued)

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor (clinical trials identifier #)	Study size	Clinical phase	Primary outcome measure
Recovery post partial meniscectomy	Allogeneic BMSC (Chondrogen™)	Placebo (hyaluronan), 50 or 150×10^6 cells	Intraarticular injection	Osiris therapeutics (Baltimore, MD) NCT00702741	50 subjects	Phase I and II	Comparison of treatment and adverse event rates
Salvage of treatment refractory GVHD patients	Allogeneic BMSC (Prochymal™)			Osiris therapeutics (Baltimore, MD) NCT00284986	30 subjects	Phase II	Primary efficacy response by 28 days
Myocardial infarction	BMSC (Provacel™)	Placebo, 0.5, 1.5, or 5.0×10^6 cells/kg body weight		Osiris therapeutics (Baltimore, MD) NCT00114452	48 subjects	Phase I	Comparison of treatment and adverse events
Crohn's disease	Allogeneic BMSC (Prochymal™)	Placebo, low, or high dose	Intravenous	NIAID NCT00609232	15 subjects	Phase III	Remission increase of ≥75% at day 28 relative to placebo
Periodontal tissue	BMSC	Ex vivo cultured MSC in platelet-rich plasma gel	Periodontal injection	Translational research informatics center and Nagoya university NCT00221130	10 subjects	Phase I/II	Alveolar bone defect
Dilated cardiomyopathy	$CD34^+$ bone marrow cells	Placebo vs. cell injection following filgastrim subcutaneous injection and apheresis	Intracoronary artery infusion	University medical center Llubljana Slovenia NCT00629018	50 subjects	Phase II	Heart failure mortality at 1 year
Posterolateral spinal fusion with instrumentation	Allogeneic mesenchymal progenitor cells (Neofuse)	Autograft bone compared to three doses of immunoselected MPCs with ceramic granules	Posterolateral spinal fusion	Mesoblast, Ltd New York, NY NCT00549913	40 subjects	Phase I/II	Safety of neofuse in combination with mastergraft resorbable ceramic granules
Liver failure cirrhosis	Autograft mesenchymal stem cells		Intravenous injection via portal vein under ultrasound guidance	Shaheed Behesti medical university, Iran NCT00420134	30 subjects	Phase I/II	Liver function and MELD scores
Treatment of decompensated cirrhosis	Autograft bone marrow mesenchymal stem cells	300×10^6 MSCs	Intravenous infusion of	University of Tehran, Iran NCT00476060	50 subjects	Phase II	MELD score, quality of life, liver volume, histology
BMSCs as a source of allogenic hepatocyte transplantation in homozygous familial hyperc-holesterolemia	Allogeneic bone marrow mesenchymal stem cells	$6–10\times10^8$ BMSCs	Intraveous portal vein infusion	University of Tehran, Iran NCT00515307	2 subjects	Phase I	Serum cholesterol and LDL levels at 6 months

(*continued*)

Table (continued)

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor (clinical trials identifier #)	Study size	Clinical phase	Primary outcome measure
Prevention of graft rejection and GVHD with allogeneic hematopoietic cell transplant	Mesenchymal stem cells		Intravenous infusion	University of Liege, Belgium NCT00504803	30 subjects	Phase II	100 day nonrelapse mortality
Treatment of acute steroid resistant GVHD or poor graft function	Mesenchymal stem cells		Intravenous infusion	University hospital of Liege, Belgium NCT00603330	100 subjects	Phase II	Efficacy at 30 and 180 days
Treatment of chronic allograft nephropathy	Mesenchymal stem cells		Intravenous infusion	Fuzhou general hospital, China NCT00659620	20 subjects	Phase I/II	Creatinine and creatinine clearance rate
Recipients of living kidney allografts	Autologous bone marrow mesenchymal stem cells		Intravenous infusion	Fuzhou general hospital, China NCT00658073	60 subjects		Incidence rate of biopsy-proven acute rejection at 1 year
Cotransplantation of islets and MSCs in type 1 diabetic patients	Autologous mesenchymal stem cells		Intravenous infusion	Fuzhou general hospital, China NCT00646724	30 subjects	Phase I/II	Exogenous insulin requirement, Hgb A1C, glucose and C peptide levels
Lupus nephritis	Autologous mesenchymal stem cells		Intravenous infusion	Organ transplant institute, China NCT00659217	20 subjects	Phase I/II	Proportion of subjects who attain and maintain remission
Refractory (acute or chronic) GVHD	Allogeneic mesenchymal stem cells	$1–2\times10^6$ MSC/kg body weight	Intravenous infusion	University of Salmanca, Spain NCT00447460	15 subjects	Phase I/II	Safety and efficacy in terms of GVHD response
Treatment of GVHD	Mesenchymal stem cells	$1–2\times10^6$ MSC/kg body weight	Intravenous infusion	Christian medical college, India NCT00314483	25 subjects	Phase I/II	Control of GVHD at 8 weeks
Treatment of distal tibial fracture	Autologous mesenchymal stem cells		Not specified	Hadassah medical organization, Israel NCT00250302	24 subjects	Phase I/II	Safety, clinical union of fracture
Cord blood expansion for myelodysplastic syndrome/leukemia	Allogeneic mesenchymal stem cells			M.D. Anderson NCT00498316	30 subjects	Phase I	Safety and feasibility of cord blood transplantation following expansion on MSCs
Refractory SLE	Allogeneic bone marrow mesenchymal stem cells	1×10^6 MSC/kg body weight	Intravenous infusion	Nanjing university, China NCT00698191	20 subjects	Phase I/II	SLE disease activity index, renal function, serology

(*continued*)

Table (continued)

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor (clinical trials identifier #)	Study size	Clinical phase	Primary outcome measure
Treatment of acute or chronic GVHD after undergoing donor stem cell transplantation	Donor mesenchymal stem cells		Infusion	Case comprehensive cancer center, Cleveland OH NCT00361049	24 subjects	Phase I	Safety
Dose escalation study to evaluate the safety and feasibility of transendocardial injection of three different doses of mesenchymal progenitor cells in subjects with heart failure	Allogeneic mesenchymal progenitor cells	25, 50, or 150×10^6 cells	Transendocardial injection	Angioblast systems NCT00721045	60 subjects	Phase II	Feasibility and safety
Osseous setting improvement in cotransplantation of osseous matrix and mesenchymal progenitor cells from autologous bone marrow	Autologous bone marrow progenitors		Combination of concentrated autologous marrow cells with Osteopure™	University hospital, Clermont-Ferrand, France NCT00557635	50 subjects	Phase II	Osseous setting evaluation at 3 month intervals relative to historical controls
Treatment of severe osteogenesis imperfecta with allogeneic bone marrow transplantation				St. Jude's childrens research hospital, Memphis, TN NCT00705120	9 subjects	Phase I	Evaluation of safety and efficacy at 1 year of allogeneic BMT in severe OI
Stromal therapy of osteodysplasia after allogeneic bone marrow transplantation	Allogeneic bone marrow MSCs	First dose 10^6 MSC/kg body weight Second dose 5×10^6 MSC/kg body weight 28 days later		St. Jude's childrens research hospital, Memphis, TN NCT00186914	8 subjects	Phase I	Evaluation of the safety of stromal cell therapy
Marrow mesenchymal cell therapy for osteogenesisimperfecta–a pilot study	Allogeneic $CD3^+$ depleted bone marrow		Secondary transplant without chemo-therapy	St. Jude's childrens research hospital, Memphis, TN NCT00187018	14 subjects		Effect on the growth rate and mineral content of bone after BMT with $CD3^+$ cell depletion
Chondrogen delivered by intraarticular injection following meniscectomy	Allogeneic mesenchymal stem cells		Hyaluronan or hyaluronan containing one or two doses of Chondrogen	Osiris therapeutics, Baltimore, MD NCT00225095	60 subjects	Phase I/II	Meniscal volume, adverse events

(*continued*)

Table (continued)

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor (clinical trials identifier #)	Study size	Clinical phase	Primary outcome measure
Combined CABG and stem cell transplantation for heart failure	Bone marrow mesenchymal stem cells		Intramyocardial	Helsinki university, Finland NCT00418418	60 subjects	Phase II	Increased ejection fraction by MRI relative to placebo control
Vasculogenesis in patients with severe myocardial ischemia	Autologous bone marrow mesenchymal stem cells		Direct intramyocardial injection	Rigshospitalet, Denmark NCT00260338	40 subjects	Phase I/II	Improved myocardial perfusion measured by SPECT over 1 year
OT1-010 for GVHD prophylaxis in patients undergoing donor peripheral blood stem cell transplantation for hematological malignancies	Autologous bone marrow mesenchymal stem cells		Intravenous infusion	Jonnson comprehensive cancer center Los Angeles, CA NCT00081055	99 subjects	Phase II	Follow-up over 3 years
Autologous MSC therapy for heart failure	Autologous bone marrow mesenchymal stem cells		Direct intramyocardial injection	Rigshospitalet, Denmark NCT00644410	60 subjects	Phase I/II	Clinical and ejection fraction improvements at 6 and 12 months
Percutaneous autologous bone marrow grafting for open tibial shaft fracture	Concentrated autologous bone marrow		Percutaneous injection 1 month following standard treatment (fixation)	University hospital, Tours, France NCT00512434	186 subjects		Proportion of subjects requiring secondary intervention due to delayed or nonunion at 1 year
Intracoronary infusion of bone marrow MSC for the treatment of idiopathic dilated cardiomyopathy	Autologous bone marrow mononuclear cells		Intracoronary artery infusion	Hospital Universitario Reina Sofia, Spain NCT00629096	30 subjects	Phase II	Improved left ventricular function at 6 and 12 months
Safety Study of allogeneic mesenchymal progenitor cells in subjects with recent acute myocardial infarction		25, 75, or 150×10^6 cells	Transendocardial injection	Angioblast systems NCT00555828	25 subjects	Phase I/II	Safety and feasibility at 30 days

6.5.2 Regenerative Medical Clinicaltrials.Gov Search Under "Adipose Stem Cells"

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor and clinical trials identifier #	Study size	Clinical phase	Primary outcome measure
Treatment of complex perianal fistulas not associated with Crohn's disease	Autologous ASCs		Intrafistula	Cellerix Universidad Autónoma de Madrid, Spain NCT00475410	207 subjects	Phase III	Fistula closure and reepithelialization up to 26 weeks
Type 1 diabetes	Autologous SVF Cells	SVFs derived from 100 to 120 mL of lipoaspirate	Intravenous	Adistem Ltd Hong Kong NCT00703599	30 subjects	Phase I/II	Lowering of insulin dependence and antihyperglycemic control medications up to 48 weeks
Lipodystrophy	Autologous transplantation of lipoaspirate enriched with ASCs		Subcutaneous	Irmandade Santa Casa de Misericórdia de Porto, Brazil Alegre NCT00715546	10 subjects	Phase I	Clinical evaluation of transplant area (1 year)
Type 2 diabetes	Autologous SVF cells	SVFs derived from 100 to 120 mL of lipoaspirate	Intravenous	Adistem Ltd Hong Kong NCT00703612	34 subjects	Phase I/II	Lowering of blood glucose (up to 48 weeks)
Treatment of patients with ST elevation myocardial infarction	Autologous adipose-derived stem and regenerative cells		Injection of lipoaspirate-derived cells	Cytori therapeutics, San Diego, CA NCT00442806 (study sites – The Netherlands and Spain)	48 subjects	Phase I	Safety – major adverse cardiac and cerebral events (up to 6 months)
Treatment of nonrevascularizable ischemic myocardium	Autologous adipose-derived stem and regenerative cells		Direct injection to the left ventricle	Cytori therapeutics, San Diego, CA NCT00426868 (study sites – Netherlands and Spain)	36 subjects	Phase I	Safety – major adverse cardiac and cerebral events
Nonsurgical treatment of complex perianal fistulas	Autologous ASCs		Autologous ASCs in fibrin glue to fistula	Cellerix Spain NCT00115466	50 subjects	Phase II	Complete closure (8 weeks)
Autologous fat enhanced with regenerative cells transplanted to reconstruct breast deformities after lumpectomy	Autologous fat enriched with adipose-derived stem and regenerative cells		Autologous fat enhanced with ADRCs	Cytori therapeutics, San Diego, CA	70 subjects	Phase IV	Patient and physician satisfaction with results (12 months)

6.5.3 Regenerative Medical Clinicaltrials.Gov Searching Under "Skeletal Muscle Stem Cells"

Disease	Stem cell type (product)	Cell dose	Cell delivery	Sponsor and clinical trials Identifier #	Study size	Clinical phase	Primary outcome measure
Myocardium post myocardial infarction	Skeletal muscle myoblasts	400 or 800×10^6 cells	Intramyocardial injection	Bioheart, sunrise, FL and Munich, Germany NCT00526253	390 subjects	Phase II/III	6 min walk test, quality of life questionaire

India, Iran, Israel, Japan, the Netherlands, Slovenia, Spain, Taiwan, and the United States, among other countries. Both university-based academic- and biotech-sponsored clinical trials are enrolling patients. Trials for acute graft vs. host disease (GVHD), Crohns disease, and perianal fistulas had advanced to Phase III clinical trials. Phase I and II clinical trial disease targets included bone and tendon repair, breast reconstruction, cardiomyopathy, diabetes, hematopoietic engraftment, liver disease, nephropathy, pulmonary disease, and systemic lupus erythematosis. Additional clinical applications are being evaluated. These include burn and skin ulcer repair, cartilage regeneration, cerebral and peripheral ischemia, and spinal injury, among others. A variety of delivery modalities have been employed, including intravenous infusion, direct tissue injection, and combination of stem cells with biocompatible matrices. It is exciting to note that some clinical trials have been approved for the use of allogeneic BMSC. It is anticipated that the use of allogeneic ASCs will be evaluated in the coming years.

6.6 Expert Opinion

Stem cells from adipose tissue, bone marrow, and skeletal muscle show significant promise for clinical application. They fulfill many of the attributes of an ideal stem cell outlined at the beginning of this chapter. Furthermore, the peer-reviewed and reproducible scientific database documenting their isolation, characterization, differentiation potential, and mechanism of action provides assurances to regulatory authorities that they can be used safely and effectively for clinical applications.

6.7 Five Year Perspective

Phase III clinical trials are now underway for the use of BMSCs in a limited number of rare but debilitating diseases. With successful outcomes, it is reasonable to expect these to be accepted by the FDA and other regulatory authorities for clinical practice. A number of promising Phase II studies are enrolling patients. It is likely that ASC applications for soft tissue cosmetic and reconstruction will become a reality in some parts of the world within the next 5 years. Such clinical investigations have advanced significantly in Japan and South Korea. Multiple groups are aggressively evaluating the use of both BMSCs and ASCs for the treatment of myocardial infarction, cardiac disease, and peripheral and central ischemia. While the application of regenerative stem cell therapies to these-life threatening disorders is expected to progress during the next 5 years, it remains to be seen whether Phase III clinical trials will be completed during this period.

6.8 Limitations/Critical View

There is sufficient basic science and preclinical data to support the translation of adult stem cells into clinical practice. Nevertheless, several limitations remain that will impede the development of regenerative medicine.

First, the health care system lacks a stream-lined infrastructure for the isolation and growth of adult stem cells [8]. So far, the isolation of clinical-grade quality-adherent adult stem cells occurs predominantly in private sector/biotech operations. While some university hospitals have invested resources in cGMP laboratory production facilities, these are the exceptions. Within

the U.S., only a few regional efforts are underway to provide these facilities at the public level. It is possible that stem cell isolation centers could be coordinated through the existing blood banking infrastructures, such as the American Red Cross or equivalent agencies serving the health care community's cell sourcing needs. In the absence of an efficient, standardized, and self-regulated infrastructure, the delivery of adherent stem cells to patients and physicians will face financial and logistical road blocks.

Second, although the International Society for Cellular Therapy has led the way, the scientific and medical communities continue to struggle with the definition and standardization of the various stem cell products [9]. It is essential to agree upon an international set of clear, reproducible, and consistent isolation protocols with identified and certified manufacturing sources for reagent materials. These guideline documents will promote the expansion of centers capable of delivering stem cells to patients in a safe and controlled manner.

Third, the preservation and packaging of adherent stem cells now requires liquid nitrogen storage in the presence of cryopreservation agents, such as dimethyl sulfoxide. In addition to the expense, storage is compounded by the necessity of weekly deliveries of liquid nitrogen to the hospital or clinic. Alternative preservation methods, such as vitrification, may allow the products to be stored at room temperature without loss of bioactivity. Vitrification approaches would reduce costs substantially and merit further investigation. Indeed, vitrification has been successfully performed with amnion-derived MSCs [31].

Fourth, while BMSCs have fulfilled some regulatory requirements allowing the initiation of clinical studies, ASCs will require additional preclinical investigation. Safety and efficacy issues remain to be proven in large -scale clinical trials. While adipose-derived SVF cells or bone marrow aspirates used directly at the point of care are subject to reduced regulatory oversight, long-term monitoring has not yet been performed on patients receiving these therapies. Further patient monitoring for unforeseen adverse events will be required for these heterogeneous cell populations as well as more purified adherent stem cells in clinical trials. Furthermore, it will be important to evaluate any combination products that use adherent adult stem cells together with biomaterials or with gene delivery vehicles.

6.9 Conclusions/Summary

Adult stem cell isolation and growth methodologies have advanced significantly since the pioneering days of Friedenstein and his colleagues. International scientific efforts are poised to translate adult stem cell science into clinically valuable products at the bedside. Nevertheless, there remains room for improvement. These include (1) the development of standardized methods, (2) the removal of nonhuman animal proteins from the expansion medium, (3) the refinement of close isolation and culture systems for tissue handling, and (4) cGMP infrastructure development at the level of university hospitals and/or regional blood banking centers. Meeting these goals will require international coordination at the scientific, business, manufacturing, and legal/regulatory levels.

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Differentiation and Plasticity of Stem Cells for Tissue Engineering

7

Yao-Hua Song, Lukas Prantl, and Eckhard Alt

7.1 Introduction

Stem cells are defined as undifferentiated cells that have the capacity to self-renew and to differentiate into various mature cells at a single cell level [118]. Stem cells support normal embryogenesis and postnatal life. Stem cells serve to renew tissue throughout an individual's postnatal life by replacing the cells that are lost owing to everyday wear and tear in our bodies. Bone marrow contains two types of stem cells: hematopoietic stem cell (HSC) and mesenchymal stem cells (MSCs). HSCs are able to give rise to all cells in the hematopoietic system [99, 100]. Injection of a single mCD34(lo/-), c-Kit+, Sca-1(+), lineage markers negative (Lin-) cell resulted in long-term reconstitution of the lymphohematopoietic system [78]. MSCs are multipotent, might be immune privileged [59, 81], and can be expanded easily ex vivo. MSCs isolated from either adult bone marrow or other origin such as adipose tissue have shown a great potential for cell therapy because these cells possess multipotent capabilities [81], proliferate rapidly, induce angiogenesis, and differentiate into myogenic and other cells [111, 115]. MSCs have been widely used for tissue engineering. In this chapter, we focus on MSCs.

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7.2 Differentiation

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria to define human MSC [22]. MSC must be plastic-adherent when maintained in standard culture conditions. MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. MSC must at minimum differentiate to osteoblasts, adipocytes, and chondroblasts in vitro.

Based on these criteria, MSCs are multipotent cells, which can replicate as undifferentiated cells. They also have the potential to differentiate with various lineages of mesenchymal tissue, including bone, cartilage, fat, tendon, muscle, ligament, and marrow stroma. Examples of differentiation among these lineages are shown in Fig. 7.1.

Another important feature of MSCs is that these cells form colonies when plated as single cell on petri dish [28]. These colony-forming cells (CFU-F) can be induced to form bone, cartilage, and fat by simple manipulation of culture conditions [28, 56, 79, 81].

MSCs cultured with transforming growth factor favor chondrogenesis [79, 81]. Ascorbic acid and dexamethasone favor osteoprogenitor differentiation with enhanced alkaline phosphatase expression, matrix production, nodule formation, and deposition of calcium, confirming the presence of osteoprogenitor cells [74, 79]. Culture in the presence of indomethacin, methyl isobutylxanthine, insulin and dexamethasone has been found to favor adipogenesis [81, 95]. The key factors involved in these differentiation pathways are summarized in Table 7.1.

Growth factors have been shown to regulate MSC colony formation and differentiation. Gronthos et al. [33] examined 25 purified recombinant growth factors

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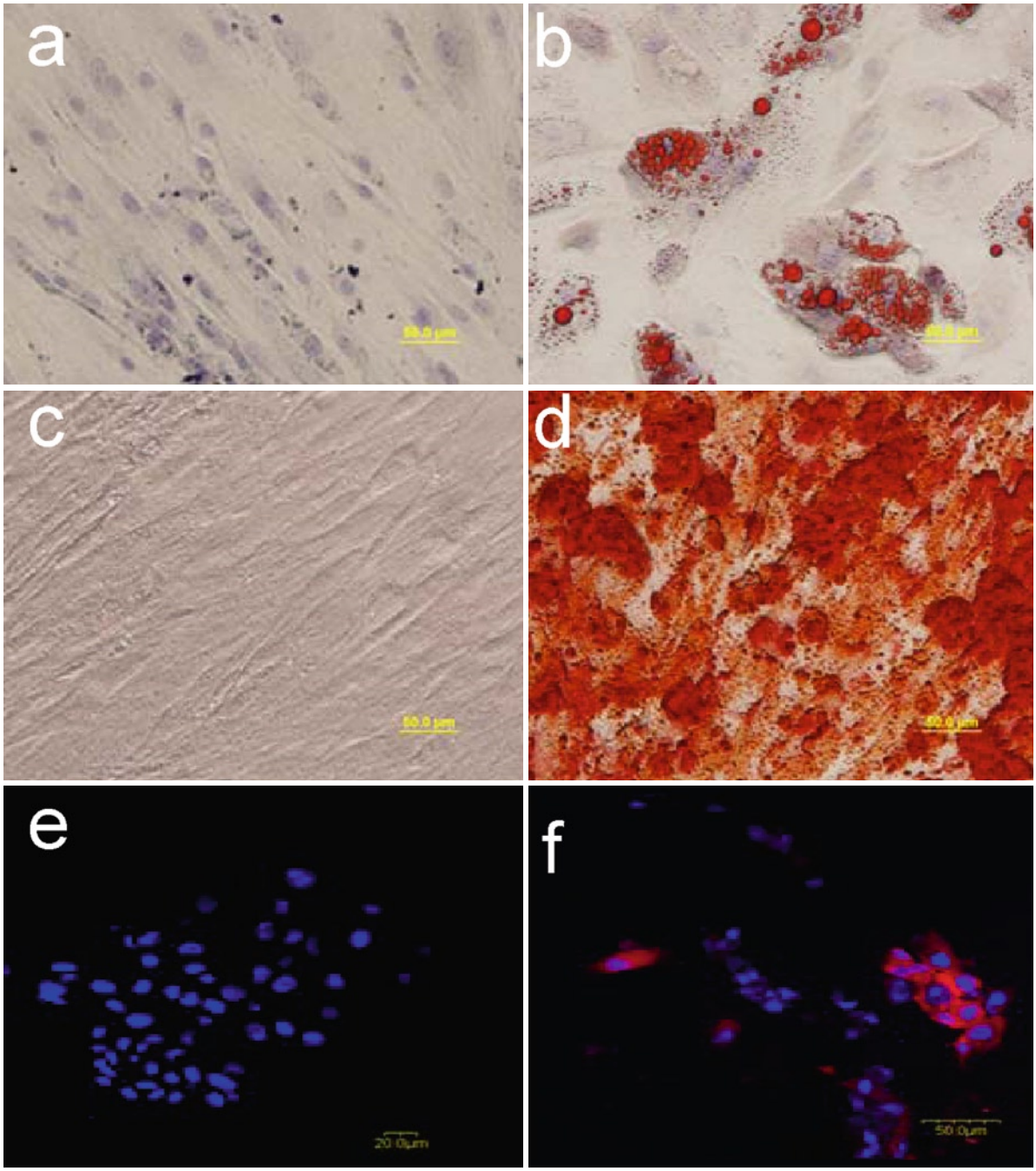

Fig. 7.1 Differentiation potential of hASCs. Cells were cultured in adipogenic (**b**), osteogenic (**d**), hepatogenic (**f**), and neurogenic induction medium (**h**) or their respective control medium (**a**, **c**, **e**, **g**). Adipogenesis and osteogenesis of hASCs were confirmed by Oil Red O staining (**a**, **b**) or Alizarin Red S staining, respectively. Hepatogenesis and neurogenesis were verified by immunofluorescence staining of hepatocytes specific antigen albumin or microtube related protein-2 (MAP-2), respectively. Scale bar = 50 μm

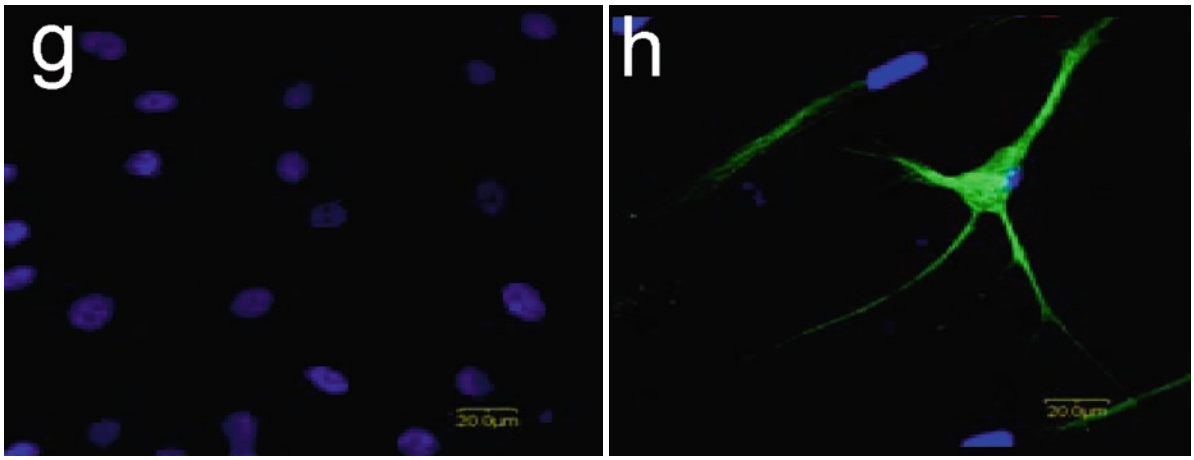

Fig. 7.1 (continued)

Table 7.1 Key factors involved in various differentiatioin pathways

Chondrogenic differentiation	Adipogenic differentiation	Osteogenic differentiation	Neurogenic differentiation	Hepatogenic differentiation
DMEM 10% FBS	DMEM supplemented with 10% FBS	DMEM plus 10% FBS	DMEM/F12, 1% FBS	DMEM 1% FBS
0.1 uM dexamethasone	100 μM l-ascorbate acid	0.1 μM dexamethasone	2% B27	10 ng/mL bFGF
25 μg/mL ascorbate-2-phosphate	1 μM dexamethasone	200 μM l-ascorbic acid	10 ng/mL bFGF	20 ng/mL aFGF
10 ng/mL transforming growth factor-3	0.5 mM 1-methyl-3-isobutylxanthine	10 mM β-glycerol phosphate	10 ng/mL tEGF	1% ITS
1 mM sodium pyruvate	100 μM indomethacin		10 μM forskolin	10 ng/mL EGF
0.4 mM proline	10 μg/mL human recombinant insulin		1 mM cAMP	10 ng/mL OSM
10 μg/mL bovine insulin			5 μg/mL insulin	20 ng/mL HGF
5.5 μg/mL transferrin			0.5 mM 1-methyl-3-isobutylxanthine	
5 ng/mL sodium selenite			1 mM 2-mercaptoethanol	
4.7 μg/mL linoleic acid			50 μM Vitamin C	
0.5 mg/mL bovine serum albumin			10 ng/mL NGF	

for their ability to initiate and support clonogenic growth of fibroblast CFU-F from adult human bone marrow. L-ascorbate and the glucocorticoid dexamethasone were found to be essential for CFU-F colony development under serum-deprived conditions. Platelet-derived growth factor-BB (PDGF) and epidermal growth factor (EGF) demonstrated the greatest ability to support colony growth.

Kuznetsov et al. [55] found that initial proliferation of an MSF precursor cell requires participation of at least four growth factors: PDGF, bFGF, TGF-β, and EGF. In mouse and human cultures stimulated to proliferate by SF-CM, neutralizing antibodies against PDGF, TGF-β, bFGF, and EGF specifically suppressed MSF colony formation. The degree of suppression was species-dependent, with the most profound inhibition

achieved in mouse cultures by anti-PDGF, anti-bFGF, and anti-EGF and in human cultures by anti-PDGF and anti-TGF-β.

Oreffo et al. [75] showed IFN-α inhibits human osteoprogenitor cell proliferation, CFU- F formation, HOP-26 expression, and alkaline phosphatase specific activity and modulates bone morphogenetic protein-2 (BMP-2) gene expression.

Wash et al. showed that FGF-2 had no detectable effect on colony formation, but markedly increased its proliferative potential and that of its immediate progeny, as shown by the increases in colony size and cell number [112]. Tsutsumi et al. suggest that FGFs play a crucial role in self-renewal of MSC since FGF-2 markedly increased the growth rate and the life span of rabbit, canine, and human bone marrow MSC in monolayer cultures [105]. Martin et al. showed that FGF-2 maintains MSCs in a more immature state allowing in vitro expansion of human osteoprogenitors, which, associated with bioceramics, can differentiate in vivo and form bone tissue [64].

The effect of TGF-β on MSC colony formation seems the opposite to that of FGF-2. Walsh et al. [113] showed that treatment with TGF-β increased the colony-forming efficiency of marrow cell suspensions but reduced the median diameter of the colonies that formed and the number of cells harvested at the end of primary culture.

Locklin and coworkers [60] showed that in the absence of fetal calf serum (FCS), TGF-β caused a dose-dependent increase in MSC growth and alkaline phosphatase activity (AP); however, in the presence of FCS, growth was inhibited at high concentrations and AP unaffected. The BMPs, member of the TGF-β superfamily, also influence MSC differentiation [114]. Rickard et al. [87] have shown that recombinant BMP-2 acted in synergy with dexamethasone to permit osteoblast differentiation.

Scutt et al. [94] showed that PGE2 induces the transition from nonadherent to adherent bone marrow mesenchymal precursor cells via a cAMP/EP2-mediated mechanism.

IL-6 maintains the proliferative and undifferentiated state of bone marrow-derived MSCs [83]. Wehling et al. [117] showed that Interleukin-1beta and tumor necrosis factor-α inhibit chondrogenesis by human MSCs through NF-kappaB-dependent pathways, which suggest that MSC-based repair of lesions in articular cartilage may be compromised in inflamed joints.

Recently, a multipotent regenerative cell preparation with defined proliferative potential has been isolated from human subcutaneous adipose tissue [31]. It has been shown that the stromal fraction of cells isolated from adipose tissue contains cells that have mesodermal and ectodermal capacity as well as cardiomyogenic potential [69, 125, 126]. A clonal analysis of the differentiation potential of this freshly isolated human adipose-derived stem cell preparation found that 52% of the cell clones differentiated into two or more of the lineages (adipogenesis, osteogenesis, chondrogenesis, and neurogenesis) [16, 31, 34]. These freshly isolated cells containing CD34+ and CD44+ as well as CD31+ cells play a role in vascular stabilization by mutual structural and functional interaction with endothelial cells [104]. Casteilla's group has shown that transplantation of cells isolated from mouse adipose tissue can efficiently rescue lethally irradiated mice, resulting in a reconstitution of major hematopoietic lineages [20]. The expression profile changes as a function of time in passage and plastic adherence [66, 68]. After two or more successive passages in culture, the adipose tissue-derived stem cells (ASCs) express characteristic adhesion and receptor molecules, surface enzymes, extracellular matrix (ECM) and cytoskeletal proteins, and proteins associated with the stromal cell phenotype. The surface immunophenotype of ASCs resembles that of bone marrow-derived mesenchymal stem or stromal cells (MSCs) [81] and skeletal muscle-derived cells [122]. Direct comparisons between human ASC and MSC immunophenotypes are >90% identical [126]. Consistent with this, the two cell populations display similar mitogen-activated protein kinase phosphorylation in response to tumor necrosis factor-α, lipolytic responses to β-adrenergic agents, and adiponectin and leptin secretion following adipogenesis [89]. Nevertheless, differences in surface protein expression have been noted between ASCs and MSCs. For example, the glycoprotein CD34 is present on human ASCs early in passage but has not been found on MSCs [66, 81]. There is a growing body of experimental evidence from both in vitro and in vivo studies demonstrating the multipotentiality of ASCs from adipose tissue isolated from humans and other species. These include the adipocyte [36, 96, 125, 126], chondrocyte [23, 119, 125, 126], hematopoietic

supporting [19, 48], hepatocyte [97, 101], neuronal-like [46, 53, 91, 126], osteoblast [35, 39, 125, 126], pancreatic [102], and skeletal myocyte [57, 125, 126] pathways. Amos et al. [7] demonstrate that ASCs express pericyte lineage markers in vivo and in vitro, exhibit increased migration in response to PDGF-BB in vitro, exhibit perivascular morphology when injected in vivo, and contribute to increases in microvascular density during angiogenesis by migrating toward vessels. We have shown that ASCs incorporated into tumor vessels [71, 80].

7.2.1 The Role of Local Environment in Differentiation

7.2.1.1 Niche: Extracellular Matrix

The maintenance, survival, and differentiation of stem cells are dependent on special microenvironmental niche, such that uncommitted pluripotent stem cells can be induced to differentiate to form a particular cell type by the nature of the environment. This microenvironmental niche represents the ECM surrounding stem cells supporting self-renewal and maintenance of stem cells.

In the skin, stem cells can be isolated from cultured human epidermis on the basis of high surface expression of beta one integrins and rapid adhesion to ECM proteins. [41, 43]. In the nervous system, tenascin C contributes to the generation of a stem cell "niche" within the subventricular zone, acting to orchestrate growth factor signaling so as to accelerate neural stem cell development [30]. In the tendon [13], stem/progenitor cells reside within a unique niche predominantly comprised of an ECM, and biglycan and fibromodulin were identified as two critical components that organize this niche.

7.2.1.2 Humoral Factors

Soluble mediators produced by local environment also regulate stem cell function. Within the *Drosophila* testis, germ line and somatic stem cells attach to a cluster of support cells called the hub. The hub specifically expresses Unpaired, which signals to adjacent stem cells by activation of the JAK-STAT pathway and induces stem cell self-renewal [47, 106].

7.2.1.3 Exchange of MicroRNA

MicroRNAs are short RNAs that regulate protein translation. Yuan et al. [123] demonstrated that embryonic stem cell microvesicles contain abundant microRNA and that they can transfer a subset of microRNAs to mouse embryonic fibroblasts in vitro. These findings suggest that stem cells can alter the expression of genes in neighboring cells by transferring microRNAs. It opens up the possibility that microRNAs may be important mediators of signaling within stem cell niches. Along this line, our group has shown that liposome-mediated transfection with extract from neonatal rat cardiomyocytes induces transdifferentiation of human adipose-derived stem cells into cardiomyocytes [93], and cardiac progenitors can be selected by a lentiviral vector expressing eGFP controlled by the Nkx2.5 promoter [10].

7.3 Plasticity

Stem cell plasticity refers to the capacity of tissue resident stem cells to exhibit a phenotypic potential that extends beyond the differentiated cell phenotypes of their resident tissue. For example, many investigators have shown that bone marrow stem cells (BMSCs) could generate nonhematopoietic cell types, such as epithelial, skeletal muscle, bone, liver, and neural cells. Toma et al. [103] injected human MSCs into the left ventricle of CB17 SCID/beige adult mice, and they observed that these injected cells differentiated into cardiomyocytes 1 week after injection. Orlic and colleagues have investigated the ability of adult mouse BMSCs to differentiate into myocardial cells within ischemia-damaged myocardium in two different studies. In one study [76], green fluorescent protein (GFP) labeled BMSCs were directly injected into damaged myocardium. In another study [77], BMSCs were mobilized by several injections of stem cell factor and granulocyte colony-stimulating factor. They found that BMSCs were able to traffic to the ischemic infarcted myocardium differentiated into cardiac myocytes and

vascular endothelial and smooth muscle cells. Valina et al. have shown that adipose tissue-derived MSCs differentiated into vascular cells when delivered to myocardium in a porcine infarction model [107]. Nuttall et al. [73] have shown that cells cultured from human trabecular bone are not only osteogenic, but are able also to undergo adipocyte differentiation under defined culture conditions.

Ferrari et al. [24] showed that transplantation of genetically marked bone marrow into immunodeficient mice revealed that marrow-derived cells migrate into areas of induced muscle degeneration, undergo myogenic differentiation, and participate in the regeneration of the damaged fibers.

Musaro et al. [72] demonstrated increased recruitment of proliferating bone marrow cells to injured MLC/mIgf-1 transgenic muscles, and these recruited cells were involved in muscle repair.

Sanchez-Ramos and colleagues [92] have shown that human and mouse BMSC can be induced to differentiate into neural cells under experimental cell culture conditions. BMSC cultured in the presence of EGF or BDNF expressed the protein and mRNA for nestin, a marker of neural precursors.

Reyes et al. identified a population of mesodermal progenitor cells (MPCs) [86]. MPCs were selected by depleting bone marrow mononuclear cells from more than 30 healthy human donors of CD45(+)/glycophorin-A (GlyA)(+) cells. Cells were cultured on fibronectin with EGF and PDGF-BB and 2% or less FCS. MPCs are CD34(−), CD44(low), CD45(−), CD117 (cKit)(−), class I-HLA(−), and HLA-DR(−). MPCs differentiated into cells of limb-bud mesoderm (osteoblasts, chondrocytes, adipocytes, stroma cells, and skeletal myoblasts) as well as visceral mesoderm (endothelial cells). In a follow-up study, the same group [42] reported that cells copurifying with MSCs termed multipotent adult progenitor cells or MAPCs differentiate, at the single cell level, not only into mesenchymal cells but also cells with visceral mesoderm, neuroectoderm, and endoderm characteristics in vitro.

However, caution should be taken when interpreting MSC differentiation. It has been shown that hepatocytes derived from bone marrow arise from cell fusion and not by differentiation [108, 116]. Nevertheless, the finding of stem cell plasticity carries important implications for the treatment of liver, heart, and neurodegenerative diseases as well as for the treatment of type I diabetes and soft tissue repair. Due to the easy access to human adipose tissue, adipose tissue-derived MSCs could be used to regenerate other organs if differentiation can be redirected.

7.4 Stem Cells for Tissue Engineering

7.4.1 Stem Cells for Cardiac Repair

MSCs are multipotent and can yield high number when cultured in vitro or in the case of adipose tissue; MSCs can be recovered without the need for culture expansion. More importantly, MSCs are initially not immunogenic because human MSCs do not constitutively express major histocompatibility complex (MHC) class II antigens when undifferentiated. Furthermore, MSCs suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo [12, 52].

For these reasons, MSCs have been used extensively for cell-based therapy in both basic cardiovascular research and preclinical studies. Amado et al. [6] reported that MSCs preprepared from an allogeneic donor engrafted the infarcted hearts and reduced scar formation and restored myocardial function in a porcine infarction model. Transdifferentiation into cardiomyocytes has been reported previously with bone marrow-derived stem cells [21, 44, 76] although negative studies have also been reported [54]. It is possible that the reported beneficial effect may be due to direct cardiomyocyte differentiation [103] or due to antiapoptotic effects [90] or differentiation into vascular cells. Therapeutic enhancement of neovascularization through transplantation of bone marrow-derived and adipose-derived stem cells has been associated with long-term salvage and survival of viable tissue [3, 51, 76]. Stromal tissue-derived cells differentiated into endothelial cells as demonstrated by an abundance of human CD31-positive cells lining in regenerated vessels in mouse hindlimbs ischemia models [67, 82, 85]. Kamihata et al. [45] have shown that freshly isolated mononuclear bone marrow cells (BM-MNC) from swine promote angiogenesis in an infarction model by secreting bFGF, VEGF, and Ang-1 as well as by direct

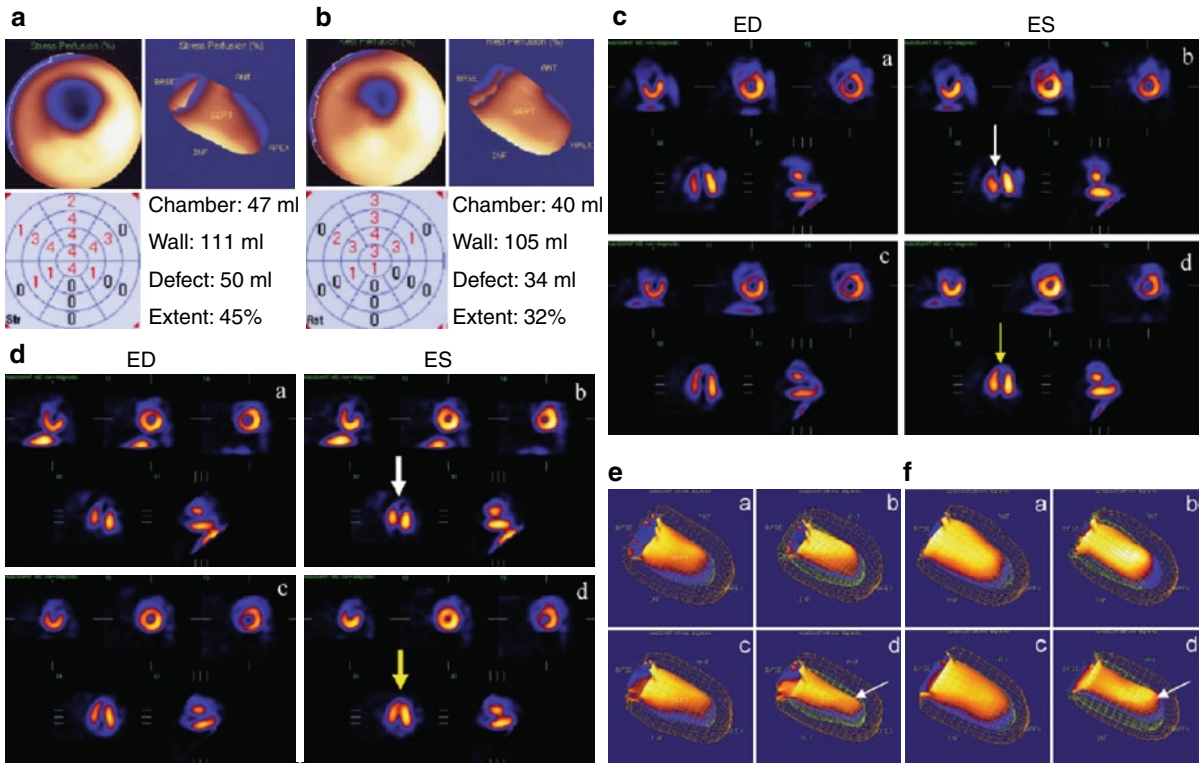

Fig. 7.2 Nuclear imaging. Bull's eye visual and semiquantitative polar plot analysis of perfusion defect (*blue zone*) in a representative pig injected with tissue-derived stem cell at base line (**a**) and follow-up (**b**). The antero-apical perfusion defect has visually decreased along with a decrease in extent from 45 to 32% of the calculated left ventricular mass on follow-up. Gated short- and long-axis images in control (**c**) and adipose tissue-derived stem cells (**d**) at baseline end-diastolic (**a**) and end-systolic (**b**) and follow-up end-diastolic (**c**) and end-systolic frames (**d**). Little improvement in perfusion defect and thickening is seen in the control pig, whereas modest improvement is seen in both parameters in an ADSC pig (*fine arrow*: control, *bold arrow*: adipose tissue-derived stem cells, *white arrow*: apex at baseline, and *yellow arrow*: at 4 weeks of follow-up). AutoQUANT derived 3D surface-rendered LVs in control (**e**) and adipose tissue-derived stem cells (**f**) at baseline end-diastolic (**a**), end-systolic (**b**) and follow-up at end-diastolic (**c**) and end-systolic (**d**). In the adipose tissue-derived stem cells pig at end systole, a resumption of inward contraction is clearly identifiable at the antero-apical infarct region when compared with the still dyskinetic wall movement in the control pig

incorporation into neocapillaries. Our group [2, 9, 107] showed that in vivo delivery of adipose tissue-derived MSCs differentiated into vascular cells and significantly improved myocardial function (Fig. 7.2).

Safety of cardiac MSC transplantation has always been a concern. There are reports that intracoronary cell injections can cause microinfarctions when large number of cells are administered [70, 110]. Breitbach et al. transplanted unfractionated bone marrow cells into infarcted myocardium and found encapsulated structures contained calcifications and/or ossifications [15]. Pak et al. reported that MSC transplantation resulted in higher and more heterogeneous sympathetic nerve sprouting than in swine without MSC transplantation. It is known that sympathetic nerve sprouting, and its heterogeneity, is one of the substrates for cardiac arrhythmia [17]. On the other hand, Fotuhi et al. showed that freshly isolated autologous ASC therapy is not proarrhythmic in pigs [25]. These results suggest again that ASCs may be a better source for cell-based therapies. A comprehensive electrophysiologic evaluation of MSCs would be necessary in order to avoid potential cardiac arrhythmia due to cell transplantation. In this regard, Bai et al. have shown that multiple functional ion

channel currents such as I(KDR), I(KCa), I(to), and I(Na.TTX) are present in undifferentiated hASCs [11] and that adipose tissue-derived human stem cells are capable to form Connesin43 [9].

7.4.2 Transdifferentiation

Recent studies have demonstrated that bone marrow-derived stem cells have a broad differentiation potential and are able to differentiate into new cardiomyocytes. However, the identity of BMSCs as true functional cardiomyocytes is still under intense debate.

Cardiomyocytes can be regenerated from bone marrow MSCs in vitro by exposing them to 5-azacytidine [62]. Because of the potential genotoxic effect of this chemical, other approaches to cardiomyocyte transdifferentiation have been explored. Shim et al. collected bone marrow from 16 patients undergoing coronary artery bypass surgery and MSCs were differentiated in a cardiomyogenic differentiation medium containing insulin, dexamethasone, and ascorbic acid included culture in medium enriched with dexamethasone and ascorbic acid [98]. The differentiated cardiomyocyte-like cells expressed multiple structural and contractile proteins that are associated with cardiomyocytes. Yoon et al. reported that MSCs pretreated with BMP-2+FGF-4 expressed sarcomeric-α-actinin and cardiac myosin heavy chain when injected into infarcted myocardium [121]. Rangappa et al. performed a coculture experiment in which human MSCs and human cardiomyocytes were mixed at a 1:1 ratio, and MSCs expressed myosin heavy chain and troponin-T after 48 h coculture [84]. Yuasa et al. reported that cardiomyocytes can be efficiently obtained from murine embryonic stem cells by stimulating them with Noggin, an inhibitor of BMPs [124]. Schimrosczyk et al. has shown that Liposome-mediated transfection with extract from neonatal rat cardiomyocytes induces transdifferentiation of human adipose-derived stem cells into cardiomyocytes (Fig. 7.3).

7.4.3 Paracrine Mechanisms

Despite initial promising reports, a recent study has shown that only a small proportion (~0.07%) of bone marrow MSCs could generate cardiomyocyte-like cells [65]. MSCs from umbilical cord and cord blood did not express cardiac markers either spontaneously or after treatment with 5-azacytidine [65]. Therefore, transdifferentiation may not account for all the functional benefits observed in studies of cardiac cell therapy. Paracrine actions may be responsible for at least some of the beneficial effect. Mangi et al. [63] showed that transplantation of MSCs overexpressing Akt into the ischemic rat myocardium inhibited the process of cardiac remodeling by reducing intramyocardial inflammation and collagen deposition. The same group later reported that paracrine action accounts for marked protection of ischemic heart by Akt-modified MSCs since Akt-MSC conditioned medium have the same effect as the cells [32].

Several growth factor therapies with antiapoptotic and proangiogenic benefits have appeared recently, including the ones with VEGF and IGF-I. However, due to their limited half-life and the fact that they are small in size, which restricts their retention within a tissue for prolonged periods, and difficulties in their administration, these proteins have shown a modest success in a number of in vivo studies even when using gene transfer [61]. For this reason, cell-based therapy has emerged since MSCs produce angiogenic and antiapoptotic factors [32]. Along this line, Sadat et al. [90] have shown that the cardioprotective effect of MSCs is mediated by IGF-I and VEGF.

A recent study by Lee et al. [58] has shown that intravenously (i.v.) infused human multipotent stromal cells (hMSCs) can enhance tissue repair without significant engraftment. The cells in lung disappeared with a half-life of about 24 h, but <1,000 cells appeared in six other tissues. The hMSCs in lung upregulated expression of multiple genes, with a large increase in the antiinflammatory protein TSG-6. After myocardial infarction, i.v. hMSCs, but not hMSCs transduced with TSG-6 siRNA, decreased inflammatory responses, reduced infarct size, and improved cardiac function. I.v. administration of recombinant TSG-6 also reduced inflammatory responses and reduced infarct size. The results suggest that improvements in animal models and patients after i.v. infusions of MSCs are at least in part explained by activation of MSCs to secrete TSG-6.

7.4.4 MSCs for Soft Tissue Repair

Both artificial implants such as nondegradable, inert, synthetic, and biodegradable implants and autologous adipose tissue have been used for soft tissue repair.

These treatments have many limitations, such as complications resulting from the toxicity of implants, unpredictable clinical results, and a low rate of graft survival [14].

Local stem cell application has recently been suggested as a possible novel therapy. ASCs are being studied intensively owing to the potential implementation in plastic and reconstructive surgery. ASCs are multipotent MSCs, which are more resistant to mechanical damage and ischemic conditions than mature adipocytes [109]. Moreover, ASCs can be readily harvested from excised human subcutaneous fat or liposuction samples and proliferate rapidly and differentiate into mature adipocytes both in vitro and in vivo [18, 125, 126]. ASCs have been increasingly reported to confer benefits in vivo as agents of angiogenesis and multilineage restoration in the face of soft tissue defects [3] [49, 50].

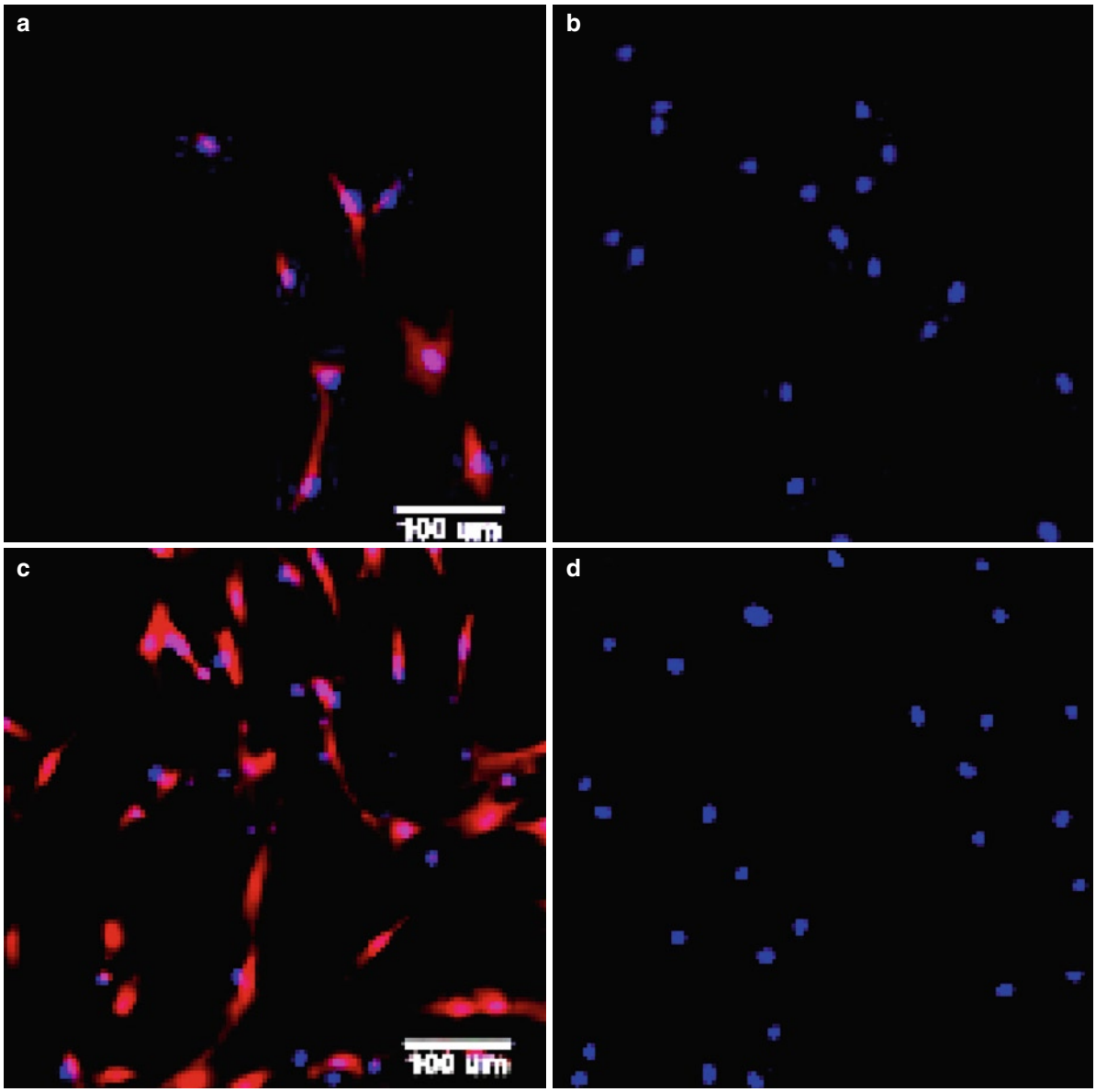

Fig. 7.3 Cardiomyogenic differentiation. ASCs were shown to express the cardiomyogenic markers Nkx2.5, c-troponin I and c-troponin T 2 weeks after transfection with nuclear proteins from cardiomyocytes or whole hearts. α-sarcomeric actin (**a**) 2 weeks, (**b**) 6 weeks; cTnT (**c**) 2 weeks, (**d**) 6 weeks; cTnI (**e**) 2 weeks, (**f**) 6 weeks; Nkx2.5 (**g**) 2 weeks, (**h**) 6 weeks

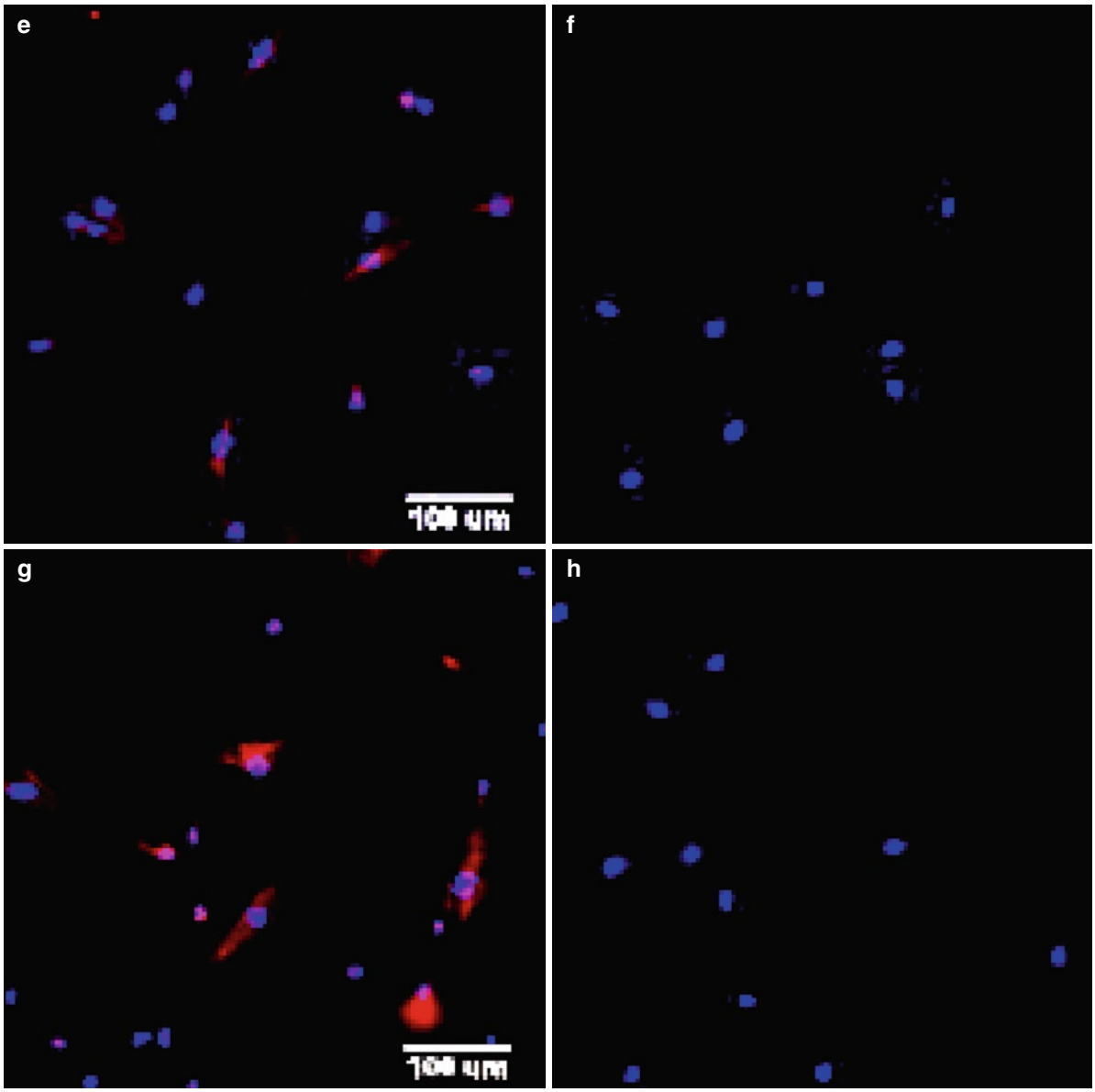

Fig. 7.3 (continued)

Altman et al. [5] evaluated the potential of a silk fibroin-chitosan (SFCS) scaffold serving as a delivery vehicle for human ASCs in a murine soft tissue injury model. GFP-labeled ASCs were seeded on SFCS scaffolds at a density of 1×10^5 ASCs per cm^2 for 48 h and then suture-inlaid to a 6-mm, full-thickness skin defect in 6-week-old-male athymic mice. Wound healing was tracked for 2 weeks by planimetry. They showed that the extent of wound closure was significantly enhanced in the ASC-SFCS group vs. SFCS and no-graft controls at postoperative day 8. Engrafted GFP cells were identified throughout the various substrata of the dermis and cutaneous appendages at 2 and 4 weeks postoperatively. Engrafted ASCs were identified, on the basis of GFP signal, at 2 weeks postoperative staining positive for the nuclear marker of proliferation Ki67, indicating ongoing proliferation of engrafted ASCs (Fig. 7.4).

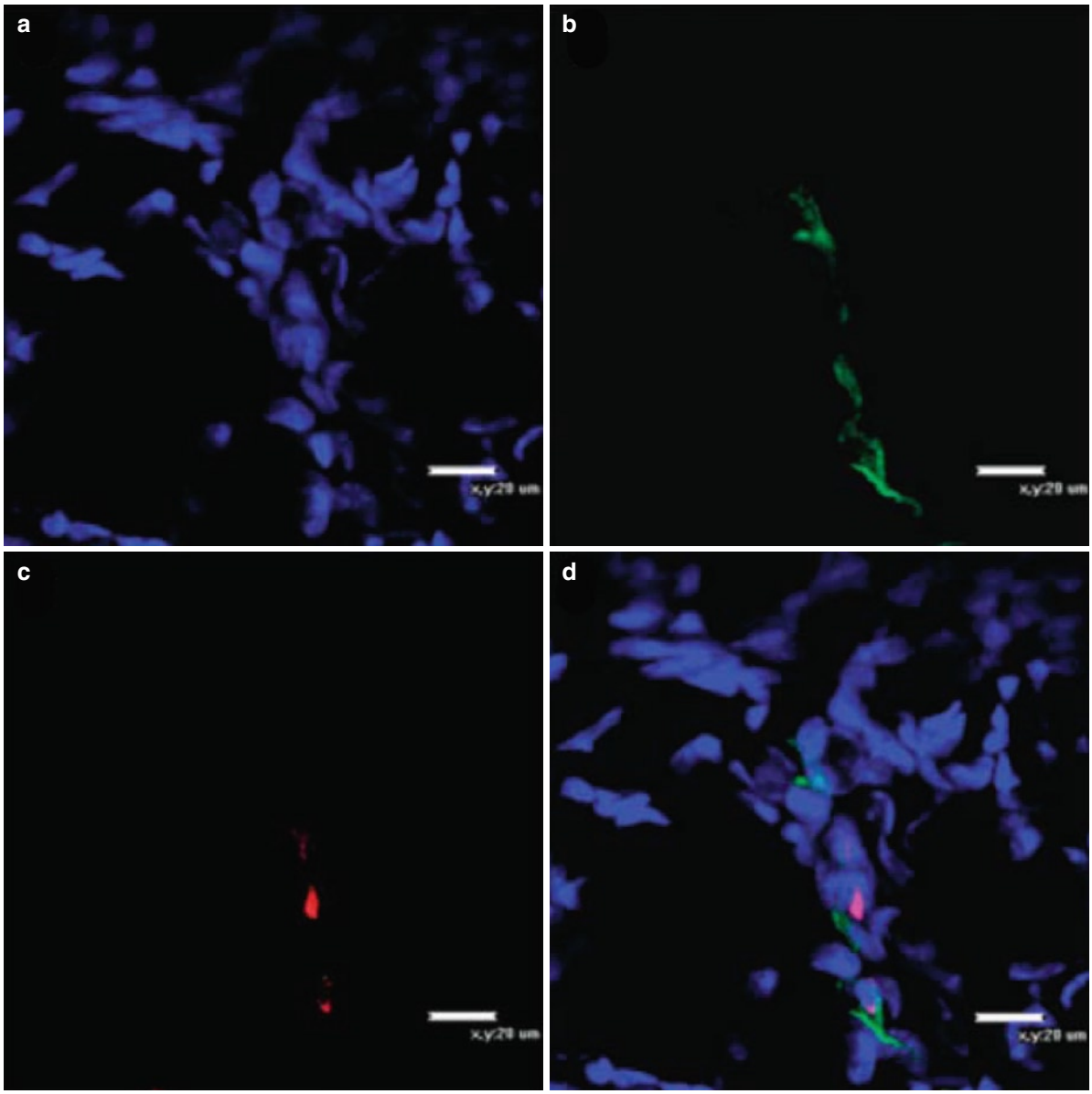

Fig. 7.4 Demonstration of engrafted human adipose-derived stem cells delivered via silk fibroin-chitosan (SFCS) scaffold in dermal tissue biopsy 2 weeks postoperative expressing the marker of proliferation Ki67. (**a**) 4,6-Diamidino-2-phenylindole staining indicates nuclei (*blue*). (**b**) Engrafted stem cells are indicated by green fluorescent protein staining (*green*). (**c**) *Red* staining indicates Ki67 signal overlapping with nucleus. (**d**) Overlay image demonstrates colocalization of all signals. Scale bars = 20 μm

Alloderm has been used as a good scaffold for skin and oral mucosa in tissue engineering, and it has been reported to organize the arrangement of seeded keratinocytes and cultured keratinocyte sheets into three-dimensional epidermal structure that has several layers, and to recreate the original rete ridges at the interface of the dermis [37]. The Alloderm has been found to produce composite oral mucosa equivalent consisting of a stratified epidermis on a dermal matrix using cultured oral keratinocytes and Alloderm [40]. Altman et al. [3, 4] showed that ASC-seeded alloderm is a pliable material with

potential dual function as a mechanical reconstructive agent and as a biolayer of stem cells for targeted delivery. The engrafted ASCs colocalized their GFP signal with a positive von Willebrand Factor signal when immunostained with the antibody against this endothelial antigen, indicating spontaneous differentiation of the engrafted cells into a vascular endothelial phenotype (Fig. 7.5).

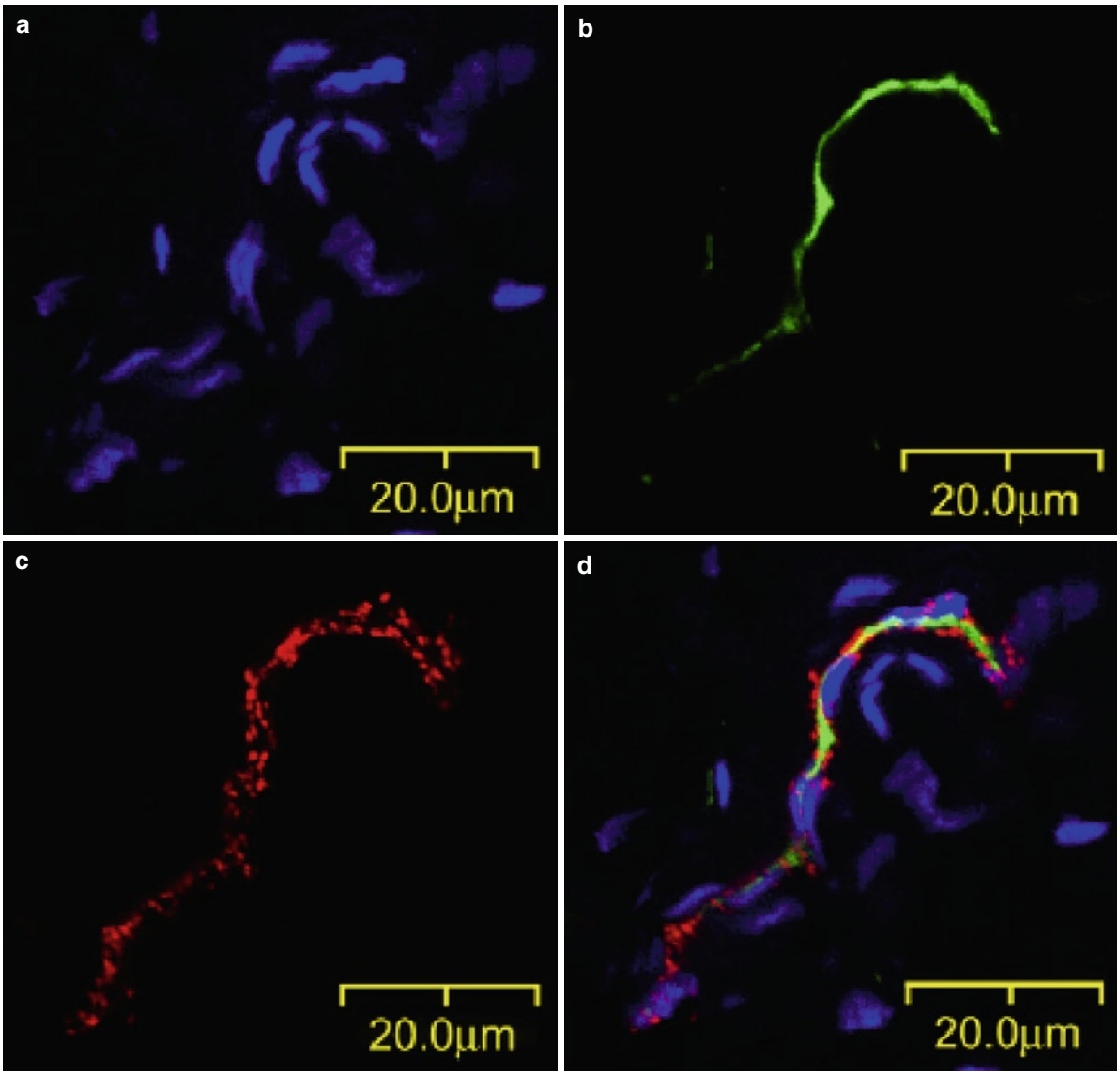

Fig. 7.5 Immunofluorescent and phase contrast examination of full-thickness skin from wound site in mice ASC–ADM engrafted. Sections are from wound biopsy at two (**a**–**f**) and four (**g**–**l**) weeks postoperatively. Hoechst staining (*blue*) indicates nuclei (**a**, **g**). *Green* staining indicates engrafted GFP-positive adipose-derived stem cells (**b**, **h**). *Red* staining indicates the marker of vascular endothelial phenotype, von Willebrand Factor (vWF) (**c**, **i**). GFP/vWF cells are seen reconstituting a serpentine linear structure consistent with reconstituted vascular tree, indicating participation of transplanted adipose-derived stem cells in vascular in-growth at the site of ASC–ADM engraftment. Scale bars represent 20 µm

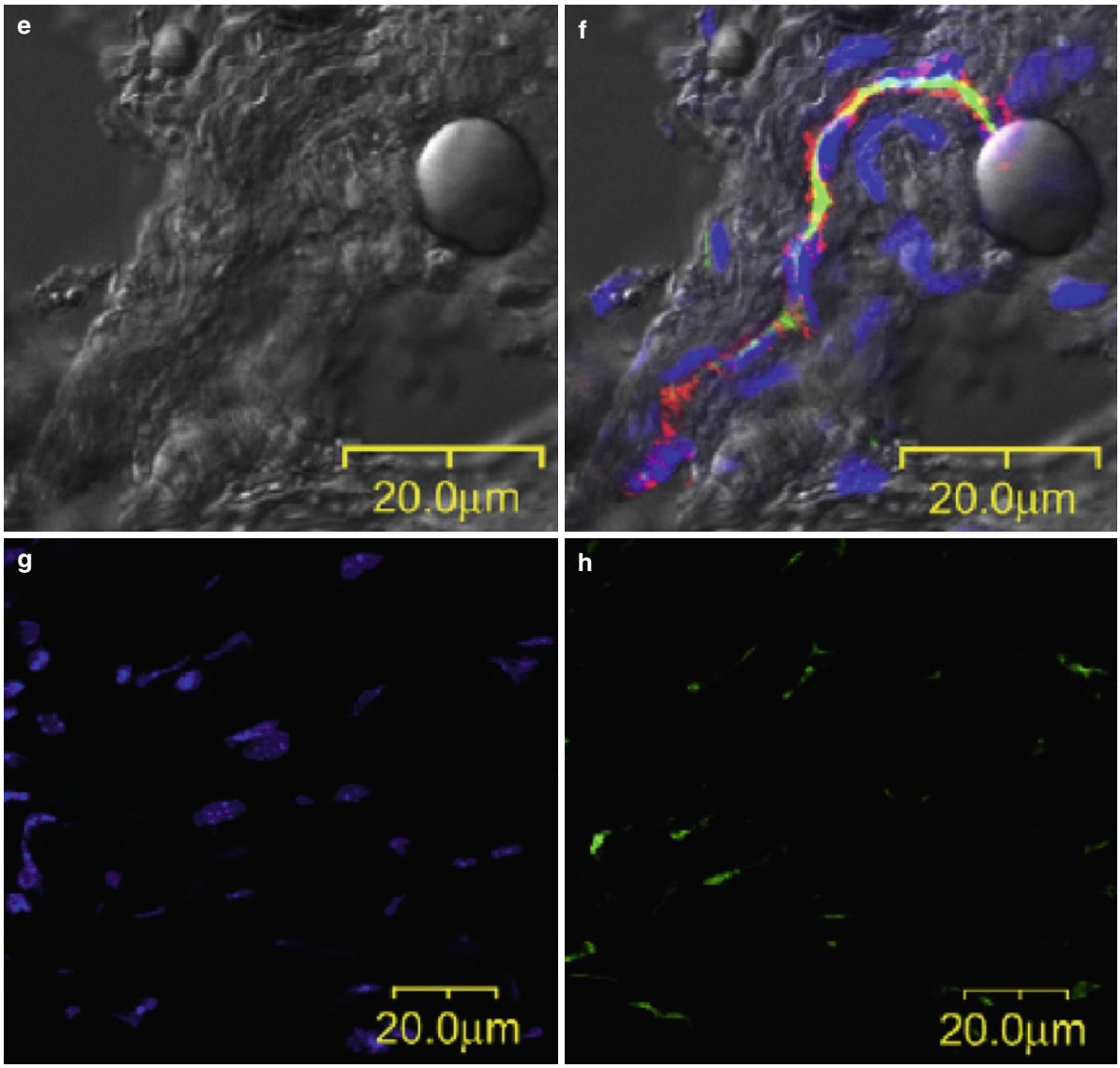

Fig. 7.5 (continued)

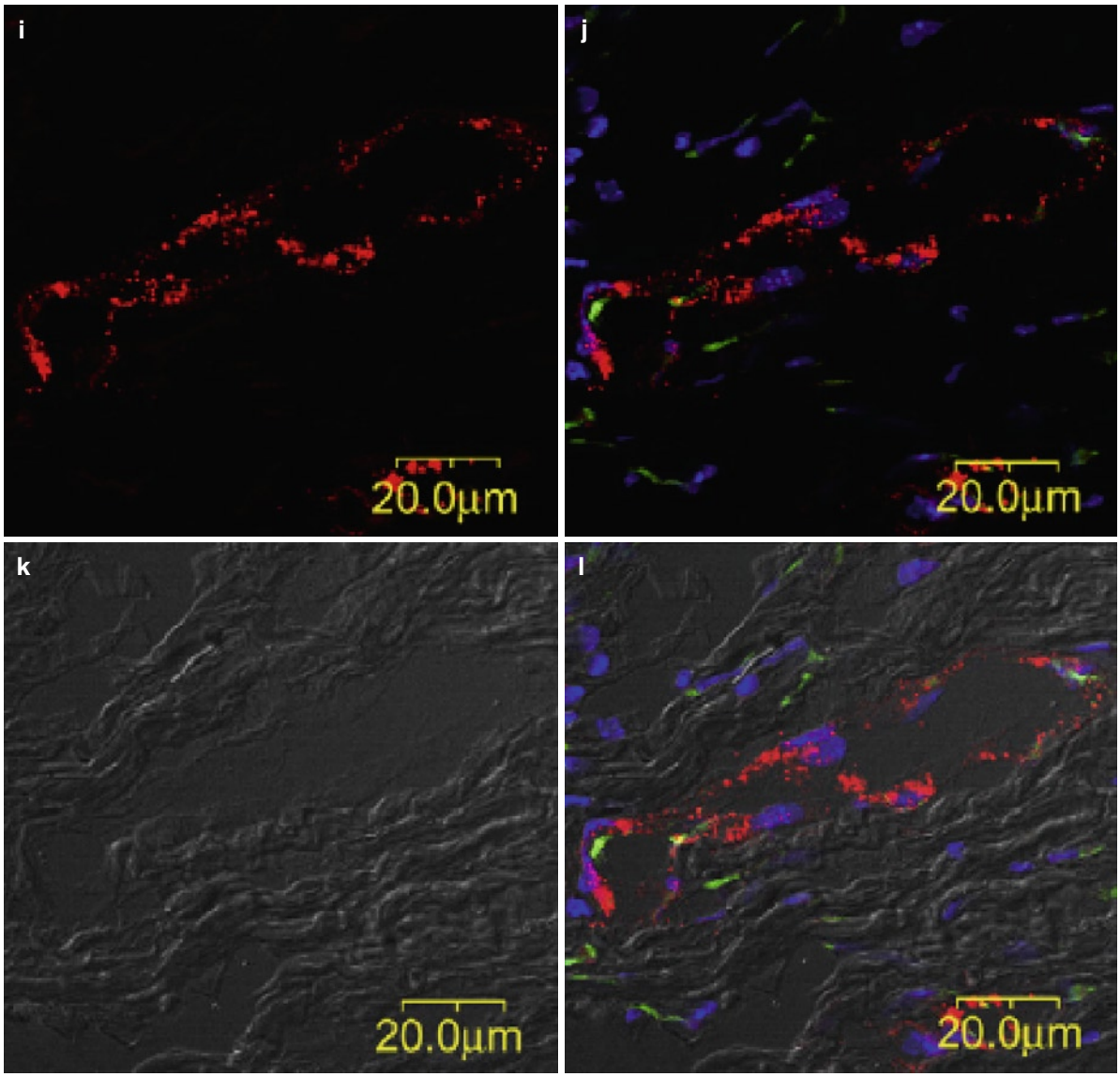

Fig. 7.5 (continued)

7.5 Future Perspectives

Over the past several years, MSC research has exploded and is getting more recognition as the research moves into the clinic. Tissue engineering in combination with stem cells could provide suitable, efficacious, alternative therapies for myocardial infarction, wound healing, and reconstructive and orthopedic applications. However, there are still issues related to stem cell transplantation that need to be resolved, including the timing for stem cell delivery and the choice of fresh vs. cultured cells for tissue repair.

Clinical studies such as the TOPCARE [8] and BOOST [120] trials infused bone marrow-derived cells within 4–5 days after MI. However, it has also been shown that myocardial interstitial edema after recanalisation of the infarct-related artery persists for at least a week [29]. The facts of a progressive increase in microvascular obstruction within the first 48 h after reperfusion [88] and limited homing after 5 days [38] might favor early cell transfer. Freyman et al. [27] injected bone marrow-derived MSCs 15 min after infarction. They found that the 14-day retention for intracoronary infusion and endocardial injection represented 6 and

3% of the administered dose, respectively. For the intravenous infusion group, none of the infarcts contained a measurable number of cells. Since the aim of that study was to compare quantitatively the three most common MSC delivery approaches following infarction in a large animal model with cellular engraftment as the endpoint, they did not report any functional analysis after stem cell administration. Furthermore, several groups have suggested that cardiac recruitment of stem cells requires both myocardial injury and expression of stromal-derived factor 1 (sdf-1) [1, 26]. Sdf-1 upregulation occurs immediately following myocardial injury [26]. Based on these previous studies, Valina et al. [107] delivered the cells immediately after infarct revascularization and demonstrated that the stem cells engrafted in the infarct region 4 weeks after intracoronary cell transplantation and improved cardiac function and perfusion via angiogenesis. Harvesting and culturing of ASCs in patients with acute infarction have only limited clinical merit since patients would not typically undergo cell harvest and culturing prior to an unpredictable event such as infarction. In order to address this issue, Alt et al. [2] evaluated the effect on myocardial infarction of a freshly isolated autologous uncultured cell preparation derived from subcutaneous adipose tissue administered into the infarct artery immediately after reestablishing blood flow. In their study [2], they demonstrate that 8 weeks after intracoronary administration of the regenerative cell preparation into the culprit vessel, left ventricular perfusion and ejection fraction are significantly improved in a pig infarction model mimicking closely the clinical scenario.

Finally, the implementation of quality assurance (QA) program based on the principles of good manufacturing practice (GMP) and a quality control system is a major requirement before starting phase I clinical trial. The development of GMP-based standard operating procedures (SOPs) for tissue and cell collection, processing, characterization, differentiation, and microbiological testing is essential to achieve a comparable high product quality.

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Animal Models for the Evaluation of Tissue Engineering Constructs

8

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8.1 Introduction

Nowadays, people reach higher and higher ages. As a consequence of this aging, more people have health problems that need to be solved by medical science. Obviously, there is a need for medical technologies to replace damaged or mal-functioning organs and tissues. At present, a wide range of synthetic materials, autologous grafts or allografts (donor tissues) or even organ transplants are available. However, most of these tissues have certain drawbacks, like availability or immunological complications. In an attempt to overcome these problems, scientists have focused upon using cells and stimulating factors to regenerate the lost or damaged tissues, the so-called tissue engineering.

When trying to regenerate any specific tissue, three basic components will be necessary for all tissue engineering approaches (tissue engineering triad); appropriate signals, cells, and a (resorbable) scaffold (Fig. 8.1). For designing a tissue-engineered substitute, two basic approaches can be defined. The first method utilizes a porous (implantable) material in which a certain growth factor (or a combination of growth factors) is incorporated. The growth factor to be used depends on the tissue required. Subsequently, the construct is implanted into the body defect to stimulate the surrounding cells to produce the extracellular matrix. The second method comprises a porous material as well; however, now (stem) cells are included. The scaffold loaded with cells will function as a reservoir, from which these particular cells will rebuild mesenchymal (muscle, bone, cartilage) or ectodermal (skin, nerve) tissues. In most cases, the cells will be cultured in the laboratory to increase in number before they are transplanted into the defect.

Obviously, tissue-engineered materials need to be tested for their safety and efficacy. When considering the testing of new materials three basic options seem to be available: performing tests on humans, animals, or tissue/cell cultures. Given that you want to find out how humans will respond to a tissue-engineered construct, performing tests on humans would be the most informative. However, for obvious ethical reasons, human testing can never be the first step in developing new materials. Also to bypass testing on

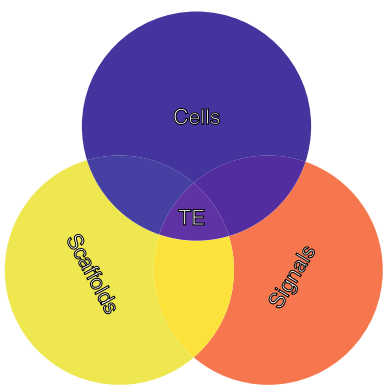

Fig. 8.1 The tissue engineering triad

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animals, human tissues or cell cultures can be used. Cell cultures and bioreactors both provide effective means for (initial) testing the safety and efficacy of a biomaterial. Another example in which cell cultures have proven to be useful is for the development and creation of vaccines. Nowadays, a (kidney) cell culture can be selected to develop a vaccine instead of using numerous animals as it was done in the past. Computer models to perform statistical testing (also known as in silico testing) are also available to reduce the need for animal testing, for example, to predict the behavior and the consequences of the substances delivered to the body. Today, computer models depend on the data sets collected from both in vitro and in vivo research done in the past. However, models may one day be used to predict the effects for which such studies have not been conducted (or not as many), thereby reducing our reliance on animal testing. Improved medical technologies, such as magnetic resonance imaging (MRI), enable researchers to investigate diseases using human scans. Also, this can be seen as a method to reduce animal testing. Despite the promising possibilities today, still (important) basic research questions cannot be (completely) answered by the previously mentioned tests. Especially, questions related to fundamental biological reactions and events to a certain material cannot be answered using any of the previously mentioned tests. Therefore research in animals is still unavoidable.

It is estimated that worldwide 50–100 million animals are used for research purposes annually. The great variance in the presented number is due to the poor documentation in some countries. In the Netherlands, the annual use of animals is gradually diminishing from 633.115 animals in 2004, 612.809 in 2005, 603.748 animals in 2006, and 597.605 animals in 2007. Most of the animals used (2007, the Netherlands) are the mouse (45.9%), and the rat (24.1%), together forming more than two-thirds of all the animals. Chicken comes in third (15.6%) and fish in fourth position (4.6%). In the Netherlands, the purposes of most research are, first academic (46.7%) and second for developing medicines, vaccines, medical devices, and so on (41.7%)[86].

At present, even though animals seem to offer the best possibility to preclinically test tissue-engineered constructs, their use is controversial. Animal experiments increasingly evoke societal debate. The stance one takes on animal experiments is largely determined by one's view on the moral status of animals. The most vehement opponents of animal experimentation believe that nonhuman animals have the same moral status as humans and that any intervention that we would not allow to be performed on humans should not be allowed to be performed on laboratory animals. The strongest defenders of animal research, on the other hand, believe that animals have no or hardly any moral status and that the likelihood of any benefit for humans is a sufficient justification for subjecting laboratory animals to experimental interventions. Most people believe, however, that although animals have a lower moral status than humans (for instance, because of our rationality or moral agency), their welfare still carries considerable moral weight. Accordingly, animal experiments, because of the suffering or discomfort they may cause, are considered to be morally problematic and are permissible only if they further a significant human interest. Moreover, the use and suffering of laboratory animals should be minimized whenever possible.

In line with this predominating view, most countries have established Animal Ethics Committees (or Animal Care and Use Committees). Researchers are legally required to have the approval of such a committee before carrying out their intended experiments. The ethical assessment of the proposed experiments by an Animal Ethics Committee typically aims to answer the following four questions:

1. Are there any alternatives to the proposed experiment?
2. What is the severity of the discomfort inflicted upon the animals by the experiment?
3. What is the benefit of the experiment for humans (and animals)?
4. Does the benefit for humans outweigh the suffering of the laboratory animals?

If there are any alternative ways available to answer the research question, then carrying out the originally proposed experiment is both morally and (at least in the Netherlands) legally prohibited. It is important to realize that the term "alternatives" in the context of animal experiments does not only refer to the avoidance of using living laboratory animals, but also, for example, using cells or computer programs instead. It refers to all the three R's of Replacement, Reduction, and Refinement (Table 8.1) [27]. If an alternative in any of these three senses is available, an Animal Ethics Committee will reject the proposed experiment (at least in its current

Table 8.1 The 3Rs; replace, reduce, refine

When considering animal studies, the guidelines of the 3Rs should be considered. The concept of the three Rs was introduced in the field of scientific experimentation by Russell and Burch [65] book "Principles of Humane Experimental Technique."
Replacement: Research has been done, and is still in progress, to investigate the possibilities to perform research without using (living) animals. Alternatives have been developed, such as the use of cell/tissue culture or the use of computer models.
Reduction: Different possibilities are available to reduce the number of animals used in research. Obviously, a good experimental setup and an accurate statistical analysis (power analysis prior to a new experiment) will allow for just the exact number of animals necessary; too many or too few animals will give unreliable test results and will imply that the test might have to be redone. Reducing the number of animals may also be accomplished by a structured research organization. For example, the first (pilot) research will be done with a minimal number of animals; in case of positive results, more sophisticated tests, possibly in other animals and/or more animals, can be done. One may also consider performing more than one test per animal; this will also reduce the number of animals, and of course, this is possible only in case of minimally invasive tests. The use of genetically identical animals is another method to reduce the number of animals. The interindividual variation will be smaller; this will prevent differences in study results because of genetic differences between the animals. Similarly, specific pathogen free, nondiseased animals also offer more reliable study results.
Refinement: Experiments can be performed in such a way that the stress and discomfort for the animals will be minimized as much as possible. For example, in case of postoperative pain, a pain killer can be given. When at several time points blood has to be taken, for example, to measure a certain hormone level, a researcher can, instead of performing several sessions in which blood will be taken, also implant a hormone-measuring sensor. Humane endpoints have to be mentioned as well. The principle is that in case of (severe) problems/suffering that eventually will lead to death, the animal can be taken out from the test at an early time point to prevent unbearable suffering. Important as well is the environment of the animal like the cage, materials within the cage, and social environment (solitary or in a group). All of these features can influence the animal's welfare and as a consequence the study outcome.

form). If there is no alternative, the Committee will turn to the weighing of the benefit for humans against the suffering of the animals. If the Committee is of the opinion that the knowledge produced by the experiment (for example, about the safety and efficacy of a tissue engineering construct) is so valuable that it outweighs the discomfort the laboratory animals are subjected to, then the proposed experiment may be carried out and the proposed animal model used.

8.2 Aim of the Discipline

As previously stated, due to aging of people there is an increasing need to restore or replace damaged/lost tissues. Scientists in the field of regenerative medicine are constantly trying to create newly developed tissues. The major targets for animal testing in tissue engineering are testing the safety and functionality of the product as also the basic biological principles of regeneration.

In line with these goals, desirable properties of an animal model include the demonstration of similarities with humans, both in terms of physiological and pathological considerations, as well as being able to observe numerous subjects over a relatively short time frame [26, 48, 69].

When deciding on the animal species for a particular study, several factors need to be considered. Logically, the first one must clearly define the research question as being addressed. Animal selection factors include: the costs to acquire and care for animals, availability, acceptability to society, tolerance to captivity, and the ease of housing [69]. Other factors comprise low maintenance care, ease of handling, resistance to infection and disease, interanimal uniformity, biological characteristics analogous to humans, tolerance to surgery, adequate facilities and support staff, and an existing database of biological information for the species. In addition, the lifespan of the species chosen should be suitable for the duration of the study.

Another important factor is to critically determine if the characteristics of the defect site are comparable to the human defect, for example, with respect to the bone density. When considering regenerative medicine using tissue engineering, one should keep in mind that surgically created defects will never fully mimic the defects caused by a certain genetic disorder or disease. Also, due to the differences in the biological systems, one has to be careful in extrapolating results from one species to another, such as humans. Obviously, the positive aspect is the possibility to create similar defects in order to observe the differences in the healing pattern after applying a range of bone substitutes or using various surgical approaches. Another aspect

is that the experimentally created defects, without any intervention, should not heal spontaneously during the course of the experiment; in other words the defects should be critical sized. Such a critical sized defect is defined as a defect, which left untreated, shows less than 10% healing of the bone during the lifetime of an animal [35]. Otherwise, the impact of the treatment might not be fully responsible for the achieved goal.

8.3 State of the Art

Because of the huge number of different models used in regenerative medicine, it is impossible to summarize all the models used; therefore, we are focusing upon our field of expertise. In this chapter, the focus is on the different animal models used for tissue engineering in dentistry. Where applicable, examples of bone tissue engineering and periodontal regeneration are highlighted. Others like Jansen et al. already gave an overview considering animal models in the complete field of regenerative medicine [37].

As previously mentioned, as tissue engineering has become popular in medical research over the last years, dentistry is in this respect no exception. In this field, research issues are mainly focused upon either the supportive hard tissues, i.e., the alveolar bone and the cementum, or the supportive soft tissue, i.e., the periodontal ligament (PDL). These tissues may be lost or injured for several reasons, such as decay, periodontal disease, trauma, genetic disorders, and continuing wear due to aging. Solutions offered today are artificial replacements such as crowns/bridges, removable partial dentures, and, as a last resort, (dental) implants. As a drawback, these solutions require regular maintenance and sometimes even replacement after several years of function. A more attractive, however, challenging solution, is to regenerate the lost tissues.

In case of periodontal disease (periodontitis), regeneration of the periodontium is an important issue. The periodontium refers to the specialized tissues that both surround and support the teeth, maintaining them in the maxillary and mandibular bones. The periodontium is composed of gingival epithelium, the cementum, which is attached onto the dental root surface and the PDL, which connects, by way of the cementum, the dental root to the alveolar bone. In case of absence of a tooth or molar, placement of a dental implant might be considered. However, in case of a bony defect, first regeneration of the alveolar ridge is demanded.

8.3.1 Animal Models Used in Dentistry

For studies investigating periodontal or bone interactions, an understanding of the species-specific bone and periodontal characteristics, such as bone microstructure and composition, as well as bone modeling and remodeling properties, are important when extrapolating the obtained results to the human situation. Finally, the size of the animal must be considered to ensure that it is appropriate for the number and size of the substitute material chosen. However, within a field of study, no single animal model will be appropriate for all purposes, nor can a model be dismissed as inappropriate for all purposes. Furthermore, multiple model systems are likely required to establish a broad body of knowledge [34].

In the next part of this chapter, according to the size of the animal, the most frequently used animal models will be described. For most of the presented models in small animals, it is possible to use the same models in larger animals as well; however, this is not done regularly due to the higher costs. A general overview of the animal models used in dentistry is depicted in Table 8.2. Additionally, the most commonly used animals will be discussed.

8.3.2 Rodent-Mouse Model

The most frequently used experimental model (as previously mentioned, 45.9% in the Netherlands), mice, are available either as regular, immune-suppressed (nude/athymic) or knock-out. Immune-suppressed implicates that the immune system is bypassed by breeding mice without a thymic gland. In knock-out mice, some specific genes are suppressed in order to study a specific disorder.

Advantages using mice could be the relatively low costs, known age, known genetic background, easy handling, and housing. However, as mice are small animals, they might present technical challenges. Like other rodents, mice differ somewhat in (dental) anatomy compared to humans, for example, the oral sulcular epithelium is keratinized while this is not the case in humans. Also, when considering the nature of the periodontal disease, the immune response in mice is different compared to humans; the neutrophils are the dominant infiltrating cells while in humans also lymphocytes, plasma cells, and macrophages are present. Therefore, the main area in which mice are used is the development of cardiac tissue and in understanding the basic molecular mechanisms

Table 8.2 Current animals models used in periodontal research

Animal	Positive aspects	Negative aspects
Nonhuman primate	Comparable dental anatomy	Expensive purchase and maintenance
	Comparable periodontal woundhealing	Hard to handle
	Suitable for studying furcation defects	Potentially infectious
	Experimentally induced defects do not spontaneously regenerate	Ethical debate
	Naturally occurring plaque and calculus	
Dog	Susceptible to periodontal disease	Much faster bone turnover rate and a different architecture and thickness of bone
	Reasonable number and size of defects	Great interanimal variability
	Suitable for studying furcation defects	Purchase and maintenance expensive
	Simple morphology of the roots	Ethical debate
	Docile temperament	
Rat	Most histological features of the epithelium and connective tissue similar to humans	Wear of occlusal surfaces (causes changes in tooth position)
		Continuous eruption
	Cheap purchase and maintenance	Small dimensions, potential surgical difficulties due to the size
		Number and size of defects limited
Minipig/ microswine	Histologically, the inflammatory process is similar to human situation	Expensive purchase and maintenance
	In older animals (>16 months), naturally occurring periodontitis with pocket depth >4 mm, can be increased using ligatures	
	Suitable for studying furcation defects	
Ferret	Occurrence of periodontal disease	Limited research has been done
	Spontaneous calculus formation	Small dimensions
	Course of periodontal lesions has similar pathway as in human	Potential surgical difficulties due to the size
		Number and size of defects limited
Hamster	Mouth opening almost 180°	Wear of occlusal surfaces (cause progressive changes in tooth position)
	Periodontitis can be introduced using ligaments	Small dimensions
		Primarily, horizontal bone loss when inducing periodontitis

that control normal heart development and subsequent congenital cardiovascular malformations [6].

In mice, an intraoral (surgical) approach is hardly possible due to the limited accessibility. However, for periodontal regeneration, tissue engineering approaches are feasible as Zhang et al. showed [85]. This study was developed to test a growth factor gene-releasing scaffold as a regenerative material for periodontal regeneration. In this scaffold, first human PDL cells were seeded and then implanted subcutaneously into mice. Results indicated that human PDL cells showed much better proliferation properties on the gene-activated scaffolds than on the scaffolds without the gene-activation. Moreover, the expression of the platelet-derived growth factor B (PDGF B) and type-I collagen appeared to be upregulated only in the gene-activated scaffolds. Implanting periodontal cells subcutaneously, thus bypassing the intraoral surgical approach, could be a good model for the preliminary testing of a newly developed material.

Inserting biomaterials subcutaneously into the back of the nude mouse is also a frequently used model in bone tissue engineering [43], because, due to the suppressed immune system, human cells can also be tested.

8.3.3 Rodent-Rat Model

The rat is the second most frequently used animal model in research, in the Netherlands even 24.1% of all animal research. Rats are inexpensive, easy to house, and might carry less societal concerns than other larger models. Several strains are available for experimental research such as albino Wistar rats and Sprague-Dawley rats, which are characterized by their calmness and ease of handling. Additionally, the so-called immune-suppressed and knock-out rats are available, therefore making them attractive candidates for bone research, for reasons as previously described.

In rats, a varied scale of (dental related) models has been proposed using not only the long bones and the calvaria, but also intraoral regions. For example, for early proof that bone morphogenic proteins (BMP) have a beneficial effect on bone regeneration, rat models were successfully used. Takagi and Urist showed that extracted bovine BMP could be used to heal large *femoral defects* in Sprague-Dawley rats [76]. These defects were created by resecting one centimeter of the middle one-third of the femoral diaphysis. This is considered a so-called critical defect [35]. The authors used a so-called omega pin and inserted it into the femoral medullary canal to distract the two ends of the femur and to block the migration of the osteoinductive and osteoconductive elements within the marrow. Although a variety of graft materials were evaluated, including variable doses of BMP without a carrier, only the defects treated with BMP and autogenous marrow healed 100% of the time.

Yasko et al. in a similar type of model found that BMP-2 combined with bone marrow in a rat *segmental femoral defect model* (5 mm segmental defect, approximately twice the diameter of the diaphysis) produced union at a rate of 100%, three times superior to the rates achieved with only autogenous cancellous graft [83].

Le Guehennec et al. compared the bone colonization of a macroporous ceramic (calcium phosphate, CaP) in three different sites; femur, tibia, and calvaria, in both rats and rabbits. The defects created in the rat were a combination of a (*lateral*) *femoral condyle defect and a* (*proximal*) *tibial defect.* The defects, in the femur and tibia, were simultaneously prepared in one surgical approach (entry), and had a 3 mm diameter and a 3 mm depth. For the rabbits, the defects were slightly larger and had a diameter of 4 mm and a depth of 6 mm. For the *calvaria defect,* two bilateral symmetrical round defects were created using a trephine drill in both parietal bones. In the rat, a diameter of 3 mm was chosen. The defects created in rabbit calvarium were 6 mm in diameter. Bone colonization in the empty and filled defects was measured. In the empty cavities, bone healing remained on the edges, and did not bridge the defects. Bone growth was observed in all the ceramic implantation sites in rats. The bone colonization appeared statistically higher in the femur of rabbits than in the tibia and calvarium sites. This slightly higher degree of bone healing was related to the differences in the bone architecture of the implantation sites. Concerning the comparison between animal species, bone colonization appeared greater in rabbits than in rats for the femoral site. For the other two sites (the tibia and calvaria), there was no statistically significant difference. The increased bone ingrowth observed in rabbit femurs was attributed to the large bone surface area in contact with the ceramic material [47].

Develioğlu et al. used a comparable type of *calvarial defect model*, as previously mentioned; instead of creating one defect in the center of the skull, two defects (diameter 5 mm) were created, one in each parietal (cranial) bone next to the midline. This study evaluated the effect of a biphasic ceramic (hydroxyapatite 65% and tricalcium phosphate 35%) compared to the empty defects on the osteogenesis. From this study, it was concluded that the test material is biocompatible, but does not offer any advantage over using hydroxyapatite ceramics as the only component. Furthermore, it had no effects on bone regeneration and it solely seemed to maintain the created space [20].

Bodde et al. created a critical sized *cranial* (*calvaria*) *defect*, in the center of the skull, 8 mm in diameter, using a trephine drill. While the previously mentioned studies created two smaller defects, in this study, one larger defect was created. Different BMP-2 loadings in a sustained release system were evaluated. The system comprised of CaP cement with BMP-2 loaded micro particles. Two evaluation times were used. Histology showed defect bridging only in the highest dose BMP-2 group. Implant degradation was limited in all formulations, but was the highest for the high-dose BMP-2 group. Already, low amounts of sustained released BMP-2 were sufficient to bridge critically sized defects. A substantial amount of BMP-2 was retained in the implants because of the slow release rate and the limited degradation (Fig. 8.2) [11].

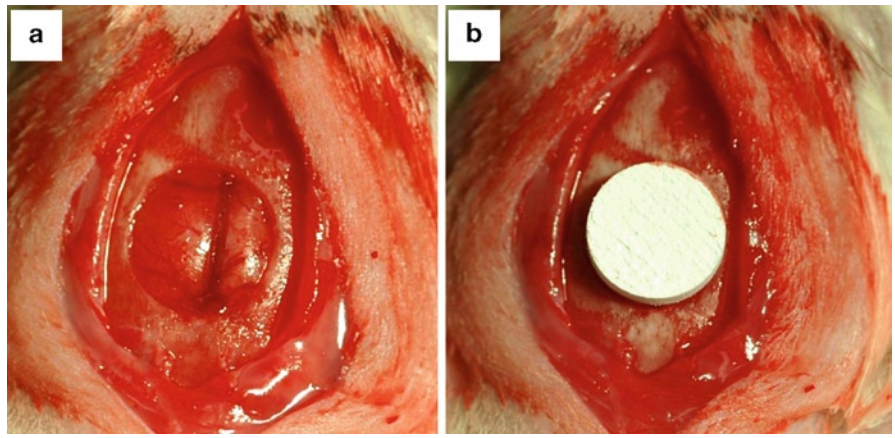

Fig. 8.2 Cranial defect in the rat. (**a**) The empty calvarial defects prepared with a trephine drill (diameter 8 mm), (**b**) the defect filled with composite (with permission [11])

Calvarial defects in rats can be used to provide good first phase (nonload bearing) bone healing models with relative biological inertness due to poor blood supply and limited bone marrow, which is thought to resemble the atrophic mandibular bone in humans [70].

For intraoral research some interesting models have been described. For example, Ohnishi et al. proposed a *guided bone regeneration* (*GBR*) *model for the rat maxilla*. Therefore, the first and second molar in the maxilla were extracted. After 1 month, an artificial bone defect in this region was created (width 1.0 mm, depth 0.5 mm, mesio-distal length 2.5 mm) by means of a round burr. In this study, the effects of membrane application periods on newly formed bone and its remodeling process after the removal of the membrane were evaluated. It was concluded that the long-term application of a membrane induces the enlargement of the bone marrow spaces. Therefore, it was suggested that the Teflon membrane removal in adequate time promotes the corticalization and maturation of the newly formed bone by the GBR technique [60].

Salata et al. used a *GBR model in the mandible*; 3-mm diameter bilateral transcortical defects were produced in the mandibular ramus [67]. This study was designed to evaluate the biocompatibility of a novel membrane. At 4 weeks, complete bony regeneration of these ramus defects had not occurred with either the membrane or the control (open) defects. However, in contrast to the control defects where newly formed bone was confined to the bony margins, the defects covered with a membrane showed evidence of bone formation centrally [68].

Donos et al. also used the mandibular ramus, but in a completely different fashion. On the lateral part of the mandibular ramus, a hemi-spherical polytetrafluorethylene (PTFE), rigid capsule was placed. Their aim was to evaluate whether the use of enamel matrix proteins (Emdogain®) with or without Bio-Oss® influences bone formation when used as an adjunct to GBR. Four groups and several time points were used; empty capsule, capsule filled with Emdogain®, capsule filled with Bio-Oss®, capsule filled with Bio-Oss® mixed with Emdogain®. It was concluded that neither the application of Emdogain® nor the use of Bio-Oss® or the combination of Bio-Oss® and Emdogain® resulted in enhanced amounts of bone formation in comparison with the GBR procedure alone [23].

Also, the *inferior border of the mandible* can be used as a location to investigate, for example, the long-term stability of bone grafts with or without the combined application of GBR [24]. In this study, a calvarial bone graft was placed at the inferior border of the mandible and was subsequently fixated. At the contra lateral side, an ischiac bone graft was positioned. There were groups with and without a GBR membrane. Width, length, and thickness/height of the bone graft were evaluated. At 5 months, both types of membrane-treated bone grafts presented an increase in all dimensions compared to the baseline. However, at 11 months, both types of membrane-treated bone grafts exhibited a decrease in their dimensions. In the control groups (without membrane), both types of bone graft presented significant resorption both at 5 and at 11 months with the ischiac bone grafts presenting more resorption in width and length than the calvarial bone grafts. It can be concluded that the long-term volume stability of the bone grafts combined with GBR is superior to that of the bone grafts alone.

Donos et al. evaluated the potential of several biomaterials for heterotopic bone formation. After an incision in the middle of the thorax the pectoral muscle was identified. *Intramuscular pouches* were created within this pectoral muscle. Three different materials (Bio-oss® alone, Bio-oss® mixed with Emdogain®, and Bio-oss® mixed with only the carrier of Emdogain® (propylene glycol alginate) were evaluated at several time points. They concluded that intramuscular bone formation did not occur in any group at any time point [25].

As can be observed from the above-mentioned studies various models can be used to study GBR. Fewer models are available for evaluating the potential of periodontal regenerative procedures. Obviously, as also in mice, the dimensions of the animal are the limiting factors concerning intraoral research. The possible dimensions of a construct or a material are limited, but not impossible as Nemcovsky et al. showed, using a surgically created *suprainfrabony periodontal defect*. The effect of Emdogain® was studied [57]. After a midcrestal incision mesial of the first maxillary molar, and raising a full thickness flap, alveolar bone mesial of this molar was surgically removed together with the PDL and the remaining cement, and an intrabony defect of reasonable dimensions was created. In the study group, Emdogain® was applied, and in the control group, only propylene glycol alginate (carrier of Emdogain®) was applied. In the study group, smaller gingival recession, deeper gingival sulcus, and shorter junctional epithelium were found. New cementum was observed only in the Emdogain® group. New bone formation was similar, however, very limited, in both the groups. From this study, it was concluded that Emdogain® enhanced periodontal healing by reducing gingival recession and junctional epithelium along the root surface and enhancing the formation of new cementum.

King et al. evaluated the effects of BMP-2 on wound healing in a rat model [40]. The buccal aspect of the molar roots was carefully denuded of the PDL through a bony window created in the mandible (the so-called *fenestration defects*). This was done from an extraoral approach. A BMP-2 collagen gel solution was placed into the surgically created defect in the test group. The control groups, either received only the collagen gel, or the defect was left completely empty. It was concluded that a single dose of BMP-2 increased the rate of normal bone formation and selectively enhanced cementum formation during early wound healing.

Using the same fenestration model, Flores et al. evaluated whether human PDL cell sheets could reconstruct damaged periodontal tissue [28]. Human periodontal cells were cultured with or without an osteogenic differentiation medium. The cell sheets were transplanted on *periodontal fenestration defects* in immunodeficient rats. Most of the experimental group specimens (osteogenic differentiation medium) exhibited a new cementum-like layer and a new attachment of collagen fibers to the layer. Histomorphological analysis indicated significant periodontal regeneration. The control group revealed dense extracellular matrix and fiber formation, but an obvious cementum layer was not present. The authors concluded that culturing PDL cells could be feasible as a new therapeutic approach for periodontal regeneration.

Although technically demanding, due to the size, several rat models, even intraoral models, can be used within the field of regenerative medicine.

8.3.4 Rabbit Model

In approximately 35% of musculoskeletal research studies, rabbits are the first choice, mainly in relation to their reasonable anatomical size and lower costs in terms of purchase and maintenance as compared to larger animals [1]. As an additional advantage, rabbits reach skeletal maturity shortly after sexual maturity, around 6 months of age [31]. As a drawback, their fast skeletal change and bone turnover makes extrapolation of the obtained results from such studies to human clinical response difficult.

Also in rabbits, the *calvarial defect model* has been used to test various biomaterials. For example, a study was performed to evaluate the effect of silk fibroin nanofiber membranes on bone regeneration [39]. In this study, 8 mm defects were created bilaterally, one on each side of the midline. Two different types of membranes were tested. At 12 weeks, in the silk fibroin nanofiber membrane group, the defect had completely healed with a new bone. The authors concluded that the silk fibroin nanofiber membrane showed good biocompatibility, which enhances bone regeneration without evidence of any inflammatory reaction.

When considering bone regeneration, loading of bone is an important factor. For this reason, various models have been proposed utilizing load-bearing

parts of the body, such as the limp. Unfortunately, till now, the rabbit does not seem to be suitable for load-bearing studies.

For example, Bodde et al. evaluated *the rabbit radial defect* (Fig. 8.3) as a potential critical size model [13]. It was hypothesized that the intact (relatively large) ulna could stabilize the radial defect, without the need of additional osteosynthesis material. In this model, the effect of poly(D,L-lactic-co-glycolic acid) (PLGA) on bone regeneration was evaluated. Rabbits received bilateral segmental radial defects of 15 or 20 mm. The osteotomy margins were marked with small titanium pins positioned at 7 mm of the defect margins (Fig. 8.3). Subsequently, the test group received the injectable PLGA/carboxymethylcellulose implants (Figs. 8.4 and 8.5), while the control group was left empty. The evaluation consisted of radiographs taken immediately after surgery and after sacrifice, as also microcomputed tomography and histomorphometry. Interestingly, the radiographs revealed (in which both the titanium pins can be spotted) that after sacrifice, the created defects were significantly reduced, pointing out the instability of the created defect. As most defects were more or less regenerated, it was concluded that both 15 and 20 mm were not of critical size. Also, PLGA did not influence bone regeneration significantly. From this study, it was concluded that 15- and 20-mm radius defects appeared not to be a suitable model for bone regeneration as these defects were neither of critical size nor stable.

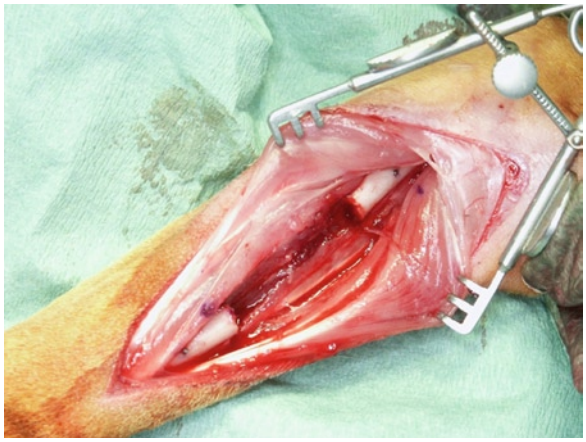

Fig. 8.3 The segmental radial defect completed; 7 mm from both the defect edges, the titanium pins can be observed. These pins are placed for defect measurements postoperatively and at sacrifice (with permission [13])

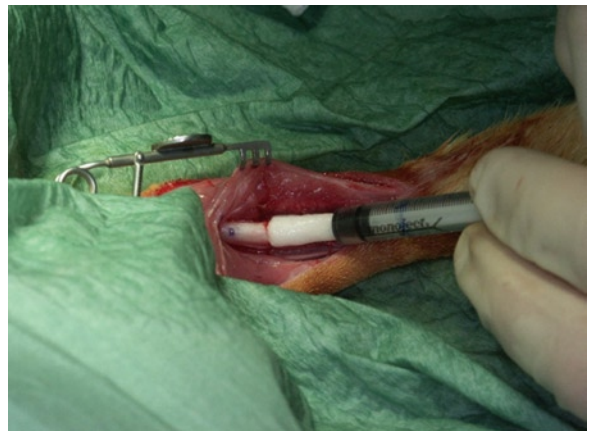

Fig. 8.4 The D,L-lactic-co-glycolic acid (PLGA) microparticle/carboxymethylcellulose material being injected with a disposable (monoject®) syringe (with permission [13])

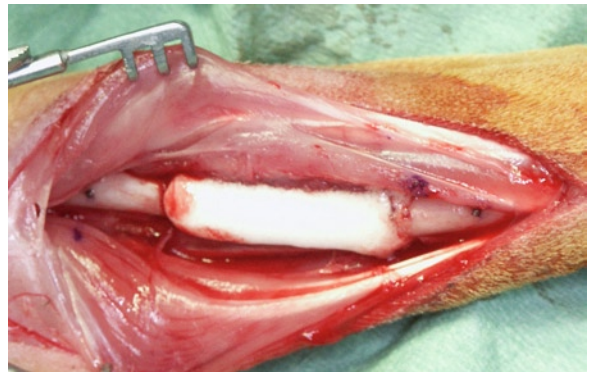

Fig. 8.5 The construct in place (with permission [13])

With respect to a critical size defect model, only 15 mm calvarial defect reveal to be reliable as such; however, this is not a load-bearing model [4, 35, 70].

The *femora of the rabbits* are suitable test sites, as shown by Voor et al. evaluating the "in vivo" performance of several different ceramic bone substitutes compared to the cancellous bone [78]. Bilaterally cylindrical defects were created in the distal femur. Defects were drilled from the lateral direction to a 10 mm depth with 5.5 mm as diameter and subsequently filled according to the study protocol. After both mechanical and histological evaluation, it was concluded that composites of hydroxyapatite cement and porous biomaterials can maintain a relatively high strength over 8 weeks in vivo, but their incorporation into a new bony structure is slower than the incorporation of the impacted cancellous bone.

In an alternative approach, Link et al. used the *rabbit femoral condyle* to create a defect, measuring a diameter of 4 mm and a depth of 6 mm [49]. In this study, the combination of a growth factor (TGT-β1) incorporated into the microparticles mixed with a CaP cement was tested. Different healing periods were analyzed. The results showed a gradual increase in mechanical strength with longer healing periods. In conclusion, microparticles containing CaP composites show excellent osteogenic properties and a rapid increase in mechanical strength. The addition of TGF-β1 significantly enhanced the bone remodeling process.

Young et al. described an accessible and reproducible, *alveolar bone defect in the mandible*, as an alternative to the existing models [84]. Two types of defect in the mandibular premolar/molar region were compared. The first defect was a bicortical 10-mm diameter defect; besides both the cortical plates, the trabecular bone and the roots were also completely removed. The second defect had the same diameter but only involved the buccal cortical bone plate, the trabecular bone, and the roots. Both the types of defect were evaluated in time (up to 16 weeks) using histology and microcomputed tomography. The results indicated that only in case of a bicortical defect a critical size defect was created.

In a comparative model, Oortgiesen et al. demonstrated that this model is not suitable for the evaluation of periodontal regeneration [61]. *Periodontal fenestration defects* were created bilaterally on the first and second molars in the mandible using an ultrasonic device to remove the alveolar bone, together with the PDL and the cementum. The dimensions of the bone window were 4×6 mm. Histology was performed to evaluate the regeneration of the periodontal defect in time. Unfortunately, due to the fast eruption of the teeth, before regeneration could take place, the created defect was already shifted to the oral cavity. Therefore, this model seems to be unsuitable for further studies in the field of regenerative periodontology.

The rabbit seems well suited for studies in the bone regenerative medicine, while it does not appear suitable for periodontal regenerative research.

8.3.5 Canine (Dog) Model

The dog is one of the more frequently used large animal species for musculoskeletal and dental research, although in the Netherlands, it is only 0.3% of the totally used animals [86]. The popularity to use dogs in dental research is probably due to the close similarity in bone composition between the dog and human. Another reason is the need for dog studies when aiming for a Food and Drug Administration (FDA) approval for certain devices. Studying the differences in bone composition, density, and quality between various species (human, dog, sheep, pig, cow, and chicken), it was concluded that the most similarity in bone composition (ash weight, hydroxyproline, extractable proteins, and IGF-1 content) is between the dog and human. Regarding bone density, beside the dog, the pig also most closely represents the human situation. The authors concluded that of the components tested, the characteristics of human bone are best approximated by the properties of the canine bone [1]. These results also supported earlier findings, where human and dog cortical and cancellous bone was found to be similar in terms of water fraction, organic fraction, volatile inorganic fraction, and ash fraction [32].

Unlike other animal species, there is a limited amount of literature comparing canine and human bone with regard to the usefulness of the dog as a model for human orthopedic conditions. Neyt et al. found that dogs and cats were used in 11% of musculoskeletal research between 1991 and 1995 [59]. This was confirmed in a later study by Martini et al., who reported that between 1970 and 2001, 9% of the orthopedic studies used dogs as an animal model [52].

Bone remodeling is a factor that should be considered as a difference between human and canine bone, especially when assessing the qualitative effect of the bone graft material. This is an important consideration as graft material-associated changes evident in a canine model may not be as apparent in humans where there is a lower rate of remodeling [8, 9].

Beagles are the dog breed most often used in animal testing, due to their size and passive nature. In 2005, Beagles were involved in less than 0.3% of the total experiments on animals in the UK, but of the 7,670 experiments performed on dogs 7,406 involved the Beagles (96.6%) (Statistics of Scientific Procedures on Living Animals Great Britain 2005).

Also for periodontal studies, although expensive, beagle dogs are the preferential model. Boyko et al. evaluated the potential use of PDL cells and gingival cells for tissue engineering [15]. In a *tooth replantation model*, first, all the premolar teeth were extracted.

Subsequently, cells from their PDL and attached gingiva were cultured in vitro. Hereafter, the lateral incisors were extracted, their PDL and pulps removed, and the root canals filled. Subsequently, these roots were demineralized and thereafter divided into three groups. Cultured gingival cells were attached to those in the first group, PDL cells to those in the second, and finally, no cells to the roots in the third (control) group. The roots were transplanted into the holes drilled in the edentulous premolar region of the mandibular alveolar process and were completely submerged during healing. All the roots exhibited ankylosis and resorption. Only the roots bearing cultured PDL cells were associated with a partial PDL.

Another study by Isaka et al. in a similar approach compared healing after extracting the roots of mandibular third premolars [36]. The roots were divided into two groups, a group in which the PDL was preserved, and a group in which the PDL was removed. Subsequently, the roots were respectively transplanted into surgically created bone cavities with additional buccal and interproximal bone defects. Both bone defects and transplanted roots were completely covered with membranes and submerged for healing. The defects were allowed to heal for 6 weeks. It was concluded that PDL preservation might act in the regeneration of PDL with new cementum formation.

Such *tooth replantation models* could be used to apply tissue-engineered constructs to roots and thereafter evaluate their potential to regenerate new periodontal tissue.

Wang et al. investigated periodontal healing after PDGF application onto the root surfaces [80]. *Fenestration defects* (4×4 mm) were surgically created on the posterior teeth. In order to create the fenestration defects a flap was reflected on the buccal aspect. With burs under continuous cooling, fenestrations were cut through the bone to expose the roots. Thereafter, the cementum was removed and thereby the dentin exposed. Subsequently, one out of four treatment modalities was performed. These were either control (saline solution) or one of the three test modalities being (1) nonresorbable membrane, (2) PDGF, or (3) the combination of a nonresorbable membrane and PDGF. The animals were sacrificed after 1, 3, or 7 days postoperatively. It was concluded that PDGF enhanced fibroblast proliferation in early periodontal wound healing, whether used alone or in combination with a nonresorbable membrane.

Akizuki et al. in a slightly different approach evaluated periodontal healing after the application of a PDL cell sheet [2]. First, using PDL cells from the extracted premolars, PDL cell sheets were fabricated. Then, *dehiscence defects* were surgically created on the buccal surface of the mesial roots of the bilateral mandibular first molars. In order to create the defects, a buccal mucoperiosteal flap was elevated, and a square-shaped dehiscence bone defect (5×5 mm) was created using burs. In the experimental group, the cell sheet in a carrier was applied and in the control group only the carrier. All the animals were sacrificed after 8 weeks. It was concluded that the PDL cell sheet has a potential to regenerate the periodontal tissue and may become a novel regenerative therapy. The advantage of a fenestration defect model is that in the absence of the oral (pathogenic) flora materials can be tested.

To mimic periodontal defects, as also found in humans, dogs are preferentially used. For example, Murakami et al. evaluated periodontal regeneration, following the application of basic fibroblast growth factor (bFGF) into *surgically created furcation defects* [56]. These were created between the roots of the mandibular first molar on both the left and right side. After flap elevation, the buccal bone was removed until the furcation region was exposed. Within the furcation, 4 mm of bone was removed in an apico-coronal direction and 3 mm in a horizontal direction towards the lingual furcation. Thereafter, the root cementum together with the remnants of the PDL was eliminated. Finally, an impression material was placed into the defect in order to create inflammation. The wound was closed and surgically reopened after 4 weeks. At this time, the area was debrided and the root surfaces were root planned. Subsequently, for histological evaluation, a notch was placed in the base of the defect. The control defects (carrier only) were compared to the defects treated with the carrier containing bFGF. Sacrifice of the animals was executed 6 weeks postoperatively. In all the sites in which bFGF was applied, PDL formation with new cementum deposits and new bone formation was observed, in amounts greater than in the control sites. From this study, it was concluded that the topical application of bFGF could considerably enhance periodontal regeneration in artificially created furcation class II defects.

Doğan et al. aimed to assess the seeding of cells derived from regenerated PDL (RPL) to promote the regeneration in *artificial furcation defects* [21]. First,

a surgery was performed to create a furcation class II defect; 5 mm of coronal bone and 2 mm of horizontal bone were removed within the furcation site of the third premolar. Then, to cover the defect nonresorbable membranes were placed. Four weeks postoperatively, the membrane was surgically removed and subsequently, biopsies were taken of the RPL that had grown underneath the membrane. By incubating these biopsies, fibroblast-like cells were obtained. For regeneration, fourth passage cells that had reached confluency were used; 2×10^5 cells were added to 0.5 mL of blood coagulum. Thereafter, class II furcation defects were induced on the second and fourth mandibular premolars. In the test group, the coagulum, containing the RPL-cells, was placed whereas in the control defects, on the contra lateral side, the furcation defect was left open. Sacrifice of the animals was executed 42 days postoperatively. From this study, it was concluded that the regeneration of furcation defects by cell-seeding techniques may be useful, but further studies are needed to determine the outcome of the procedure. A similar cell approach was described by Doğan et al. in 2003 [22]. Now *artificial fenestration defects* were regenerated. These fenestrations were surgically created by removing the buccal alveolar bone, 5×5 mm, with bone chisels, while keeping a 3 mm of marginal bone intact at the coronal border of the defects. Also in this study, they concluded that seeding of cells from PDL may be promising to promote periodontal regeneration, but needs to be investigated in further studies.

The defects in the previous study were surgically created. Another option is to try to create defects by inducing disease instead. For example, Kinoshita et al. examined periodontal regeneration after applying BMP-2 in a *ligature-induced periodontitis model* [41]. These periodontal defects were created around the second premolars by placing silk ligatures around the teeth until the bone loss exceeded half of the root length. The distance from the cemento-enamel junction to the remaining alveolar crest was approximately 5 mm, as confirmed on radiographs. Then the ligatures were removed. To combat this acute phase of inflammation, a plaque control regime was executed for 5 weeks. Then, flap surgery was performed and the exposed cementum removed. For reconstruction, two different materials were used; a carrier material with BMP-2 or solely the carrier. These materials were placed inside the furcation area surrounding the roots. The flaps were repositioned to cover the roots and materials completely. Twelve weeks postoperatively, the animals were sacrificed. Considerable new bone formation was observed in the BMP-2-treated sites. It was concluded that the application of BMP-2 could produce considerable periodontal tissue regeneration, even in cases of horizontal circumferential defects.

Instead of inducing periodontal defects by applying silk ligatures, one can also allow periodontitis to develop spontaneously, when using older beagle dogs [51]. It was hypothesized that the combination of two growth factors, PDGF and insulin growth factor (IGF) might enhance periodontal regeneration. Prior to surgery, the dogs were subjected to supragingival cleaning. Two weeks later, conventional periodontal surgery was performed on all four quadrants in beagle dogs with *naturally occurring periodontal disease*. Radiographic evaluation showed an average bone loss in between 30 and 60% (measured from the cement-enamel junction to the alveolar crest divided by the total length of the root). Also, the majority of dogs revealed class III furcation defects. If necessary, the preexisting defects were standardized by removing more bone during surgery. At the time of surgery, a gel, containing both the growth factors, was applied in two quadrants, while the contra lateral quadrants received only the bare gel. Sacrifice of the animals was executed after 2 and 5 weeks. It was concluded that the application of PDGF and IGF could enhance the formation of the periodontal attachment apparatus during early phases of wound healing. Interestingly, the clearance of in situ growth factors was measured by labeling them with ^{125}I.

Besides studies in the field of (regenerative) periodontology, the dog is also commonly used in the field of dental implantology and regenerative bone grafting procedures. For example, Meijer et al. first extracted all the mandibular premolars [53]. After a healing period of 3 months, four implant types were compared, three at each side of the mandible (Fig. 8.6). They concluded that using a flexible bone bonding polymer, implants can be constructed, which have a mobility resembling natural teeth, thereby reducing undesired peak stresses.

Simion et al. evaluated different grafting procedures in a *vertical ridge augmentation* (*GBR model*) model [72]. Defects were produced by the bilateral extraction of all the four mandibular premolars and subsequently reducing the alveolar ridge about 7–10 mm in height.

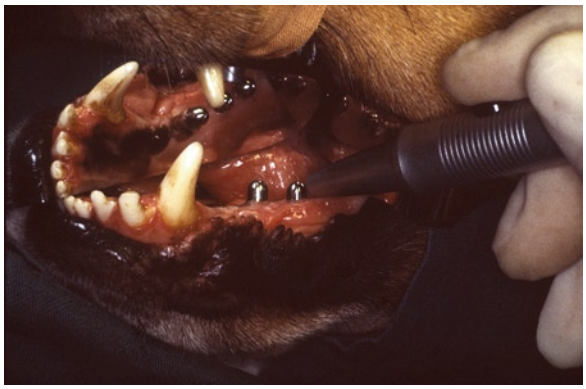

Fig. 8.6 Three implants placed in the beagle dog (with permission [53])

After 3 months of healing, the area was prepared for the experimental grafting procedure with one of the three different combinations. In the first group, a deproteinized bovine bone block in combination with a collagen membrane was used, and in the second group, the same bone block, but infused with PDGF without a membrane. In the third group, the bone block was infused with PDGF and subsequently covered by a collagen membrane. All the bone blocks were stabilized by the use of two (dental) implants. Healing was allowed for 4 months and after sacrifice histology was performed. Histological examination revealed that in the second group, large amounts of newly formed bone could be observed in the grafted area. It was concluded that the combination of a PDGF with a bone block could regenerate a new bone.

Sverzut et al. aimed to investigate the influence of polylactide membrane permeability on the fate of iliac bone graft used to construct mandibular segmental defects [75]. Unilateral 10-mm-wide *segmental defects* were created in the mandibles in mongrel dogs. In order to create these defects, both the fourth premolar and the first molar were extracted. After a healing period of 2 months, the corpus of the mandible was approached extraorally. Subsequently, a 10-mm-wide segmental defect was created by cutting through the buccal and lingual cortical bone plates, and removing the bone segment in between. All the defects were mechanically stabilized using osteosynthesis material. Six different treatment modalities were evaluated. All the animals were sacrificed after 6 months. Finally, it was concluded that the combination of a specific membrane and iliac bone graft efficiently delivered increased bone amounts in segmental defects as compared to the other treatment modalities. It has to be mentioned that a remarkable number of animals were taken out of the test early due to severe complications, such as wound infections and wound dehiscences.

Jovanovic et al. evaluated local bone formation following the application of BMP-2 in an absorbable collagen sponge carrier with or without a membrane [38]. Surgically induced, mandibular alveolar ridge *saddle-type defects* were created. In preparation of the defect, the mandibular premolars and the first molar were removed. After a healing period of 3 months standard bone defects were created. Full thickness flaps were elevated on the buccal and lingual side; thereafter, two cuts (10×15 mm, depth × length) were sawed in the edentulous region, approximately 5–10 mm apart. Subsequently, the bone segments were removed by means of a chisel, resulting in two saddle-type bone defects. Different combinations of BMP-2 and membranes were tested. All the animals were sacrificed after 12 weeks. The authors concluded that BMP-2 appears to be an effective alternative to GBR in the reconstruction of advanced alveolar ridge defects. Combining BMP-2 with GBR appeared to be of limited value due to the potential for wound failure or persistent seromas.

8.3.6 Sheep and Goat Models

During the period from 1990 to 2001, sheep were used in 9–12% of orthopedic research involving fractures, osteoporosis, bone lengthening, and osteoarthritis, in comparison with just over 5% in the period from 1980 to 1989. Goats were also used for 8.2% of animal studies published in the Journal of Orthopedic Research (John Wiley and Sons, Hoboken, NJ) between 1992 and 1996. Mostly, they were used in the studies of cartilage, meniscal, and ligamentous repair [3]. Within the Netherlands, goats and sheep respectively contribute 0.1% and 0.7% of the totally used animals within research.

Although literature reports that the dog is more suitable as a model for human bone from a biological standpoint than the sheep, however, adult sheep and goats offer the advantage of being of a more similar body weight to humans and having long bones of dimensions suitable for the implantation of human implants and prostheses [4, 58].

Regarding bone remodeling, some studies argue that the sheep is still a valuable model for human bone turnover and remodeling activity. For example, a study by Willie et al. observed bone ingrowth into porous implants placed into the distal femur of sheep (a weight-bearing model), and showed that sheep and humans have a similar pattern of bone ingrowth into porous implants over time [81]. Although sheep are shown to have a larger amount of bone ingrowth than humans, it is proposed that this is due to the greater amount of cancellous bone in the distal femur of sheep, in comparison with humans. Also, in the femurs of goats, the osteointegration and osteoinductive potential of the implants installed transcortically (Fig. 8.7) can be investigated [33]. Compared to sheep, also goats are considered to have a metabolic rate and bone remodeling rate similar to that of humans [3].

Gerhart et al. evaluated four different treatment modalities in a *femoral segmental defect* [29]. Defects were created in the center of the right femur by removing a 2.5 cm bone segment. Stabilization was achieved by means of an osteosynthesis material. Four different groups were used; empty defects, demineralized bone matrix, combination of demineralized bone matrix and BMP-2, or autogenous bone. Postoperative healing was evaluated at 3 months. In the empty defect and the demineralized bone graft treated group, no bridging of the defect was observed. However, in the BMP-2 (mixed with demineralized bone) and the autogenous bone group bridging of the defect did occur. Because the BMP-2 group had higher bending strength compared to the autogenous group, it was suggested that adding BMP-2 to an appropriate carrier could be an alternative to grafting with autogenous bone.

Bodde et al. compared bone regeneration after the use of two different ceramic (particulated) materials [14]. These ceramic particles were implanted in the sheep trabecular bone of *the femoral medial condyle*. After exposing the medial condyle, two holes were drilled into the condyle using gradually increasing different diameter drills until a final hole with a diameter of 6 mm and a depth of 9 mm was reached; these holes were at least 10 mm apart. Thereafter, the holes were filled with either one of the materials (Fig. 8.8). Different healing periods were used; 3, 12, and 26 weeks. From this study, it was concluded that both the ceramics are biocompatible and osteoconductive.

Due to the fact that goats and sheep are large animals, implantation studies in the long bones can be combined with subcutaneous studies. For example, Bodde et al. also evaluated the osteoinductive potential of porous CaP cement [12]. First, the skin was shaved, washed and disinfected; thereafter, a 3 cm skin incision was made in the center of the back. Subsequently, a *subcutaneous pouch* was created by blunt dissection and immediately, the (prehardened) cement was placed (Fig. 8.9). Implantation time was 3 or 6 months. In conclusion, no bone formation was found, and it was hypothesized that the fast degradation and thereby the collapsing of the porous structure of the CaP cement implant might have prevented osteoinduction. Kruyt et al. evaluated the potential of tissue-engineered bone [44]. Tissue-engineered constructs were prepared from goat bone mesenchymal stem cells and porous calcium phosphate scaffolds. These tissue-engineered constructs (TE) or empty scaffolds (without cells) were implanted paired in a *critical sized iliac wing defects* or in a *intramuscular defect*. After 9 or 12 weeks the animals were

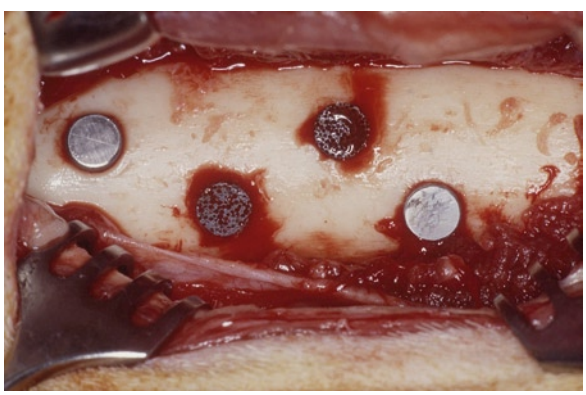

Fig. 8.7 Four implants are transcortically placed into the femur (goat) (with permission [33])

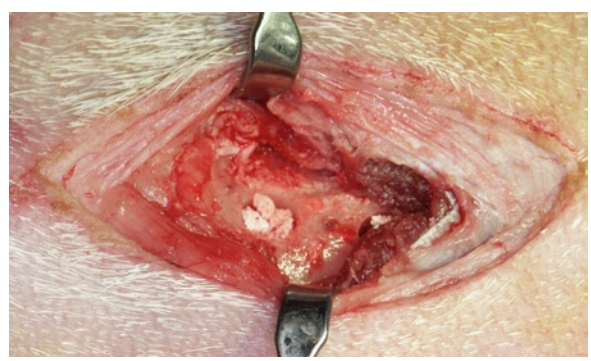

Fig. 8.8 The two different ceramic materials placed into the femoral defects in sheep (with permission [14])

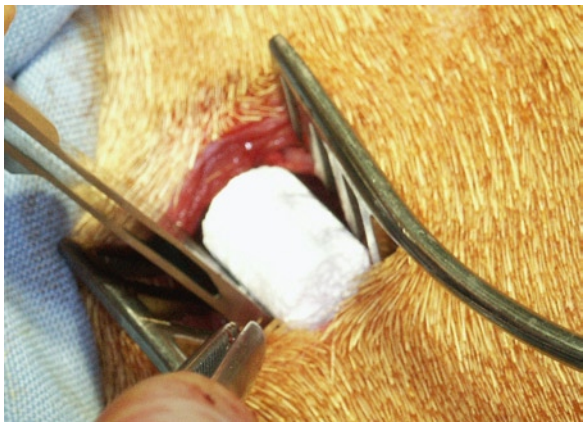

Fig. 8.9 The porous calcium phosphate (CaP) cement just before delivering it into the pouch (with permission [12])

sacrificed. After 9-weeks implantation in the iliac wing defect, significantly more bone apposition was found for the TE group, however after 12 weeks no difference was found between the groups anymore. This is in contrast with the intramuscular samples where the TE group showed significantly more bone at both time points. The authors concluded that, bone TE is feasible however, the effect of cell seeding may be temporary.

Research performed in goats has mainly concentrated upon bone regeneration and orthopedic research although Suzuki et al. published a paper on the possibilities of using a miniature (Shiba) goat for periodontal studies [74]. This study describes the possibilities, but no treatment has been performed, and one could consider using this animal for tissue engineering approaches. They observed spontaneous vertical alveolar bone loss and the downgrowth of the epithelium. In goats, a pathological microflora similar to humans was present. It was concluded that the clinical, histopathological, and microbiological features of periodontitis were quite similar to those found in human periodontitis. They postulated some advantages; the size of the oral cavity is suitable for treatment, and handling and housing are easy. According to the authors, these results suggest that the Shiba goat could be a useful animal model for studying periodontitis and possible therapies.

Pinholt et al. evaluated whether alveolar ridge augmentation could be induced using different osteoconductive materials [63]. A vertical incision was made in the midline of the mandible in the inferior sulcus, and from this incision, tunnels were created subperiostally toward the distal side on both sides of the incision. The tunnels, or *subperiostal pouches*, are located in the naturally edentulous region in between the incisors and the first molar. Within the same animals, another defect was created in the front limb, a so-called *intramuscular pouch*. Allogenic, demineralized, and lyophilized dentin or bone was implanted. The final conclusion was that no osteoinduction could be found using these materials.

Mohammed et al. evaluated the effect of TFG-β1 on wound healing in standardized class II furcation defects [55]. Two weeks prior to the regenerative procedure, a class II furcation defect was surgically created. The defect was created on the second two-rooted mandibular premolar. In order to be able to surgically approach this area, sometimes the cheek had to be incised. The teeth were cleaned with a curette, and thereafter using adhesive dentistry, the knot of a 20 mm long piece of 3–0 silk suture material was attached to the buccal surface of the tooth near the furcation region. Subsequently, a full thickness flap was elevated on the buccal side, 5 mm of bone was removed in a corono-apical direction, and 3 mm bone was removed horizontally into the furcation (in the lingual direction). The free end of the suture material was then packed into the created bone defect, as an adjunct to create inflammation, and thereafter the flap was sutured. Two weeks after the creation of the defect, the surgical area was reopened and debrided. One of the three different treatments were performed; either carrier alone (control group), or TGF-β1/carrier, or TGF-β1/carrier covered with a membrane. Two different healing periods were used, either 2 or 6 weeks. This study concluded that TGF-β1 encouraged bone regeneration in Class II furcation defects in sheep, an effect enhanced by the presence of a barrier membrane.

Oortgiesen et al. (unpublished data) evaluated an injectable CaP cement in *a sinus model.* The sinus region was approached extraorally via a skin incision directly to the lateral wall of the sinus. A round osteotomy with a diameter of 12 mm was created using a drill, until the schneiderian membrane was reached. Thereafter, the bone wall was carefully detached from this membrane. The membrane was internally elevated and two holes were drilled to place experimentally designed implants, and thereafter, the CaP cement was injected, surrounding the implants and filling the pouch underneath the membrane. The bone wall, which was removed externally, was subsequently replaced (Fig. 8.10). The major drawback in this study was the presence of several septa within the sinus, which made it very hard to preserve the integrity of the schneiderian membrane.

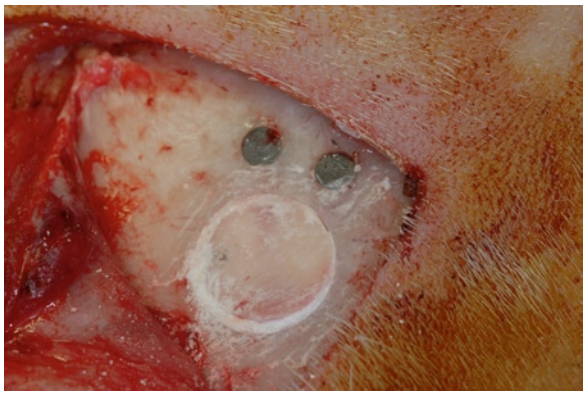

Fig. 8.10 Two implants placed into the CaP cement augmented sinus in a goat

8.3.7 Porcine (Pig) Model

Pigs are used as experimental animals, as they have quite similar anatomy and physiology to that of humans. A disadvantage associated with the use of pigs is that they are uncooperative without full general anesthetic [64]. Another disadvantage of pigs is their large growth rates and excessive final body weight. Nowadays, micropigs and miniature pigs are available which can overcome this problem to some extent [79]. However, it has to be noticed that these types of pigs are (very) expensive.

Several studies described pigs as having bone remodeling processes similar to humans. In a study comparing the bone regeneration rate of dogs, pigs, and humans, the authors found that pigs have a more similar rate of bone regeneration to humans than do dogs (dog, 1.5–2.0 mm/day; pig, 1.2–1.5 mm/day; human, 1.0–1.5 mm/day) [45]. Another study evaluating the effects of fluoride on cortical bone remodeling in growing pigs showed that control animals have a similar cortical bone mineralization rate to humans [42].

Lang et al. induced periodontal bone defects and evaluated whether replanted cultured cells from the periodontium could contribute to new attachment formation [46]. *Experimental periodontal defects* were induced by placing orthodontic elastics around the premolars and molars; in addition, metal wires were adhesively attached to the teeth with the apical end located at the entrance of the furcation. These actions will lead to periodontal breakdown. The elastics and metal wires were removed after 62 days. At this time, primary cell cultures from alveolar bone and PDL were obtained as well. The defects were treated according to the study design by flap surgery alone, or replantation of alveolar bone cells and a membrane, or replantation of PDL cells and a membrane, or carrier material without cells and a membrane, or flap surgery and a membrane, or, finally, no treatment at all. The study showed that the replantation of cultured alveolar bone cells leads to the formation of new cementum and bone, which, in turn, leads to the formation of a new attachment. It is likely that the cells stabilize the tissue formation in the defect or on the root surface in the early phase of wound healing and prevent epithelial down growth.

A faster method of creating periodontal defect is to surgically *remove the bone and thereafter induce inflammation* like Liu et al. did [50]. They explored the potential of using periodontal ligament stem cells (PDLSCs) to treat periodontal defects. The periodontal lesion was generated in the first molars area of miniature pigs by surgical removal of the bone and subsequent positioning of a silk ligament suture around the cervical portion of the tooth. PDLSCs were obtained from the extracted teeth of the miniature pigs and then expanded ex vivo to enrich PDLSC numbers. When transplanted into the surgically created periodontal defect areas, PDLSCs were capable of regenerating periodontal tissues, leading to a favorable treatment for periodontitis. This study demonstrates the feasibility of using stem cell-mediated tissue engineering to treat periodontal diseases.

Also, GBR studies have been performed in *standardized bone defect.* For example, Strietzel et al. evaluated the barrier function of seven different membranes, investigated their structural changes at different time points, and finally characterized tissue composition and reaction adjacent to the barrier [73]. The membranes were solely used; in other words, no bone substitute material was placed underneath the membrane. The defects were created from an extraoral approach. The right inferior margin of the mandible was exposed, and thereafter, with drills, eight standardized defects were created, measuring 4 mm in diameter and 8 mm in depth. Samples of the defect areas with membranes were harvested after 2 days, 4 and 8 and 12 weeks. In case of using collagen or synthetic polymer barriers for GBR, it was suggested to also apply bone or bone substitutes to prevent membrane prolapse into the defect.

8.3.8 Primate (Monkey) Model

From both an anatomical and physiological standpoint, primates resemble humans more closely than any other type of animal. The wide range of nonhuman primate species allows appropriate selection for different investigations. The size of the animal and thereby the possibilities to study relatively large defects is a positive feature as well. Due to the lack of dramatic growth, they are ideal for very long-term studies, usually over several years. However, ethical considerations restrict their use in experimentation in numerous countries especially when other species can be used [82]. Additionally, they are expensive to purchase and to maintain when compared to other animals species.

An example of a monkey study which evaluated the use of two different bone graft materials is the experiment performed by Cancian et al. [16]. In their study, the biologic behavior of two filler materials, BioGran® and Calcitite®, was compared in surgically created cavities in four adult monkeys (Cebus apella). The surgical cavities were prepared through both the mandibular cortices, with a diameter of 5 mm, in the angle region. Two cavities were prepared on the right side and one on the left and divided into three groups: (1) sites filled with bioglass (BioGran®), (2) sites not filled, and (3) sites filled with hydroxyapatite (Calcitite®). After 180 days, the animals were sacrificed and the specimens were removed for histological processing. Results showed no bone formation when the cavities were left empty. BioGran®-treated sites showed bone formation and total repair of the bone defect; the bioglass particles were almost totally resorbed and substituted by bone. The few remaining crystals were in intimate contact with the newly formed bone. Calcitite® did not allow bone formation, and granules inside the cavities were involved by connective tissue. Based upon those results, the authors concluded that the use of bioglass resulted in the total obliteration of the surgical cavity with bone. However, hydroxyapatite was present in a large amount and involved by connective tissue, without bone formation.

Rutherford et al. evaluated a combination of PDGF and dexamethasone with an insoluble collagen carrier matrix in an *experimental periodontitis model* [66]. In this study, periodontitis was induced in the second premolar and the first and second molar in each quadrant by placing ligatures around the teeth and the weekly injections of a known periodontal pathogen; Porphyromonas gingivalis. This led to considerable attachment loss after 3–4 months. One week prior to the periodontal surgery, the ligatures were removed and the teeth were carefully cleaned in order to reduce the inflammation. A full thickness flap was elevated and the defects were debrided and the teeth were scaled and planed. Four different treatment modalities were tested. Postoperative evaluation was done at 4 weeks, showing that periodontal regeneration was present only in the PDGF, dexamethasone, and carrier treatment modality. The presence of substantial amounts of regenerated periodontium suggested that this combination may provide a new therapeutic agent for the regeneration of lesions of periodontitis.

Teare et al. evaluated transforming growth factor-beta 3 (TGF-β3) for its ability to induce periodontal tissue regeneration [77]. This study uses a heterotopic site to develop a material later to be transplanted into periodontal defects. Heterotopic ossicles, for transplantation to furcation defects, were induced within the rectus abdominis muscle by TGF-β3. *Pouches in the rectus abdominis* were prepared by a sharp incision and thereafter blunt dissection. Within these pouches different materials were implanted. *Class II furcation* defects were created bilaterally in the maxillary and mandibular molars. These furcation defects were surgically created, on the first and second mandibular molars, by removing the bone. In the bucco-lingual direction, at least 10 mm of bone was removed in the furcation. Subsequently, the teeth were scaled and planed to remove the remnants of the original periodontium. Three different materials were compared; TGF-β3 in a carrier, or TGF-β3 plus minced muscle tissue, or the ossicle (induced by TGF-β3). Sacrificing was executed after 60 days. It was concluded that substantial regeneration was observed in defects implanted with fragments of the ossicles and with TGF-β3 plus minced muscle tissue.

Blumenthal et al. compared regenerative outcomes in 2- or 3-wall intrabony defects, using various regenerative therapies [10]. Interproximal, *intrabony defects* were surgically created, with different defect morphologies, either three bony walls or two bony walls remaining. These defects were surgically created distal of the lateral incisor and at least one more site per quadrant, where it would be possible to remove 3 mm of bone in a bucco-lingual direction

and 5 mm of bone in a corono-apical direction, resulting in 3-wall defects. In the opposing jaw, the remaining buccal wall was removed as well, to create the 2-wall defect. Thereafter, ligatures were placed into the defect to create inflammation. After 8 weeks the ligatures were removed, and after a healing period the teeth were cleaned. The defects were thereafter surgically treated with various treatment modalities. Sacrifice of the animals was done 6 months postoperatively. From this study, it was concluded that a defect morphology-directed rationale should be used for periodontal intrabony therapy.

Sculean et al. evaluated immunohistochemically the expression of matrix molecules associated with periodontal tissues reformed after regenerative periodontal treatment [71]. *Chronic intrabony defects* were created by the surgical removal of 6–8 mm of alveolar bone distal of both maxillary and mandibular central incisors and first premolars. At the time of the surgery, metal strips were adhesively attached to the teeth in the region of the defect, in order to create inflammation. A period of 4 weeks without any oral hygiene measures followed; thereafter, the metal strips were removed. Three weeks later, the defects were treated with guided tissue regeneration, enamel matrix proteins, the combination of both, or access flap surgery. Healing period was 5 months. It was concluded that even after 5 months postoperatively, extracellular matrix molecules associated with wound healing and/or remodeling are more strongly expressed in regenerated than in intact tissues. Additionally, once an environment for periodontal regeneration has been created, the expression of extracellular matrix molecules associated with the healing process seems to display the same pattern, irrespective of the treatment modality.

8.4 Clinical Application

In general, animal models are used to test human clinical issues, for example, how to regenerate a bone defect. As a result, various products can be developed.

For example, in the USA and Canada, recently (2006) a tissue engineering product was approved by the FDA, combining a (osteoconductive) CaP scaffold with a growth factor (rhPDGF-BB) (GEM21S®). This is one of the first products available relying upon the principles of tissue engineering, combining a scaffold with a signaling factor. For approval, this product was preclinically tested using several different animal models besides (numerous) in vitro studies, such as the *furcation defect* in the beagle dog [18, 62], a *vertical ridge augmentation defect* in foxhound dogs [72], a *peri-implant defect* in the dog [54], and the *ligature-induced periodontitis model* in nonhuman primates [30].

8.5 Expert Opinion

To start with, it would be advantageous if tissue-engineered products for a specific application like periodontal or bone regeneration would all be tested in a similar pathway of experiments (series) . Testing materials in a similar track makes it easier to compare the results of the products. In addition, if a product becomes available, then it is possible to compare the clinical outcome to the earlier preclinically obtained results. In case of a positive outcome in these "screening experiments," more specific models can be designed that match the eventual clinical application more. As such, within a certain limit of time a more or less efficient pathway can be designed.

Keeping in mind that research also has financial consequences, a first step into designing such a pathway could be a pouch model (subcutaneously or intramuscularly) in either the mouse or rat to check tissue responses to a certain tissue-engineered material. Positive aspects of these rodents, besides the reasonable costs and good availability, are the opportunities to use immune-suppressed animals, giving the possibilities to use cell-based constructs, which even do not necessarily have to be taken from the same animal or even the same species. A next step for the use of a bone substitute could be using a cranial defect in the rat, either one in the center or two defects one on each side of the midline, as previously described. If the ultimate goal of the specific construct is the application in load bearing parts of the body, this also can be tested, for example, by using a segmental bone defect, such as in a limp of a rabbit, dog, or sheep/goat. Dogs in different models are commonly used in periodontal and bone regenerative research, because of the (intraoral) size. Like the (miniature) pig the major disadvantage are the costs. Therefore, using large animals is one of the last steps in a series of experiments showing consistent positive results

with a certain material. In addition, it is of great importance to evaluate the (clinical) human results and to use these results to adjust the series of animal experiments.

8.6 5-Year Perspective

The development of new technologies and substitutes as an alternative to animal testing is important to enhance our knowledge of medicine as well as prevent and treat disease without the use of animals. As the need for alternatives increases, more funding will hopefully be directed to the development of animal testing alternatives, which means successful new treatments for disease without the use of animals in testing.

Reduction of the number of laboratory animals may not only be achieved by the restriction of the number of model species used and by replacement with in vitro and in silico tests within the field of tissue engineering, but also by realizing the potential that tissue engineering constructs themselves have as alternatives to animal experiments. Several tissue-engineered skin models (namely EPISKIN, EpiDerm and SkinEthic) have already been validated by the European Center for the Validation of Alternative Methods (ECVAM) as alternatives for the tests of skin irritation and corrosivity, replacing the Draize test, which uses living rabbits. Other tissue engineering products for toxicological application are being developed: apart from the products based on tissues similar to human epidermis like corneal and oral epithelium, there are promising advances in the field of liver and kidney tissue engineering [7]. But tissue engineering constructs may also be used for other applications. Because of their 3D structure and the possibility to use human cells, they are highly suitable both as tissue models for studying normal human physiology [5] and as disease models [17, 19]. In conclusion, tissue engineering itself might make a significant contribution to the expansion of the field of alternatives to animal experiments.

8.7 Limitations/Critical View

Animals can never represent the exact human situation; however, they offer the possibilities to get background information about biological principles. In most regenerative periodontal research, the defects that are treated do not originate from an inflammation causing periodontal breakdown, but rather from (manually) created defects that only resemble the natural occurring defects.

Only some studies use animals that actually develop periodontitis spontaneously, for example, the (older) beagle dog. Besides the positive point that these defects are naturally caused, it poses negative aspects as well. The defects will obviously never be standardized, so it will be challenging to compare treatment outcomes.

In bone regenerative surgery, more or less the same problems occur; defects that have to be treated in humans arise from congenital deformities, accidents, and (malignant) diseases, and therefore are never standardized. Human reconstructive surgery after a car accident can hardly be compared to any animal model. But, one has to keep in mind that in order to develop materials, animals offer valuable possibilities to unravel biological events on the road to developing the ultimate material for a specific purpose.

A critical note should be that after a product is commercially available, generally, the results obtained in the animal studies are not (often) compared to the results in the human studies. The knowledge from these comparisons could be useful in designing pathways to more effectively test products in the right models, thereby making the preclinical studies more valuable.

8.8 Conclusions/Summary

When thinking of, or actually creating, tissue-engineered constructs, sooner or later the need for clinical testing occurs. Obviously, besides testing for safety, also the effectiveness of the created material has to be studied. If possible histology is preferred as the ultimate proof. That is why animal models are still unavoidable and useful. Often, it is difficult to choose the appropriate animal, and thereafter, it might be even more difficult to choose the best suitable defect model. In other words, it is highly dependent on the study of what is the best animal to use, since the universally "gold animal model" does not exist, and then the defect site still has to be chosen. Another important issue, although it has nothing to do with the validity of research, is the financial consequence of the choice. For example, it could be advantageous to use the nonhuman primate

for periodontal regenerative research, but on the other hand, if you can get results in a rat experiment, it would be possible to conduct, with the same amount of money, more experiments. The challenge is that, you have to find a model that will present results representative for the human application, spending as little money as possible.

Various interesting models are currently used in regenerative medicine, all with their specific positive and negative aspects. One has to try to find and use the best compromise overlooking all these aspects in a well-designed (series of) experiment(s), giving the best results using as little animals as possible.

8.9 Literature with Abstracts

Please observe the reference list.

Suggested Readings with Abstracts

For examples please read

Link DP, van den Dolder J, van den Beucken JJ, Cuijpers VM, Wolke JG, Mikos AG, et al. Evaluation of the biocompatibility of calcium phosphate cement/PLGA microparticle composites. J Biomed Mater Res A. 2008;87(3):760–9.

In this study, the biocompatibility of a calcium phosphate (CaP) cement incorporating poly (D,L-lactic-co-glycolic acid) (PLGA) microparticles was evaluated in a subcutaneous implantation model in rats. Short-term biocompatibility was assessed using pure CaP disks and CaP disks incorporating PLGA microparticles (20% w/w) with and without preincubation in water. Long-term biocompatibility was assessed using CaP disks incorporating varying amounts (5, 10, or 20% w/w) and diameter sizes (small, 0–50 μm; medium, 51–100 μm, or large, 101–200 μm) of PLGA microparticles. The short-term biocompatibility results showed a mild tissue response for all implant formulations, irrespective of disk preincubation, during the early implantation periods up to 12 days. Quantitative histological evaluation revealed that the different implant formulations induced the formation of similar fibrous tissue capsules and interfaces. The results concerning long-term biocompatibility showed that all the implants were surrounded by a thin connective tissue capsule (<10 layers of fibroblasts). Additionally, no significant differences in capsule and interface scores were observed between the different implant formulations. The implants containing 20% PLGA with medium- and large-sized microparticles showed fibrous tissue ingrowth throughout the implants, indicating PLGA degradation and interconnectivity of the pores. The results demonstrate that CaP/PLGA composites evoke a minimal inflammatory response. The implants containing 20% PLGA with medium- and large-sized microparticles showed fibrous tissue ingrowth after 12- and 24-weeks indicating PLGA degradation and interconnectivity of the pores. Therefore, CaP/PLGA composites can be regarded as biocompatible biomaterials with the potential for bone tissue engineering and advantageous possibilities of the microparticles regarding material porosity.

Jansen JA, Vehof JW, Ruhé PQ, Kroeze-Deutman H, Kuboki Y, Takita H, et al. Growth factor-loaded scaffolds for bone engineering. J Control Release. 2005;101(1–3):127–36.

The objective of the study presented here was to investigate the bone inductive properties as well as release kinetics of rhTGF-β1- and rhBMP-2-loaded Ti-fiber mesh and CaP cement scaffolds. Therefore, Ti-fiber mesh and porous CaP cement scaffolds were provided with these growth factors and inserted in subcutaneous and cranial implant locations in rats and rabbits. In vitro, a rapid release of rhTGF-β1 was observed during the first 2 h of the Ti-fiber mesh scaffolds. During this time, more than 50% of the total dose of rhTGF-β1 was released. Following this initial peak, a decline in the level of rhTGF-β1 occurred. After 1 week, the entire theoretical initial dose was observed to have been released. This in contrast to the rhTGF-β1 and rhBMP-2 release of the porous CaP cement scaffolds. Here, no substantial initial burst release was observed. The scaffolds showed an initial release of about 1% after 1 day, followed by an additional marginal release after 1 week. Histological analysis revealed excellent osteoconductive properties of nonloaded CaP material. Inside nonloaded Ti-mesh fiber scaffolds, bone ingrowth also occurred. Quantification of the bone ingrowth showed that bone formation was increased significantly in all scaffold materials by the administration of rhTGF-β1 and rhBMP-2. Consequently, we conclude that the release kinetics of growth factors from porous CaP cement differs from other scaffold materials, like metals and polymers. Nevertheless, orthotopic bone formation in a rabbit cranial defect

model was stimulated in rhTGF-β1- and rhBMP-2-loaded CaP cement and Ti-fiber mesh scaffolds compared with nonloaded implants.

Fritz ME, Jeffcoat MK, Reddy M, Koth D, Braswell LD, Malmquist J, et al. Implants in regenerated bone in a primate model. J Periodontol. 2001;72(6):703–8.

Background: Earlier publications from our laboratory described the use of guided bone regeneration to fill large bone voids in the mandible created through en bloc resection in primates. The present report describes the placement of implants into the regenerated bone with subsequent prostheses construction and loading. *Methods*: Lesions were created in the mandibles of nine monkeys in a standardized mandibular defect of 8×19 mm. Reinforced expanded polytetrafluoroethylene membranes were placed in the animals and held in place with mini screws and sutures for anywhere from 1 to 12 months. No material was added to the defect. In each animal, a root-form implant was placed 12 mm distal to the abutment teeth into the regenerated bone and was loaded with a prosthesis for 12 months. These implants were compared to the original implants placed in the same monkeys years earlier in the same location in a nonregenerated bone. Digital radiology and histomorphometry are described. *Results*: The results show that root-form implants placed in the regenerated bone show the same radiological and histomorphometric characteristics as in the normal bone when loaded. In addition, the percentage of bone contact with the implants seen in the regenerated bone vs. nonregenerated bone is the same when both are loaded ($65 \pm 13\%$ SD in regenerated bone vs. $59 \pm 15\%$ SD in nonregenerated bone). *Conclusions*: In a primate mode,l root-form implants placed in the regenerated bone and prosthetically loaded show no difference when compared to root-form implants placed in the nonregenerated bone and prosthetically loaded.

Rajnay ZW, Butler JR, Vernino AR, Parker DE. Volumetric changes following barrier regeneration procedures for the surgical management of grade II molar furcation defects in baboons: I. Overall defect fill. Int J Periodontics Restorative Dent. 1997;17(4):378–91.

A computer imaging technique has been advocated for measuring the volumetric fill in furcation defects. Histologic material for this investigation was obtained from an animal study using five adult baboons (Papio anubis). The photographed histology was converted into digitized electronic information, and a computer calculated the overall volume of the defect fill for the treated and the untreated control sites. All the volumetric measurements were expressed as a percentage of the original surgically created defect size, with 100% indicating complete healing of the defect. The results indicate that none of the defects achieved complete healing. Teeth that had received flap debridement had the most overall defect fill (79.50%). Teeth that received a biodegradable barrier (Epi-Guide) showed a mean overall defect fill of 74.98%, while sites treated with an exclusion barrier (Gore-Tex) showed 70.75% overall fill. The untreated control teeth showed a mean overall fill of 78.70%. A variety of statistical tests revealed no significant differences among the teeth within the same animal and between treatments and controls. The following conclusions were drawn: (1) digital imaging technology is a useful research tool for determining the volume of the defect fill in surgically created grade II molar periodontal furcation defects in the baboon model; and (2) no significant differences were found among the treatment modalities and the untreated control sites.

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Biomedical Imaging and Image Processing in Tissue Engineering

9

Haiou Shen, Aaron S. Goldstein, and Ge Wang

9.1 Introduction

Cell-based tissue engineering efforts, involving the incorporation of primitive (e.g., undifferentiated, pluripotent, stem) cells into biomaterial scaffolds, represent a significant research thrust in the field of regenerative medicine [7, 55, 65, 77]. The ability for these engineered tissues to regenerate functional tissues or organs hinges on the ability of the cellular component to differentiate, organize into tissue-like structures, and provide physiological signals to guide integration, vascularization, and normal remodeling processes in vivo. Despite the promise of primitive cells in tissue-regeneration strategies, they are highly complex and dynamic, and their behavior – even in well-controlled studies – is difficult to predict. Consequently, their use demands extensive characterization of both cell/biomaterial interactions in vitro, and engineered tissue/host interactions in vivo. For example, cells may be seeded into biomaterial matrices and allowed to proliferate, migrate, and mature (i.e., to differentiate and acquire specialized functions) over many days in a bioreactor. During this culture period, the ability to monitor and evaluate changes in phenotype and function, noninvasively, in detail, and in real-time could translate into significant improvements in the engineered tissue products.

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Because the size of engineered tissues – typically, several millimeters to a few centimeters – compares well to that of small rodents, many of the biomedical imaging techniques developed for the study of small animals may be used to characterize engineered tissue development in both in vitro and in vivo. These techniques include but are not limited to the following: X-ray computer tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET), optical coherence tomography (OCT), fluorescence molecular imaging (FMI), bioluminescence imaging (BLI), diffuse optical tomography (DOT), and ultrasound imaging. These imaging modalities can be classified into two general groups: (a) tomography to recover internal tissue and organ structures and (b) molecular probes to characterize tissue and organ function.

Traditional clinical-structured tomographic modalities, such as X-ray CT and MRI, rely on nonspecific physical properties. For example, X-ray CT images rely on the different linear attenuation coefficients of X-rays between different tissues. In principle, these tomographic modalities can be miniaturized to fit the size and resolution needs of small animal and tissue engineering studies. Although the scaling of these technologies is straightforward, significant challenges remain to achieve ultrahigh-resolution images, which are necessary to obtain meaningful structure information for those small specimens – millimeters to centimeters in size. New technologies, such as higher-resolution sensors, better contrast agents, powerful sources, and new reconstruction algorithms, will advance the current state-of-the-art imaging systems to micrometer resolution and even submicrometer resolution.

In contrast to those clinical-structured tomographic modalities, molecular imaging technologies, such as fluorescence and BLI, use genetic and molecular probes to identify gene expression and molecular

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function. Fluorescence imaging employs fluorophores (e.g., quantum dot, fluorescent dye, or fluorescent protein), while bioluminescence uses enzymes (e.g., luciferase) to produce light that can be detected by a camera. Molecular probes can be conjugated to antibodies that accumulate in specific tissues. Reporter genes have been developed to encode the fluorophore or enzyme and can be delivered into cells to identify subcellular structures or cell phenotypes. Thus, the advantage of molecular imaging technologies over structure tomographic methods is that they can identify specific molecules associated with cell and tissue function and provide micrometer length scale resolution without compromising cell and tissue viability. Consequently, molecular imaging strategies have become increasingly common in basic cell research and hold promise for early detection, characterization, and treatment of diseases. In addition, these types of technologies can be directly applied to tissue engineering applications [10, 15, 28, 29, 32, 53, 63, 68, 70].

With recent advances in the field of biomedical imaging, scientists are now able to perform several important tasks that are pivotal in the advancement of tissue engineering research. These tasks include the ability (1) to image cells in a nondestructive and minimally disturbing manner; (2) to track the migration of cells in engineered tissue; (3) to monitor the structure of engineered tissues (especially the scaffold) at high resolution (submicron and nanometer resolution); (4) to image multiple molecular events or multiple cell types simultaneously; (5) to monitor the dynamic of a process; and (6) to integrate multiple imaging modalities.

Molecular imaging will be instrumental in revolutionizing medicine. The combination of fluorescent and bioluminescent probes with optoelectronics will lead to the development of powerful optical molecular imaging tools. These tools will allow the visualization and deeper understanding of biologic interactions at the cellular and molecular levels, over time, and in complex systems. Progress in molecular imaging has continued and will continue to drive a paradigm shift from the exclusive reliance on simple cell-based in vitro assays to sophisticated analyses of 3D in vitro models and, most importantly, in animal models of human diseases. This type of paradigm shift has now influenced the development of tissue engineering. In particular, optical molecular imaging has enormous unexplored potential as an enabling technology for regenerative medicine.

Because it is not possible to cover the entire biomedical imaging field in one chapter, this chapter will focus on optical molecular imaging, especially bioluminescence tomography (BLT). The photon propagation modeling techniques for optical molecular imaging will be discussed first, followed by BLT in detail. Because of the similarity between bioluminescence and fluorescence tomography, fluorescence molecular tomography (FMT) will be presented. Finally, the trend for optical molecular tomography will be discussed.

9.2 Light Transportation Model

There are two major optical molecular tomography modalities: bioluminescence and fluorescence, but the objective of these tomographies is the same: to recover the 3D optical light source distribution in tissue/animal based on the surface optical light signal. Let us use BLT as an example. Cells labeled with bioluminescent probe emit bioluminescent light, which is absorbed and scattered by the tissue while the light propagates in the tissue. A part of the bioluminescent light can reach the surface of the tissue/animal and is then captured by a camera as a planar image. BLT recovers the internal source distribution based on the captured surface planar image. Predicting the relation between source position and photon fluence distribution is the core technique in solving this type of tomographic problem.

Biological tissue is a turbid medium that both scatters and absorbs photons. However, if the optical properties of the tissue are known, then the movement of a photon can be predicted using a tracing technique. This approach can be extended to large number of photons by tracing the individual photons one by one using the Monte Carlo simulation [35, 36, 47, 56, 61, 89, 91]. However, this type of tracing technology is not efficient in optical molecular tomography, in which the tracing – a technique to solve the forward problem – must be applied for many different locations and conditions. Therefore, statistical models have been developed to describe photon propagation in a turbid medium. Within this approach, the propagation of photons in tissue is modeled with the radiative transfer equation (RTE) [12, 16, 17, 48, 51, 72], and approximation methods have been developed to solve the RTE efficiently [5, 22–24, 50].

9.2.1 Radiative Transport Equation

The steady state RTE describes the variation of the photon radiance: $L(\vec{\mathbf{r}},\hat{\mathbf{s}})$ at position $\vec{\mathbf{r}}$ and along the direction $\hat{\mathbf{s}}$ in biological tissue [48, 50, 51, 72] equals

$$[\hat{\mathbf{s}}\cdot\nabla+(\mu_a+\mu_s)]L(\vec{\mathbf{r}},\hat{\mathbf{s}})=\mu_s\int_{4\pi}L(\vec{\mathbf{r}},\hat{\mathbf{s}}')p(\hat{\mathbf{s}},\hat{\mathbf{s}}')d\hat{\mathbf{s}}' \quad (9.1)$$
$$+\frac{1}{4\pi}Q(\vec{\mathbf{r}},\hat{\mathbf{s}}),\quad \vec{\mathbf{r}}\in\Omega,$$

where Ω is the problem region; $Q(\vec{\mathbf{r}},\hat{\mathbf{s}})$ the light source distribution; μ_a the absorption coefficient, which defined as the probability of photon absorption along a unit length; and μ_s the scattering coefficient. RTE considers the Rayleigh scattering (the deflection of light), which is an elastic scattering of light with photon energy unchanged. μ_s is defined as the probability of scattering per unit length. The scattering phase function $p(\hat{\mathbf{s}},\hat{\mathbf{s}}')$ gives the probability of photons coming in the direction $\hat{\mathbf{s}}'$ that is scattered into the direction $\hat{\mathbf{s}}$. The exact form of the phase function $p(\hat{\mathbf{s}},\hat{\mathbf{s}}')$ is unknown in practice and may vary from tissue to tissue.

In 2005 and 2007, Klose et al. presented an optical molecular tomography algorithm for fluorescence and BLT using RTE directly [49, 51]. There is no analytical solution to the photon propagation problem for heterogeneous media and complex geometry. Numerical methods are needed to solve the RTE. In the 2005 paper, the researchers employed a finite-difference discrete-ordinates method to convert the integro-differential equation into a system of algebraic equations [51], as follows:

$$\mathbf{AR}=\mathbf{BR}+\mathbf{S},$$

where $\mathbf{A}$ is the discretized streaming and collision operator matrix, $\mathbf{B}$ the discretized integral operator, $\mathbf{S}$ the source term, and $\mathbf{R}$ the radiance. A source iterative refinement step ($\mathbf{AR}_{i+1}=\mathbf{BR}_i+\mathbf{S}$) was used iteratively to compute the radiance from an initial radiance distribution $\mathbf{R}_0$. A major difficulty in RTE is that the size of the problem is too large. In addition to discrete locations, the direction also needs to be replaced with a set of discrete ordinates. For biological tissue, about >48 discrete ordinates are needed [51]. The size of the system matrices of $\mathbf{A}$ and $\mathbf{B}$ can easily exceed $100{,}000\times100{,}000$. Klose et al. demonstrated the direct RTE solver on a phantom with accurate reconstruction results, but the computation time was about 25 h on a Linux cluster of 10 Intel Pentium III 2.4 GHz Xeon computation nodes [51]. Therefore, the RTE is still not practical even on a current high-end desktop workstation.

9.2.2 Diffusion Approximation

By making appropriate assumptions about the scattering behavior of photons, the number of independent variables can be reduced, making the problem much easier to solve. The widely used phase function in optical molecular imaging is the Henyey–Greenstein phase function:

$$p(\cos(\theta))=\frac{1-g^2}{4\pi(1+g^2-2g\cos(\theta))^{3/2}},$$

where θ is the angle between $\hat{\mathbf{s}}$ and $\hat{\mathbf{s}}'$. This phase function has the following Legendre polynomial expansion:

$$p(\cos(\theta))=\sum_{n=0}^{\infty}\frac{2n+1}{4\pi}g^n P_n(\cos(\theta)),$$

where $P_n(x)$ represents the Legendre polynomials. The diffusion approximation is the P_1 approximation of the phase function.

Diffusion approximation to the RTE has been widely used in biophotonics [5, 6, 11]. It is one of the most important approximation methods in BLT [1, 18, 20, 21, 25, 27, 37, 57–59, 75, 81, 86, 87, 93]. The major requirement for diffusion approximation is that the scattering should be the major factor in the transportation ($\mu_s >> \mu_a$) [5]. Bioluminescence probes, such as FLuc, produce major energy at red and NIR ranges. In these ranges, photon scattering dominates absorption in the biological tissue, and diffusion approximation can be used to describe photon propagation in tissue with good accuracy [27]):

$$-\nabla g(D(\vec{\mathbf{r}})\nabla\Phi(\vec{\mathbf{r}}))+\mu_a(\vec{\mathbf{r}})\Phi(\vec{\mathbf{r}})=Q(\vec{\mathbf{r}}),\text{for }\vec{\mathbf{r}}\in\Omega, \quad (9.2)$$

where $D(\vec{\mathbf{r}})=1/(3(\mu_a(\vec{\mathbf{r}})+(1-g)\mu_s(\vec{\mathbf{r}}))$, g is the anisotropy parameter (the average cosine value of the deflection angle), and $\Phi(\vec{\mathbf{r}})$ represents the photon fluence. In the bioluminescence experiment, the

specimen is placed in a completely dark environment; thus, no external photon can travel into the specimen through the boundary. The boundary condition for (9.2) can be expressed as:

$$\Phi(\vec{\mathbf{r}}) + 2A(\vec{\mathbf{r}}, n, n')D(\vec{\mathbf{r}})(v(\vec{\mathbf{r}})\,\mathrm{g}\,\nabla\Phi(\vec{\mathbf{r}})) = 0, \text{for } \vec{\mathbf{r}} \in \partial\Omega,$$

where n and n' are the refractive indices for the specimen and the surrounding medium, which is air in most cases and $A(\vec{\mathbf{r}}, n, 1) = (1 + R(\vec{\mathbf{r}}))/(1 - R(\vec{\mathbf{r}}))$, and $R(\vec{\mathbf{r}}) = -1.4399n^{-2} + 0.7099n^{-1} + 0.6681 + 0.0636n$ [27, 74]. The photon density at the boundary is still not the measurement of the charge-coupled device (CCD). We can assume that the CCD measurement is directly proportional to $\Phi(\vec{\mathbf{r}})$. It is also possible to use the angle between the normal vector of the surface and the axis of the camera to map the CCD readout to $\Phi(\vec{\mathbf{r}})$.

The DA model can be converted into a system of linear equations [27]:

$$\mathbf{M}\Phi = \mathbf{F}\mathbf{P}, \tag{9.3}$$

where $\mathbf{\Phi}$ is the discretized photon fluence on the nodes of the finite element mesh, $\mathbf{M}$ and $\mathbf{F}$ are two matrices, and $\mathbf{P}$ is the source power vector. The matrix $\mathbf{M}$ is a sparse positive definite symmetric matrix. $\mathbf{F}$ is also a sparse matrix, and each column of the matrix corresponds to a unit source at one of the finite elements (or nodes). If we remove the source power vector, $\mathbf{P}$, the linear system will be a system with multiple right-hand-sides (RHS): $\mathbf{M}\Phi = \mathbf{F}$.

Two major types of methods solve the linear equation: the iterative method (such as Conjugate Gradient method) and the direct solver (such as Choleskey factorization method). The iterative method is fast but cannot efficiently utilize the shared information between multiple RHS. Choleskey factorization is one of the fastest methods to solve a sparse positive definite symmetric equation, and there are multiple high performance commercial and open source implementations of this method. Let $\mathbf{L}\mathbf{L}^{\mathrm{T}} = \mathbf{M}$ is the Choleskey factorization of $\mathbf{M}$, where $\mathbf{L}^{\mathrm{T}}$ is the transport of $\mathbf{L}$, a lower triangular matrix with positive diagonal entries. An additional reordering step can be performed on $\mathbf{M}$, before the factorization, to improve the equation-solving performance, but we skipped the reordering step to simplify the following presentation. Thus, the equation can be solved by forward and backward substitutions:

$$\begin{aligned}&\mathbf{L}\mathbf{L}^{\mathrm{T}}\Phi = \mathbf{F}\\ &\Rightarrow \mathbf{L}^{\mathrm{T}}\Phi = \mathbf{L}^{-1}\mathbf{F}\\ &\Rightarrow \Phi = (\mathbf{L}^{\mathrm{T}})^{-1}(\mathbf{L}^{-1}\mathbf{F})\end{aligned}$$

There are several advantages for the diffusion approximation and the direct solver: the matrices $\mathbf{M}$, $\mathbf{F}$ and $\mathbf{L}$ are highly sparse; thus, the DA can handle large finite element mesh without running out of memory; the system matrix $\mathbf{M}$ is a symmetric positive definite matrix, and highly efficient Cholesky factorization can be used to solve the equation; the factorization only needs to be computed once for multiple RHS; the matrix $\mathbf{F}$ is sparse, and a great deal of computation can be saved in the forward and backward substitution steps; the substitution steps can be easily paralleled across the multiple RHS to utilize the multicore architecture of current high-end workstation fully.

9.2.3 Simplified Spherical Harmonics Model

The major drawback to diffusion approximation is accuracy. Significant errors have been observed in the strong absorption, near source, and thin tissue cases [6, 9, 35, 67, 69, 71, 74]. Many biological tissues have strong absorption in the UV, blue, green, and yellow spectral ranges [19, 90], and many commonly used fluorescence and bioluminescence probes have major energy in that spectral range [44, 95, 96]. Therefore, the diffusion approximation will not work well but will introduce significant error in some optical tomography cases.

To overcome this drawback, some researchers were trying to compensate for the error of the diffusion approximation or use a complex approximation method. The diffusion approximation is the P_1 approximation of the phase function. Higher order approximation of the phase function can be used to improve the accuracy but will be about 200–400 times slower than diffusion approximation [50]. Recently, [50] presented an extensive study on a simplified spherical harmonics (SP_N) method up to order $N = 7$ for photon transportation in biological tissue with strong forward

scattering [50]. The numerical experiment showed that the SP_N model was significantly more accurate than diffusion approximation in large absorption, near source, and near boundary cases with only a small increase in computation cost.

To derive the SP_N method, [50] first derived the P_N approximation under 1D case. The P_N approximation could then be extended into multidimensional cases, but it involved expansion of the angular variable in terms of the spherical harmonic functions, which greatly increased the complexity of the multidimensional P_N approximation. To simplify the extension, the researchers replaced the 1D diffusion operator with its 3D counterpart and gained three simplified spherical harmonics approximations of RTE: SP_3, SP_5, and SP_7.

The 2D numerical simulation results of SP_N approximation showed significant improvement over diffusion approximation in term of accuracy under broad situations, especially for partially reflective boundary condition, large absorption, and anisotropic scattering. The processing time of SP_N is still within the same magnitude of diffusion approximation: SP_3, SP_5, and SP_7 are about 2.5, 5, and 10 times more computationally consuming than the diffusion equation in 2D case.

9.2.4 Phase Approximation for RTE

The phase-approximation model, which is based on a generalized Delta-Eddington function, outperformed the diffusion approximation consistently and significantly in the difficult cases mentioned above [22–24]. As a result, we can effectively simplify the radiative transfer equation into an integral equation with respect to the photon fluence rate without introducing substantial errors. Different from the diffusion approximation derived from the first order approximation of the photon radiance, the phase approximation makes a reasonable estimation to the phase functions and performs the computation of the photon radiance in the biological tissue.

The biological tissue scatters light strongly in a forward direction, and the scattering phase function can be modeled by a generalized delta-Eddington function ([22–24]), as follows:

$$p(\hat{\mathbf{s}},\hat{\mathbf{s}}') = \frac{1}{4\pi}[(1-f)+2f\delta(1-\hat{\mathbf{s}}\cdot\hat{\mathbf{s}}')],$$

where $f \in [-1,1]$ is the anisotropy weight factor measuring the anisotropy of photon scattering, which is a unique optical parameter of the phase-approximation model. The new phase function is a mix between the strongly forward scattering and isotropy scattering. With the Delta-Eddington phase function, (9.1) can be formulated as an integral equation ([22–24]):

$$\Phi(\vec{\mathbf{r}}) = \frac{1}{4\pi}\oint_{4\pi}\int_0^R [\tilde{\mu}_s(\vec{\mathbf{r}}-\rho\hat{\mathbf{s}})\rho(\vec{\mathbf{r}}-\rho\hat{\mathbf{s}})] \exp(-\int_0^\rho \tilde{\mu}_t(\vec{\mathbf{r}}-t\hat{\mathbf{s}})\mathrm{d}t)\mathrm{d}\rho\,\mathrm{d}\hat{\mathbf{s}} + \oint_{4\pi} L(\vec{\mathbf{r}}-R\hat{\mathbf{s}},\hat{\mathbf{s}})\exp(-\int_0^R \tilde{\mu}_t(\vec{\mathbf{r}}-t\hat{\mathbf{s}})\mathrm{d}t)\mathrm{d}\hat{\mathbf{s}},$$

where $\tilde{\mu}_s = (1-f)\mu_s$ is the reduced scattering coefficient, and $\tilde{\mu}_t = \mu_a + \tilde{\mu}_s$ the reduced attenuation coefficient.

To solve the photon fluence rate, the finite element method will be applied to the problem. The problem domain will be discretized into tetrahedral finite elements. The photon fluence can be approximated as:

$$\Phi(\vec{\mathbf{r}}) = \sum_{j=1}^{N}\Phi(\vec{\mathbf{r}}_j)\varphi(\vec{\mathbf{r}}_j),$$

where $\mathbf{N}$ is the number of nodes and $\varphi(\vec{\mathbf{r}}_j)$ the basis function. Then, the PA model can be converted into a system of linear equations ([22–24]):

$$(\mathbf{I}-\mathbf{M}-\mathbf{B}) \quad = \mathbf{S},$$

where $\mathbf{\Phi}$ is the discretized photon fluence on the nodes of the finite element mesh, and $\mathbf{S}$ is the source vector. The photon fluence $\mathbf{\Phi}$ can be found by solving the equation:

$$\mathbf{\Phi} = (\mathbf{I}-\mathbf{M}-\mathbf{B})^{-1}\mathbf{S}.$$

The components of $\mathbf{M}$, $\mathbf{B}$, and $\mathbf{S}$ are defined as ([22–24]):

$$\begin{cases} m_{i,j} = \dfrac{1}{4\pi}\displaystyle\int_\Omega \tilde{\mu}_s(\mathbf{r}')\varphi_j(\mathbf{r}')G(\mathbf{r}_i,\mathbf{r}')\mathrm{d}\mathbf{r}' \\ b_{i,j} = \dfrac{\mathbf{r}_d}{4\pi(1+\mathbf{r}_d)}\displaystyle\int_{\partial\Omega}\varphi_j(\mathbf{r}')G(\mathbf{r}_i,\mathbf{r}')\beta\mathbf{g}\mathbf{n}\mathrm{d}\mathbf{r}' \\ s_i = \displaystyle\int_{\partial\Omega} S(\mathbf{r}')G(\mathbf{r}_i,\mathbf{r}')\beta\mathbf{g}\mathbf{n}\mathrm{d}\mathbf{r}' \end{cases}$$

where $G(\mathbf{r},\mathbf{r}') = \dfrac{1}{|\mathbf{r}-\mathbf{r}'|^2}\exp(-\displaystyle\int_0^{|r-r'|}\mu_t(\mathbf{r}')(\mathbf{r}-t\beta)\mathrm{d}t)$

and $\beta = \mathbf{r}-\mathbf{r}'/|\mathbf{r}-\mathbf{r}'|$.

The PA model is much more accurate than the DA model while the absorption coefficient is large. Many bioluminescence and fluorescence probes have major energy in the spectrum of 400 and 600 nm, in which the absorption coefficient is relatively large. Based on recent numerical experiments [22–24], while albeto $\mu_s'/\mu_a \geq 10$, DA model can give satisfactory result. When $\mu_s'/\mu_a < 10$, PA will have better results. However, the PA model has two major drawbacks:

1. the parameter f used in PA is different from other optical models. Currently, there is no easy way to computer f from μ_a, μ_s, and g directly. Additional experiments and computations, as shown in Cong's [22, 23] paper, are needed to compute **f**.
2. The PA model is a time- and computational-consuming process. Even PA is easier than RTE; however, it is still more difficult than the DA model. When converting the DA model into the finite element model, the system matrix is a sparse positive definite real symmetric matrix, which can be solved by a direct sparse solver using Cholesky factorization. The PA's system matrices are dense matrices, which are much harder to solve. Another computational problem for PA is that the system matrix is difficult to construct for a heterogeneous object domain. For each element in the system matrix, there is a nontrivial integration needed to compute. For complex geometry, this integration itself is not an easy job.

9.3 Bioluminescence Tomography

BLT is a form of optical molecular imaging method to recover 3D bioluminescence source distribution in living tissue/animal based on the surface bioluminescence light signal. A bioluminescent probe is a genetic light emission reporter probe [43]. Bioluminescence light is the result of a chemical reaction, which needs luciferase enzyme, substrate luciferin, and ATP (oxygen). The luciferase enzyme is from the labeled cells, which contain a *luc* gene that is genetically inserted. Before each BLI experiment, substrate luciferin is injected into the animal or tissue to make a bioluminescence reaction happen. The bioluminescence source propagates with the labeled cells as the cells migrate and multiply inside an animal or engineered tissue. By tracing the bioluminescence light externally, we can view the cells labeled with the bioluminescence probe, which means that we can study the migration and the growth of those labeled cells without sectioning the animal or tissue. The ability to trace living cells in living tissue and even living animals has changed and speeded up the research in many fields.

In comparing BLT to planar BLI, planar imaging is a qualitative analysis and BLT, which can give location and source power, is a quantitative analysis. This is because the strength of the surface bioluminescence signal is not in direct proportion to the power of the internal source, which may have different location and depth. With the development of BLT, we can now recover the location and power (the place and quantity of cells with bioluminescence labeling) of the internal bioluminescence light source, which is useful for quantitative study.

BLT and FMT have similarities in their theoretical frameworks because both use the surface light measurement to inverse the internal source location. But the two techniques represent different inverse problems: the BLT is a problem of internal source inversion with passive measurement, while the FMT is a problem of internal source aided by external excitation. Compared with FMT, the BLT problem is less accurately posed and significantly more challenging as shown in the first paper on BLT [84]. In other words, any measurement noise, data inconsistency, model mismatch, and characteristic uncertainties will significantly compromise the reconstruction quality and may even render the solution useless.

9.3.1 Overview of Bioluminescence Tomography

In 2002, Dr. Wang's group from the University of Iowa invented the BLT using a modality fusion approach as a key to compensate for the optical heterogeneity of the mouse for 3D reconstruction of an internal bioluminescence source distribution from external bioluminescent signal [82, 83].

Since 2002, Wang and collaborators have published over 20 journal articles in the field of BLT based on the heterogeneous mouse model using the modality fusion framework. As shown in Fig. 9.1, three imaging modalities were needed in the process: CT/MRI/OCT

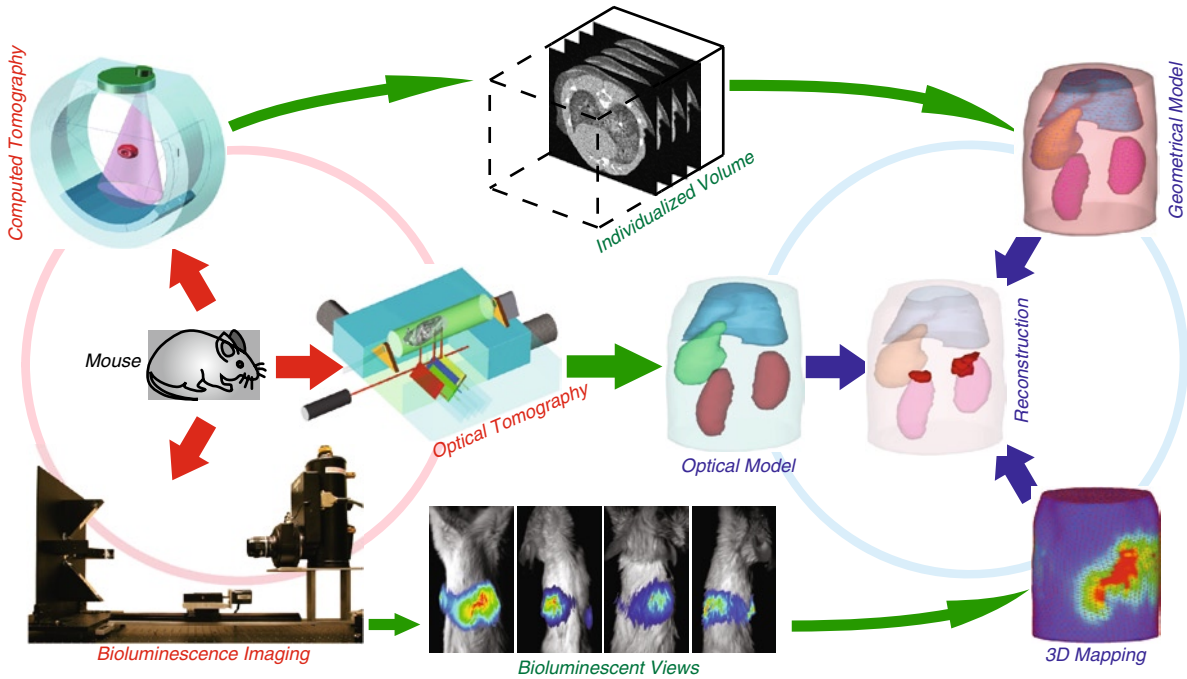

Fig. 9.1 Flowchart for bioluminescence tomography (BLT). A bioluminescent source active mouse is imaged in a bioluminescent mode to capture bioluminescent views around the mouse, in a tomographic mode (computer tomography (CT) or magnetic resonance imaging (MRI)) to reconstruct an anatomical volume and in an optical mode to estimate optical parameters to each component. Then, a linear imaging model is built between the bioluminescent measurement and source distribution, and inverted for 3D localization and quantitative analysis of the underlying molecular/cellular activities (Molecular imaging advance watches tumors grow, shrink. RSNA News. 2007;17(11):10–11. Copyright by Biomedical Imaging Division, Virginia Tech-Wake Forest University School of Biomedical Engineering & Sciences.)

for 3D volume information, diffuse optical tomography for on-site optical parameter identification, and BLI for measurement of bioluminescent signal on tissue/animal surface. Based on the volume structure information, the optical parameters, and the surface bioluminescence measurement, BLT algorithm can recover the 3D bioluminescence source distribution. In 2004, Wang et al. published the first journal article about BLT [84]. In the article, Wang et al. established the first BLT framework using the well-known diffusion approximation for forward photon propagation modeling and reviewed the inverse source methods for the reconstruction. They also proved the solution's uniqueness for BLT under practical constraints, such as: the source permissible region and source structure, despite the ill-posed inverse problem in the general case for BLT. The results of this theoretical paper confirmed the necessity for modality fusion in the development of BLT. In 2005, a finite element implementation of diffusion approximation and a maximum-likelihood optimization approach for BLT was proposed by Wang's group and demonstrated in a heterogeneous phantom experiment [27]. In 2006, the reconstruction method was applied to an in vivo mouse study using a prostate cancer mouse model with promising reconstruction result [81]. In 2006, Cong et al. proposed a multispectral BLT algorithm and demonstrated the algorithm in numerical simulation with promising results [21, 25]. Introducing multispectral BLT and the temperature dependence of a bioluminescence signal [96] enabled temperature-modulated BLT [86, 87], which greatly stabilized the BLT reconstruction. The introduction of multispectral BLT required accurate photon transport modeling technique for the wavelength of <600 nm at which diffusion approximation would fail. In 2007, Cong et al. introduced the phase-approximation method, an accurate photon modeling method for higher absorption coefficient cases (<600 nm), and demonstrated the reconstruction in numerical simulation with promising result [22–24]. In addition to the reconstruction method, the development of BLI system for BLT also gained the attention

of researchers. The BLT algorithm needed the surface bioluminescence signal around the specimen for the interested spectral channels. In 2006, Wang et al. introduced a multiview BLI system to capture multiple bioluminescence views simultaneously [86, 87] to overcome the dynamic change of the bioluminescence signal [80]. In 2008, Wang et al. proposed a multispectral imaging extension of the multiview imaging system to enable the multispectral BLT [88]. Wang's group also studied the other possible BLT reconstruction schemata, including a boundary integral method [21, 25]) and a Born-type approximation method [26].

Wang collaborated with Han (2006) on the theoretical analysis for BLT and multispectral BLT [38–40]). They considered combining BLT and diffuse optical tomography (DOT), used for the reconstruction of tissue optical parameters, to improve the source reconstruction accuracy [41, 42]. Wang's group collaborated with Tian's group, a medical imaging group from Chinese Academy of Science, to develop a mouse optical simulation environment (MOSE) [56]. MOSE is a Monte Carlo simulation program to investigate bioluminescent phenomena in a complex geometry such as a mouse. MOSE can act as a golden standard to check the soundness and accuracy of other approximation and direct RTE solvers. In addition to this, the two groups developed a multilevel adaptive finite element algorithm for diffusion approximation to reduce the total computation cost of BLT [59, 60].

Independently, in 2002, Xenogen (currently Caliper Life Sciences) made original contributions to the development of BLT [76]. The Xenogen group presented a reconstruction method based on a homogeneous mouse optical model in their patent application and developed two commercial BLI systems with tomography imaging capability: the IVIS 3D (discontinued) and the IVIS Spectrum. In 2007, Kuo et al. [52] presented a fast analytical tomography method based on single-view multispectral bioluminescence images. In that research, a Green's function-based tangential planar (TP) approximation, which treated the local surface as an infinite plane boundary, was used to predict the surface photon measurement with minor computational costs of photon propagation. Multiple wavelength data were used to reduce the condition number of the Green's kernel matrices, which, in turn, stabilized the bioluminescence reconstruction. After reconstruction, the system mapped the source locations to the 3D anatomical positions in the mouse. There were two major differences between Xenogen's reconstruction method and the modality fusion framework proposed by Wang's group. First, Wang's group used a heterogeneous model instead of the homogeneous mouse model used in Xenogen's imaging system. Based on recent studies, the accuracy of optical parameters is important for the correctness of reconstruction [2, 3, 93]. The homogeneous model will introduce much larger error than the heterogeneous model. Secondly, Wang's group used a modality fusion framework in the early stages of their invention to compensate for the optical heterogeneity. Because Xenogen used a homogeneous model, there was no requirement to use modality fusion.

Starting in 2004, other research groups entered in the BLT field. The group led by Jiang published a model-based reconstruction method using diffusion approximation and demonstrated the algorithm on a homogeneous phantom with a near boundary source condition [37]. They extended their algorithm to deal with larger permissible source regions (the entire imaging domain) [57]) and sources with different luminescence concentration [58]. In 2008, they proposed a new method to combine on-site DOT and BLT to improve the reconstruction accuracy and experimented with the method in a phantom study [93]. In 2005, Leahy's group proposed a multispectral BLT method [18]. They introduced a multiview imaging system for BLI and presented a finite element reconstruction method for multispectral BLT. Extensive numerical simulations, phantom studies, and in vivo nude mouse studies with brain tumors were conducted using their multiview BLT system. They showed that the multispectral information added independent information and made the inversion less ill-posed. In 2007, Dutta et al. presented a faster algorithm based on a perturbative forward model [33]. This algorithm was an extension of Xenogen's algorithm [52] but dealt with heterogeneous tissue models. In 2008, Ahn et al. introduced a fast iterative reconstruction method for BLT and FMT [1]. The finite element method for BLT is a computation-consuming process; fast algorithm is needed for BLT. Ahn et al. presented a Cholesky factorization and substitution-based iterative solver.

Most of the reconstruction methods above were based on the diffusion approximation of the RTE. Diffusion approximation is fast but with relatively large approximation error. RTE is the best to describe photon transportation in biological tissue, but it is

highly computationally expensive in terms of computation time and memory. In 2005, Klose et al. applied a direct RTE solver on a computer cluster for fluorescence tomography [51]. In 2007, Klose introduced the direct solver for BLT [49]. In 2006, Klose introduced a new faster approximation of RTE into optical molecular imaging [50]. This approximation method had much better accuracy than diffusion approximation with the same magnitude of computation costs. Instead of solving the RTE directly, Monte Carlo simulation can be used to solve RTE with high accuracy. In 2006, Zhang et al. introduced a table-based random sampling Monte Carlo simulation method for BLT [94]. There was also a trend to combine the BLT with other tomography methods such as PET and MRI to improve the accuracy of BLT [2–4].

9.3.2 Bioluminescence Imaging System

Figure 9.2 shows a basic BLI system [82]. The commercial BLI systems, such as IVIS series BLI systems from Caliper Life Sciences, have similar structures [52]. The system is comparable to a photography setup: a camera, a lens, and a light enclosure box. The major task of a BLI system is to capture the surface bioluminescence light distribution. The major challenge in BLI is the weakness of the bioluminescence signal. For a bioluminescence source with 1 mm^3 cells, the power of this bioluminescence source is between nW and pW. If the light can be collected without leaking, it will be very strong. But the light source is in a tissue, and tissue will strongly diffuse and absorb the bioluminescence light. Therefore, only a small portion of the bioluminescence light reaches the tissue surface and is detected by the camera. This is also true for deep tissue fluorescence imaging. A BLI system should be able to collect as much of the signal as possible. There are three things that increase the signal strength: (1) increasing the sensitivity of the imaging sensor; (2) using longer exposure times; and (3) improving the collection efficiency of the optical system.

Currently, a highly sensitive full-frame, back-illuminated CCD is one of the best choices for BLI. One of the major advantages of this silicon-based detector is its high sensitivity in visible and near-infrared range; in fact, the top quantum efficiency can reach >95%. Because the newly developed cooled CCD is so sensitive, it can detect even a single photon. To reach this sensitivity, the CCD chip must be cooled to a very low temperature to at least −100ºC. This is because the thermal noise within the silicon chip results in constant release of electrons and the release rate is doubled for every 7ºC increase in temperature. For BLI, the CCD is usually cooled to −110ºC to reduce the dark current to near negligible levels (<1e-/p/h) to support long exposure (several minutes to 1 h) experiments. This kind of CCD sensor usually has a large pixel size and results in a large CCD size (>20×20 mm) because larger pixel sizes mean more light can be incidental on a pixel if the other optical conditions are the same. To

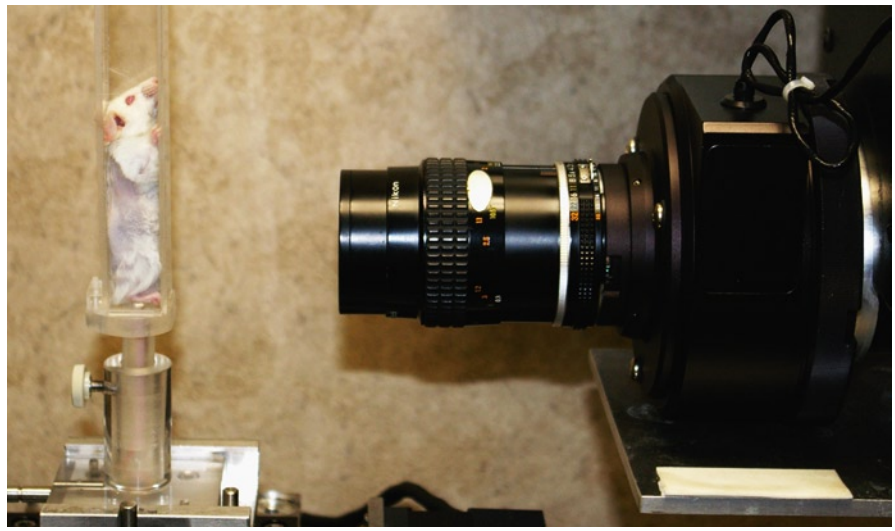

Fig. 9.2 Basic bioluminescence imaging (BLI) system used in the first BLT system with a cooled charge-coupled device (CCD) camera, a Nikon 55 mm lens, and a rotation mouse stage

keep a balance between resolution and sensitivity, most highly sensitive CCD cameras provide a hardware binning function to combine several pixels into a super-pixel to increase the sensitivity of the sensor. Obviously, binning is an efficient method of improving the sensitivity because reducing half of the resolution can improve the sensitivity four times. If resolution is more important, the user can always reduce the binning size. As shown in the following section, BLT has a resolution of roughly 1 mm. The surface measurement with better than 0.5 mm resolution will not help the reconstruction. We can utilize the extra resolution to increase the sensitivity.

Longer exposure times can integrate more photons over time and can increase the signal-to-noise ratio of the image. But long exposure times are constrained by the thermal noise of the CCD; if the thermal noise is comparable to the signal strength, increasing exposure time will not increase the signal-to-noise ratio. In this case, deep cooling is very important for ultraweak signal detection. Another factor, not discussed in the literature, restricts the long exposure time experiment, the light-sealing performance of the imaging enclosure. If using a custom-built light enclosure, attention should be paid to the sealing problem. Even a tiny light path on the enclosure may ruin the experiment.

Another way to collect more photons is by using a large aperture lens system. Current commercial systems, such as the IVIS 100 and the IVIS 200 from Xenogen, use an f/1.0 lens for small animal study. An f/1.0 lens has a short depth of field (the portion of a scene that appears clear and sharp in a photography). For curved surface, the short depth of field may introduce problems for high-resolution imaging. If the object is small enough (compared to the size of the CCD sensor) and has a flat surface, a customized lens system with an even higher aperture (<f/1.0) can be used in this situation. Figure 9.3 shows an extreme design, which can collect almost all the photons. This design, borrowed from a so-called "Mirage Mirror" toy design, needs two large parabolic mirrors. There are two holes in the center of the two reflectors. The specimen is placed on the focus point of the upper parabolic mirror. This parabolic mirror converts the diverse light from the specimen to a parallel beam, which is then focused by the lower parabolic mirror to the CCD. This design can collect much more light than an f/1.0 lens, which means it can target in extreme low light conditions or for fast dynamic monitoring. The only requirement of this design is the specimen must be flat and small (compared to the size of the CCD).

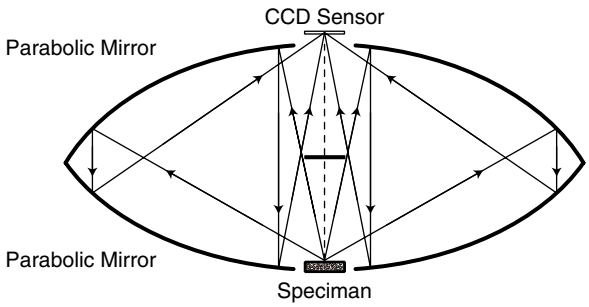

Fig. 9.3 Matched parabolic mirror design to collect as much light as possible. The two parabolic mirrors with similar shape and focus length are coupled to form a ~1:1 copy lens

9.3.2.1 Multiview and Multispectral Bioluminescence Imaging System

To improve the BLT reconstruction, we can sample the bioluminescence spectrum into a number of bands or channels for multiple wavelength imaging and then perform the probe source reconstruction. Results showed that multispectral bioluminescence tomography (MSBT) could improve the BLT reconstruction stability and transform the ill-posed problem into a better-posed setting [2, 18, 21, 25, 30, 38–40, 52]. After the injection of luciferin substrate, the bioluminescent signal will increase while the substrate is diffused to the mouse body within 10–20 min. Then, the strength of the bioluminescent signal will decrease over several hours [80]. To avoid the dynamic change of bioluminescent signals, most bioluminescence experiments will allow a 15-min uptake time. In multispectral BLI, most current and commercial BLI systems use a filter wheel to capture the multispectral bioluminescence signal spectrum by spectrum, sequentially, each time with a different filter [18, 21, 25, 52]. This approach assumes that the bioluminescence signal is stable in the data acquisition process. Unfortunately, this assumption is only realistic for BLI experiments with a short experimental time. Several studies have shown that the time course of the bioluminescence signal could have a broad range of dynamic behaviors during the BLI/BLT experiments [80]. This assumption was clearly violated, and it became very difficult to improve the fundamentally compromised data quality

retrospectively. Hence, this kind of measurement will introduce significant inconsistencies in the resultant datasets and most likely render the reconstruction useless [80]. For current commercial BLI systems, to capture multispectral signal with multiple views, we need to open the door of the imaging system and flip the mouse manually. This will introduce even more problems, even longer imaging time, and the change of the mouse position. The latter problem makes the image registration even harder. To solve this problem, Caliper introduced an IVIS 3D system (discontinued). In this system, the mouse or specimen was placed on a flat transparent glass plate, and a mirror system rotated around the specimen to capture multiple views without adjusting the posture of the specimen. For multiview and multispectral BLI, the IVIS 3D system still needed to capture those views and spectral channels one by one, and it was almost impossible to compensate the time course of the bioluminescence signal. In 2005, a multiview system was introduced to capture multiple views of a small animal simultaneously by placing several mirrors around the mouse [18]. But the virtual images for the four views are not on the same focus plane, and the distance between the four views ranges from a few centimeter to more than a decimeter. For low light imaging with an aperture of f/1.0 used in a experiment, this multiview imaging system could not get a clear image for all four views because a large aperture has a very shallow depth of field. Even using a larger pixel size, the depth of field is less than a few millimeters.

Recently, a multiview imaging system was introduced to capture multiple views simultaneously without those problems [86, 87]. As shown in Fig. 9.4, four mirrors are placed around the specimen symmetrically to reflect the bioluminescence photon to the camera. Since the mirrors are symmetrical and the angle between the mirror and the axis of the specimen is 45°, the four virtual images formed in the four mirrors will be in one imaging plane and normal to the axis. Hence, the camera can focus on all the four views and capture the bioluminescence views simultaneously. In this case, we do not need to compensate the signal difference intrinsic to the sequential collection mode. The multiview design can be refined to fit the need of the tissue to have more or fewer mirrors surrounding the object. For example, a two-mirror design is best suited for a thin-sheet engineered tissue.

The major advantage of this system design is that it can capture multiple views simultaneously. This is crucially important for BLT, which needs multiple bioluminescence views for reconstruction. Another advantage for multiview design is using longer imaging times. For example, in a single-view design, an imaging experiment needs 20 min (4 views × 5 min per view). For a four-view design, we can use a single 20-min exposure to capture all four views. There is also a drawback in this design; the camera

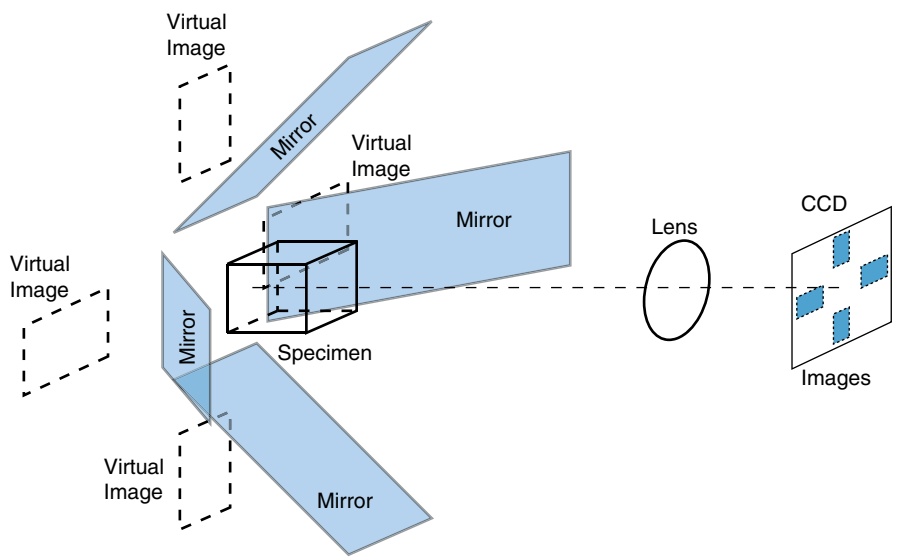

Fig. 9.4 Four-view system design for BLI. Four mirrors are placed around the specimen to reflect the bioluminescence signal to the camera. The four virtual images are on the same focus plane

needs to cover all four views, which will reduce the resolution for the surface bioluminescence image. In ultralow-signal cases, single-view BLI can use this extraresolution to trade for sensitivity by hardware pixel binning.

With the multiview setting, we still need to change filters for different spectral bands and still need to have a method to compensate for the signal decay for different spectral bands. In photography field, the most common way to capture multispectral image is by placing a tiny filter directly in front of each pixel of the CCD/CMOS sensor. The adjacent pixels have different filters for different wavelength (red, green, and blue), and several pixels can be combined as a superpixel with color information. Since the color-filter array is mounted directly in front of the sensor, it is not convenient to change the filter for different wavelengths. Another technique called three-CCD technology is used in some cameras and camcorders [54], which relies on a special dichroic prism to divide the spectrum and direct each spectral component to its corresponding CCD. For different applications, we can change the prism for different wavelengths. Since more than one CCD is required in this design, the hardware expense is high. But this design has the potential to be adapted for multispectral BLI. Recently, Foveon Inc. (http://www.foveon.com/) introduced a new type of imaging sensor, with three layers for three spectral bands because red, green, and blue signals penetrate silicon to different depths, which means that the spectral partition is fixed. Nikon has an alternative design, which puts a microlens and microdichroic array in front of the sensor. Since the spectrum-splitting device is rigidly attached to the sensor, the spectral partition cannot be changed flexibly. All the above designs have some difficulty to apply to BLI.

There is a digital-spectrum-separation method, which can recover the signal decay automatically between different spectral bands [88]. The core component of digital spectral separation is a customized dichroic beam splitter. Called a spatially translated spectral-image mixer (SSM), it features a glass plate with a dichroic coating on one side and a mirror coating on the other. For example, if the bioluminescence spectrum can be partitioned into two bands, red and blue, the dichroic coating at the front surface can be a perfect long-pass dichroic, which passes red wavelength and reflects blue wavelength. The reflected blue spectrum will form a virtual image, and the transmitted red spectrum will be reflected by the mirror coating on the second surface of the glass plate. The reflected red spectrum passes through the dichroic coating and is then captured by the camera. The red spectrum forms another virtual image. The two virtual images have a spatial shift, a common unpleasant effect in optics called a *ghost image*. Normally, the ghost image is eliminated by using antireflective coating and thinner component. In the digital spectral separation, we create and utilize the ghost image to separate different spectra. However, the ghost image in the SSM component is different from the ordinary ghost image. The SSM component separates two spectral channels, shifts one of the channels in space, and then mixes them together. A digital separation algorithm unmixes the two spectra digitally (Fig. 9.5).

By modeling the spectra separation, shifting, and mixing of the SSM component, the composite image can be expressed as a linear equation. Two composite

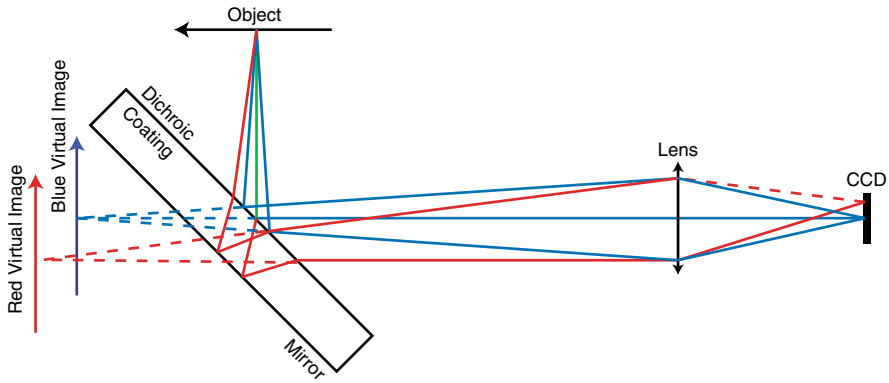

Fig. 9.5 Illustration of optical path for spectral-image mixer (SSM) component. SSM component separates the mixed spectra and translates one of the spectral channel in space and remixes the two spectral channels together

images collected by two different SSMs will have different linear equations and a nonnegative linear least squares problem can be constructed based on the two images. The solution of the nonnegative linear least squares problem is the unmixed spectral images.

The four-view BLI system can be incorporated into the digital spectral separation technique by replacing two of the mirrors with two SSM components. The two SSMs are of the same type, which redirect two views with remixed spectral components. The whole setting can be rotated 90° to acquire two independent views from each orthogonal direction around the specimen surface. The resulting datasets will offer sufficient information to recover the two spectral components for all the four views.

9.3.3 Bioluminescence Tomography Algorithm

Given the geometrical model, physical model, in vivo optical parameters, and spectra of the bioluminescence probe, we can discrete the photon transportation equation (either diffusion-approximation method, phase-approximation method, or any other approximation method) into a linear algebraic system. Using the diffusion equation as an example, the geometrical model of the mouse can be discretized into finite elements with n vertex nodes. The photon fluence rate Φ is solved by

$$\mathbf{M}\Phi = \mathbf{F}\mathbf{P} \Rightarrow \Phi = \mathbf{M}^{-1}\mathbf{F}\mathbf{P} \Rightarrow \bar{\Phi} = \bar{\mathbf{K}}\mathbf{P}.$$

From this formula, we can compute the surface fluence rate for any bioluminescence source inside the tissue. This produces the following formula:

$$\bar{\Phi} = \begin{bmatrix} \bar{K}_1 & \cdots & \bar{K}_j & \cdots & \bar{K}_n \end{bmatrix} \begin{bmatrix} P_1 \\ \vdots \\ P_j \\ \vdots \\ P_n \end{bmatrix} = \bar{\mathbf{K}}\mathbf{P},$$

where $\bar{\Phi}$ is a vector corresponding to the surface fluence, $\bar{K}_j$ is the surface fluence vector corresponding to a unit power source at finite element j, and P_j is the power for a bioluminescence source at finite element j.

Therefore, the source reconstruction of MSBT is reduced to reconstruct the bioluminescent source distribution $\mathbf{P}$ from the measured photon fluence rate data Φ^m from the surface of the tissue, and the problem is equivalent to find a nonnegative linear combination of $\bar{K}_j$ to fit Φ^m. Since the measured photon fluence rate is a 2D dataset and the bioluminescent source distribution is 3D, the number of unknown variables for the bioluminescence source distribution is more than the number of truly independent measures on the mouse body surface. Hence, BLT is a typical underdetermined problem. To solve the BLT problem, most BLT algorithms compute a set of photon fluence rates for all the possible source locations. This will greatly increase the computational costs.

9.3.3.1 Objective Function

The most common objective function to describe the fitness of reconstructed source and the measurement is the least squares error function [18, 30, 37, 81]:

$$\min_{\mathbf{P}\in\text{constrain}} \Theta(\mathrm{P}) = \left\| \Phi^m - \bar{\mathrm{K}}\mathrm{P} \right\|^2. \tag{9.4}$$

To stabilize the optimization process further, regularization and penalty functions are used with the object function [18, 30, 37, 81]. This linear least squares problem can be solved by using a nonnegative least squares solver or a gradient-based optimization solver, such as the Newton method. Because of the measurement noise and model mismatch, the deterministic optimization methods cannot solve this optimization problem efficiently and often result in local minimal, which is far away from the true source distribution.

Besides the use of the least square error function as the objective function, other objective functions can be used in BLT. Cong et al. [27] used a Maximum-Likelihood approach:

$$\min_{\mathbf{P}\in\text{constrain}} \Theta(\mathrm{P}) = (\Phi^m - \bar{\mathrm{K}}\mathrm{P})^T \mathrm{W} (\Phi^m - \bar{\mathrm{K}}\mathrm{P}),$$

where $\mathbf{W}$ is a diagonal matrix from the covariance matrix of the measurement data. This maximum-likelihood approach can be seen as a weighted least squares error function and, similarly, we can use gradient-based optimization method to solve the optimization problem.

It is also possible to use other objective functions for this inverse problem. For example, Alexandrakis et al. applied an expectation maximization (EM) algorithm to a multiple spectral channel BLT [2]). In addition to refining the objective function, additional constraints can greatly help to reduce the problem search space. We can use a so-called *permissible source regions strategy* to limit the possible source region [27, 81]. From bioluminescent views taken by the CCD camera, possible source clusters should be closer to the place with bioluminescence signal than other surface places. Thus, with this hint, we can generate permissible source regions where a bioluminescent source may exist. Applying the permissible source region can significantly reduce the number of unknown source variables. If we can limit the size of permissible region lower than the independent surface measurements, deterministic optimization solver can give good results, as shown in the article of Wang et al [81]. One very important assumption for BLT involves the number of isolated bioluminescence sources, which is usually a very small number (<10). Based on bioluminescent views, we can estimate a rough number of bioluminescent sources, which are then used as a constraint for optimization. We may assume different source numbers for BLT reconstruction to compare the resultant errors of the objective function and find optimal results.

9.3.3.2 Stochastic Algorithm

In 2006, Klose introduced a stochastic-based iterative algorithm that used an evolution algorithm to guide the stochastic selection and alteration mechanism [49]. The search algorithm had the following steps:

(1) Initial a source distribution solution pool: SP.
(2) Evaluate the objective function for every solution in SP.
(3) Alter solutions in the pool.
(4) Evaluate the objective function for the new solutions.
(5) If the new solutions are better than the old solutions, replace them in SP.
(6) If the improvement between two iterations is smaller than a predefined value, return the best solution; otherwise, go to step (3).

In this algorithm, stochastic and evolution mechanisms play important roles; the initial source distribution pool is generated randomly, and the alteration is randomly selected (with bias). This guarantees the search algorithm can travel most of the problem space instead of resulting in a local minimum. The evolution strategy, a well-established method in many fields, is a powerful search method for complex optimization problems. The evolution algorithm was inspired by natural selection, a biological process in which the stronger are more likely to be the survivors. The evolution algorithm uses a direct analogy of this natural process. The most popular genetic operations are *mate* and *mutate*. *Mate* means exchanging the gene information between parents to generate children. In Klose's research, the *Mate* was implemented by adding two solution vectors and dividing by two. *Mutate* means perturbing genes randomly. The candidates for the *Mate* and *Mutate* operators are randomly selected. Klose tested the stochastic algorithm in a 2D numerical phantom extensively with accurate reconstruction results. More testing in 3D cases is needed to verify the performance of this algorithm.

9.3.3.3 Multispectral BLT

BLT research groups realized the importance of multispectral information [1, 2, 18, 21, 25, 30, 33, 34, 38, 52, 60, 86–88]. The reason for multispectral imaging is that the optical parameters of biological tissue depend on the wavelength of the light. Hence, we can produce a set of independent system matrices and linearly independent measurements. By using two spectral channels, we can double the independent measurements with the same number of unknowns. Numerical simulations, phantom, and in vivo studies confirmed the advantage of multispectral BLT [1, 2, 18, 21, 25, 30, 33, 52].

Actually, the BLT algorithm above is applicable to any spectral band. At each wavelength of interest λ_i, we have

$$\bar{\Phi}_{\lambda_i} = \bar{K}_{\lambda_i} P_{\lambda_i}.$$

If there is only one type of bioluminescence probe in the specimen, we can utilize this information to enhance the reconstruction. Since for each type of bioluminescence probe, the weight for each spectrum is fixed

$$\bar{\Phi}_{\lambda_i} = \bar{K}_{\lambda_i} P_{\lambda_i} = w_{\lambda_i} \bar{K}_{\lambda_i} P,$$

where w_{λ_i} is the weight for the spectrum λ_i in the bioluminescence probe. In the optimization step, we can combine the equations for all spectra:

$$\min_{S\in constrain} \Theta(P) = \sum \left\| \left\{ \Phi_{\lambda_i}^m \right\} - w_{\lambda_i} \bar{K}_{\lambda_i} P \right\|^2.$$

The multispectral BLT algorithm can be extended for multiprobe BLT [21, 25, 86, 87]. If there is more than one type of bioluminescence probes in the specimen, we need to solve the reverse problem for each spectrum. That information can then be used to determine the distribution for each type of probe. If we have three types of bioluminescence probes in one specimen, we need to divide the spectrum into at least three spectral channels to cover the three probes spectral range. Let P_{λ_i} be the source distribution for λ_i. Based on the known spectra of the bioluminescence probes and the reconstructed source distributions in every spectral band, we have the following relationship between the sources P_{λ_i} and probes $\mathbf{b}_i$, $i = 1, 2, 3$:

$$\begin{bmatrix} w_{11} & w_{12} & w_{13} \\ w_{21} & w_{22} & w_{23} \\ w_{31} & w_{32} & w_{33} \end{bmatrix} \begin{bmatrix} \mathbf{b}_1 \\ \mathbf{b}_2 \\ \mathbf{b}_3 \end{bmatrix} = \begin{bmatrix} \mathbf{P}_{\lambda_1} \\ \mathbf{P}_{\lambda_2} \\ \mathbf{P}_{\lambda_3} \end{bmatrix},$$

where b_i is the number of cells tagged with bioluminescent with the ith probe, and w_{ij} the ith spectral component from the jth probe measured in the experiment (after normalization). The equation can be solved to obtain the probe distributions b_i, for $i = 1, 2, 3$.

9.3.3.4 Temperature-Modulated Bioluminescence Tomography

As shown in several research articles, the emission spectra of bioluminescence probes are modulated by temperature [43, 96], which provides a major opportunity to overcome the inherent ill-posedness of BLT. In 2006, temperature-modulated bioluminescence tomography (TBT) [86, 87] was proposed to utilize the temperature dependence of bioluminescence for superior BLT performance. As shown in last section, the BLT uses a 2D surface measurement to reconstruct the 3D source distribution. Even if we can use the surface measurement to predict the permissible regions (regions may have bioluminescence sources), the permissible regions are still too large, and multiple sources may exist in one permissible region. If we can reduce the size and number of permissible regions, the BLT problem will be much easier to solve. In TBT, a focused ultrasound array is used to heat a small volume of interest (VOI). If there is a bioluminescence source inside the VOI, the surface optical signal will change, and the change will only relate to the source in the heating region.

In the TBT process, we first record bioluminescent data on the body surface of a mouse at a lower body temperature and may perform a regular BLT reconstruction. Based on the information we collect, we can determine which regions should be heated for improvement of the BLT reconstruction. For example, we may evaluate all the elements in the permissible region via a cluster analysis, which is a well-established methodology in pattern recognition to merge finite elements of bioluminescent sources into localized groups. Then, we may use minimal spheres to cover identified clusters and heat each sphere to collect bioluminescent data again. If there is a significant difference between the data before and after heating, a TBT reconstruction is performed on the difference signal for the heated volume. If the heated volume does not produce a significant change in the bioluminescent signal, the previous BLT reconstruction in that region is considered invalid, and we need to select a different permissible region. Any body region **R** in a mouse can be heated to determine if a bioluminescent source exists; if so, we can perform a TBT reconstruction based on the difference data measured on the mouse body surface before and after heating. The surface fluence rate at reference temperature (lower temperature) is:

$$\bar{\Phi} = \sum_{i=1}^{n} w_{\lambda_i} \bar{\mathbf{K}}_{\lambda_i} \mathbf{P}_I + \sum_{i=1}^{n} w_{\lambda_i} \bar{\mathbf{K}}'_{\lambda_i} \mathbf{P}_O,$$

where the $\mathbf{P}_I$ and $\mathbf{P}_O$ are the source power vector inside and outside the heating range, respectively. After heating, the surface fluence rate will change to:

$$\bar{\Phi}_H = t \sum_{i=1}^{n} w'_{\lambda_i} \bar{\mathbf{K}}_{\lambda_i} \mathbf{P}_I + \sum_{i=1}^{n} w_{\lambda_i} \bar{\mathbf{K}}'_{\lambda_i} \mathbf{P}_O,$$

where t is the normalized total energy at the elevated temperature. Hence the signal difference is:

$$\bar{\Phi}_D = \bar{\Phi}_H - \bar{\Phi} = \sum_{i=1}^{n} (t w'_{\lambda_i} - w_{\lambda_i}) \bar{\mathbf{K}}_{\lambda_i} \mathbf{P}_I.$$

By heating a relatively small region and extracting the difference signal, the permissible region for

bioluminescence source reconstruction is dramatically reduced to the heat region. This helps regularize an ill-posed BLT problem into a better-conditioned TBT framework.

9.3.4 Discussion

The main advantage of BLI/BLT is the sensitivity. Recent study showed that BLI had the sensitivity to trace ~100 photons/s/cm^2/sr, which were about 10–100 labeled cells under subcutaneous situations [73, 79]. Since there is no need to have an external excitation light source, the bioluminescence signal is virtually background-free. Second, BLI is easy to perform and can be used to validate hypotheses rapidly in nondestructive and in vivo experimental models. Also, the cost of BLI is relatively low compared to micro-CT and micro-MRI. A customized BLI system can be constructed for under $70K, and the everyday running cost is very low. Third, compared with other optical-imaging method, BLI has the most penetration depth; a 1–2 cm penetration depth for normal biological tissue is achievable. Finally, the BLT can be used to recover the 3D location and quantity of the labeled cells, which is more informative than the planar-imaging modality.

However, the BLI/BLT has several major drawbacks. (1) Since the BLI relies on the bioluminescence labeling probes, which could not be directly used in human, this prevents the direct clinical use of BLI. (2) The energy in currently available bioluminescence probes is in the visible spectrum range, which is strongly absorbed and scattered by the biological tissue. This limits the accuracy and the penetration depth of this modality. Current BLI technology can have a penetration depth of 1–2 cm depending on the strength of the bioluminescence sources. More synthesized bioluminescence probes with NIR spectrums have better penetration depth. (3) The BLT can help identify the 3D position of the bioluminescence source, but with a low resolution. The reconstruction resolution depends on the depth of the bioluminescence sources. Currently, state-of-the-art bioluminescence algorithms can have a 1–2 mm resolution in small animal studies [85, 92]. We can have better resolution for small-size engineered tissue. (4) There is no commercial BLT system, and extra effort is needed to apply this technology.

9.4 Fluorescence Imaging

There is a large family of fluorescent probes (fluorescent proteins, dyes, quantum dots, and nanosensor) to cover the spectra from UV to NIR and the ability to tag cellular and molecular processes in vivo. Fluorescence imaging received particular attention in studying gene expression/function and molecular interactions [62, 66]. Fluorescence is a process wherein some molecules can absorb light with certain wavelengths and then emit light of different wavelengths. To utilize the fluorescence in an imaging system, an excitation light source and an optical delivery system are used to illuminate the fluorescence-labeled molecule or cell; an optical system collects the emitted light and filters out the excitation light; and a sensor converts the light signal to an electric signal. The most commonly used optical molecular imaging technique in a biological laboratory is fluorescence microscopy, which has long been the gold standard for in vitro and ex vivo application in molecular and cellular biology. There are several fluorescence microscopy techniques, including the ordinary fluorescence microscopy, confocal fluorescence microscopy [31], total internal reflection fluorescence microscope [8], second-harmonic generation fluorescence microscopy [13, 14, 64], and multiphoton fluorescence microscopy [31, 32, 45, 78]. Recently, fluorescence microscopy was extended in in vivo imaging for deep tissue using nonlinear light-matter interactions, including two-photon absorption, second-harmonic generation, and coherent anti-Stokes Raman scattering. Especially, the two-photon excitation fluorescence imaging technique provides a powerful tool to study the fluorescence probe distribution, which can be labeled with a particular cell or molecule, in tissue sample or even living animals in great detail at depths of up to 1 mm [45]. The major problem for those microscopy techniques is the shallow penetration depth (within 1 mm).

Planar whole body or large-scale fluorescence imaging are the most common method for large specimen and deep tissue imaging [66]. In this method, an external excitation light source shines on the surface of the specimen and penetrates into the tissue. The diffused excitation light excites the fluorophore, which emits light in different wavelengths (usually longer wavelengths). The emission light can then reach the specimen surface where it is collected by the camera

as a planar image. Compared to fluorescence microscopy, planar fluorescence imaging can have much deeper penetration depth but lacks accurate depth information. To overcome this problem, fluorescence tomography was introduced to recover the 3D internal fluorescence distribution.

9.4.1 Fluorescence Tomography

Fluorescence tomography provides a nondestructive method to study the fluorophore distribution in engineered tissue noninvasively. The basic idea of fluorescence tomography is similar to BLT. The fluorescence tomography is an inverse problem aided by an external excitation light source. In comparing the fluorescence tomography with fluorescence microscopy, the fluorescence tomography deals with much larger and deeper tissues with a relatively lower resolution (submillimeter resolution).

As shown in Fig. 9.6, a typical fluorescence imaging system is similar to a BLI system but with an excitation light source and a set of filters to prevent the excitation energy going into the camera. The major difference between different fluorescence imaging system is the excitation light delivery and emission light collection methods. As shown in Fig. 9.6a, the epi-illumination illuminates light from the same side where back-emission light is collected. The excitation laser delivery method shown in Fig. 9.6b is called transillumination, in which the illuminate light is on one side of the specimen and collects the transmitted emission light on the opposite side of the specimen [66]. In comparing the two illumination methods, the epi-illumination favors the fluorescence source near the camera, while the transillumination method does not have this problem.

Recently, the multiview collection method, which collects multiple views of the emission fluorescence signal simultaneously, was used in some fluorescence imaging systems, such as the DyCE in vivo fluorescence imaging system from Cambridge Research & Instrumentation [46]. In Fig. 9.6c, four or eight mirrors are placed around the tissue. Excitation light shines on the mirrors and be reflected to the tissue (or direct shines on the tissue surface). The mirrors also reflect the emission light to the camera. This is equivalent to a mix of epi-illumination and transillumination with multiview enhancement. In Fig. 9.6d, the excitation light is delivered directly to the bottom of the tissue, and the camera collects the emission light around the tissue.

In a regular fluorescence tomography experiment, an excitation light with known power, shape, and wavelength shines on the surface of the tissue. The excitation light propagates into the tissue, some of the light escapes from the tissue surface and is captured by a camera to form an excitation planar image. The light excites the fluorophore in the tissue, and the emission fluorescence photon propagates in the tissue. Some of the fluorescence light can reach the surface of the tissue and is then captured by a camera to form an emission planar image (a filter is needed to filter out the excitation light).

9.4.1.1 Fluorescence Tomography Algorithm

Figure 9.1 showed the schema for BLT and fluorescence tomography has a similar schema. Three types of information are needed to solve the internal source inverse problem: (1) the 3D structure information, (2) the optical parameters of the specimen for each structure domain, and (3) the excitation and emission surface fluence measurements.

Fluorescence tomography aims at 3D reconstruction of the internal fluorophore distribution in tissue based on light measurements at the tissue surface. A light propagation model is needed, as shown in Sect. 9.2, to describe the excitation and emission photon propagation in biological tissue. A linear equation can be used to describe the photon fluence rate: $\Phi = \mathbf{KP}$. Let $\mathbf{P}_x$ be the excitation light source, $\mathbf{K}_x$ be the system matrix for the excitation wavelength, then $\Phi_x = \mathbf{K}_x\mathbf{P}_x$ is the photon fluence for excitation light. We can then link the excitation and the emission in the following formula:

$$\Phi_e = \mathbf{K}_e\mathbf{P}_e = (\mathbf{K}_e\{\Phi_x\})\hat{\eta}\mu,$$

where $\{\Phi_x\}$ is a diagonal matrix with diagonal elements as the excitation photon fluence from Φ_x, μ the absorption coefficient of the fluorescence probe, and $\hat{\eta}$ the fluorescence quantum yield, which is directly proportional to the fluorophore concentration. In $\mathbf{C} = \mathbf{K}_e\{\Phi_x\}$, furthermore, removing those rows of $\mathbf{C}$ that correspond to the internal photon fluence rate

values and keeping the rows that correspond to the surface nodes, $\mathbf{C}$ becomes $\overline{C}$. Accordingly, we obtain the linear equation system linking the unknown fluorescence source density and measurable photon fluence rate on the external surface of the geometrical model: $\overline{\Phi}_e = \overline{\mathbf{C}}\hat{\eta}\mu$, where $\overline{\Phi}_e$ is the surface photon fluence of the emission fluorescence. Therefore, the reconstruction of fluorescence distribution is reduced to reconstruct the emission light source from the measured photon fluence data Φ^m based on the linear model, which is similar to BLT, and the optimization algorithms discussed in BLT can be applied to fluorescence tomography.

If the excitation light position changes, $\{\Phi_x\}$ will change nonlinearly; hence, multiple excitation positions will result in multiple measurements with some kind of independency. Assume there are l measurements each with different excitation positions, and there are l different linear systems, which can be combined to form a nonnegative least square problem:

$$\min_{\eta\geq 0}\Theta(\eta) = \min_{\eta\geq 0}\left\|\begin{bmatrix}\Phi^{m,1}\\ \vdots \\ \Phi^{m,l}\end{bmatrix} - \begin{bmatrix}\mathbf{K}_e\{\Phi_x\}^1\\ \vdots \\ \mathbf{K}_e\{\Phi_x\}^l\end{bmatrix}\hat{\eta}\mu\right\|^2,$$

where $\Phi^{m,i}$ is the ith fluorescence image mapped on the surface and $\{\Phi_x\}^i$ the diagonal matrix formed from ith fluorescence excitation.

In the algorithm above, one assumption is that the concentration of the fluorophore is relatively low to ensure the light absorption of fluorophore is insignificant compared to the tissue absorption coefficient, which means the fluorophore distribution will not change the optical parameters of the tissue significantly. In case the fluorophore concentration is high, an iterative method can be used to refine the result of fluorescence tomography. In the algorithm, an initial absorption coefficient for the tissue can be used without considering the fluorophore concentration to solve the fluorescence tomography. The resulting fluorophore concentration can then be used to update the absorption coefficient and solve the fluorescence tomography again. The process can be repeated until the change of the reconstructed fluorophore distributions between two iterations is less than a threshold.

9.4.1.2 Multispectral FMT

There are two multispectral techniques that can be used in fluorescence tomography: (1) sample the emission spectra in multiple channels, which are the same as multispectral BLT; (2) use different excitation wavelength light sources. Since many fluorescence probes, such as quantum dots, have a very broad excitation wavelength range, multiple excitation light sources with different wavelength can be used one at a time. Assume the emission spectrum is partitioned into n channels ($\lambda_{e,i}$, $1\leq i\leq n$) and m excitation wavelengths ($\lambda_{x,j}$, $1\leq j\leq m$) are used in the multispectral FMT experiment, for a specific excitation wavelength $\lambda_{x,j}$ and emission spectral channel $\lambda_{e,i}$, the emission image $\Phi_{e,\lambda_{e,i}}$ is governed by the following equation:

$$\Phi_{e,\lambda_{e,i}} = (\mathbf{K}_{e,\lambda_{e,i}}\{\Phi_x\}_{\lambda_{x,j}})\hat{\eta}\mu_{\lambda_{x,j}}w_{\lambda_{e,i}},$$
$$\text{for } 1\leq i\leq n \text{ and } 1\leq j\leq m,$$

where $\mathbf{K}_{e,\lambda_{e,i}}$ is the system matrix for the emission wavelength $\lambda_{e,i}$, $\{\Phi_x\}_{\lambda_{x,j}}$ the diagonal excitation fluence matrix for excitation wavelength $\lambda_{x,i}$, $\mu_{\lambda_{x,j}}$ the absorption coefficient of the fluorescence probe for excitation wavelength $\lambda_{x,i}$, $w_{\lambda_{e,i}}$ and the normalized weight for emission spectral channel $\lambda_{e,i}$. In combining these equations with multiple excitation sources, multiple excitation wavelengths, and multiple emission spectral channels, a nonnegative least square problem can be constructed.

The above multispectral fluorescence tomography algorithm is for single type of fluorescence probe. In case the experiment needs to label several types of cells with different type of fluorescence probes, the selection of fluorescence probes should avoid or minimize emission spectral overlap between different types of fluorescence probes. In case there is overlap between the emission spectra of different fluorescence probes, multispectral fluorescence tomography can unmix the probe distributions.

$$\Phi_{e,\lambda_{e,i}} = (\mathbf{K}_{e,\lambda_{e,i}}\{\Phi_x\}_{\lambda_{x,j}})\mathbf{P}_{\lambda_{x,j}} \text{ for } 1\leq i\leq n \text{ and } 1\leq j\leq m.$$

In the equation, $\mathbf{P}_{\lambda_{x,j}} = \sum_{k=1}^{l}\hat{\eta}_k\mu_{k,\lambda_{x,j}}w_{k,\lambda_{e,j}}$, there are a total of l fluorescence probes, $\hat{\eta}_k$, $\mu_{k,\lambda_{x,j}}$, and $w_{k,\lambda_{e,j}}$ are the corresponding parameters for probe k. After solving $\mathbf{P}_{\lambda_{x,j}}$, a linear equation can be constructed to solve the $\hat{\eta}_k$ for each fluorescence probe.

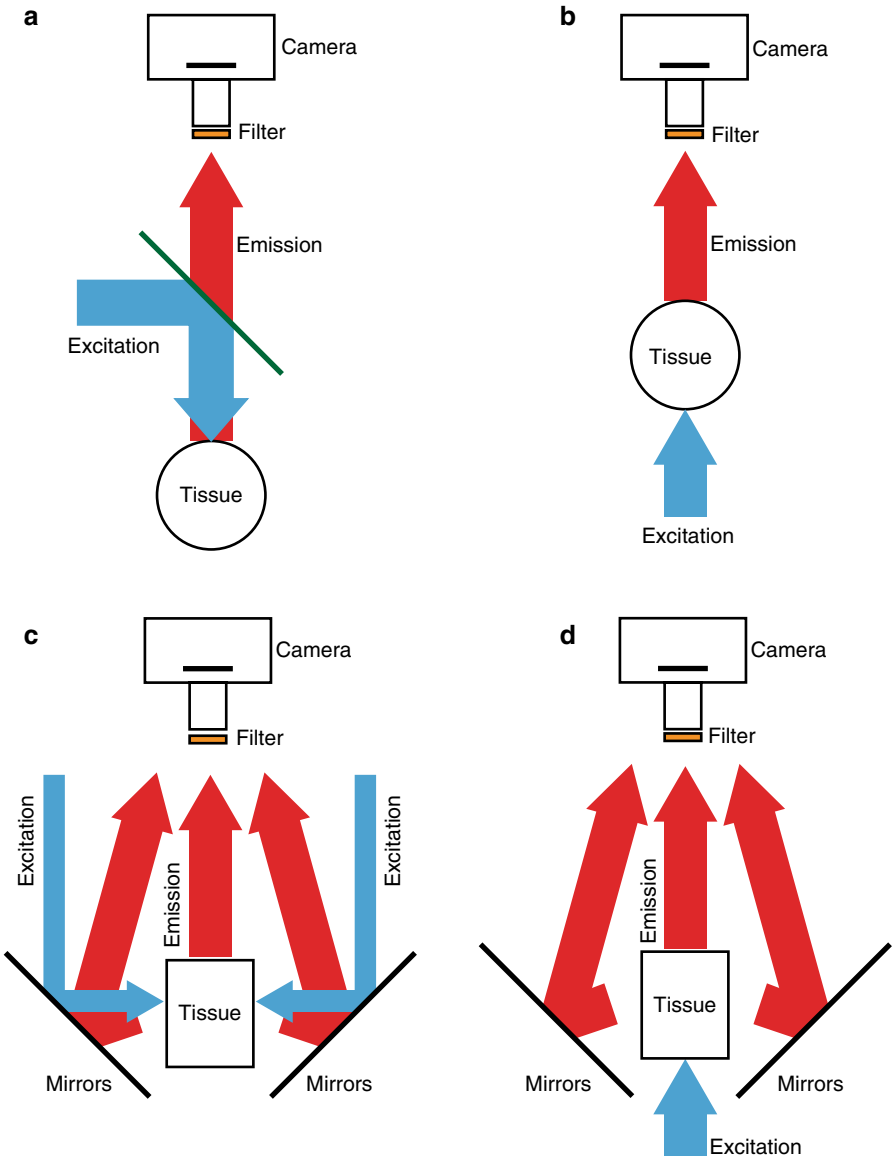

Fig. 9.6 Planar fluorescence imaging scheme. (**a**) Epi-illumination imaging schema, (**b**) transillumination imaging schema, (**c**) and (**d**) multiview imaging schemes

9.4.2 Discussion

Compared with BLT, fluorescence tomography has several advantages: (1) since the fluorescence signal is much stronger than a bioluminescence signal, the fluorescence tomography can resolve much higher temporal resolution. Less than 1 s temporal resolution is possible using a high speed EMCCD camera. (2) While the fluorescence is not very deep in the tissue, the reconstruction resolution of fluorescence tomography is higher than bioluminescence because multiple external excitation positions can be used, which results in more independent measurements. (3) There are more types of fluorescence probes than bioluminescence probes. The spectrum of those fluorescence probes covers the whole UV, visible, and NIR ranges, and those fluorescence probes have relatively narrow emission bands compared to bioluminescence. We can easily trace multiple fluorescence probes simultaneously. It also has several drawbacks: (1) It is hard to deliver the excitation light to a deep location (>1 cm). (2) Because many materials in tissue engineering have fluorescence/autofluorescence, sometimes, it is impossible to avoid this kind of background fluorescence noise. The sensitivity of fluorescence imaging/tomography will be lower than BLT.

9.5 Five-Year Perspective

Biomedical imaging has many potential applications in tissue engineering: studying the biological function in the engineered tissue, monitoring and controlling the tissue-regeneration process, verifying specific hypotheses of biological study, and helping drug designs using engineered tissue as a test bed. Biomedical imaging, especially optical molecular imaging, can play a major role in this exciting development.

Optical molecular tomography has been developed for more than a decade mainly on small animals in cancer studies and drug designs; recently, researches have showed that reliable reconstruction can be achieved under controlled situations. The error in tomographic reconstruction is mainly introduced by inaccurate photon propagation modeling, structure model mismatch, inaccurate optical parameter, measurement noise, and inherent ill-posed condition of inverse problem. Engineered tissue is a perfect field for application of optical molecular tomography. (1) The engineered tissue has a much simpler structure (internal and boundary) than a mouse and can greatly reduce the effort of creating an on-site finite element method for engineered tissue. (2) Engineered tissue has a better boundary condition, and high performance optical imaging system can be customized for nondestructive tissue engineering study. (3) The optical conditions of engineered tissue are simpler, and the optical parameters of engineered tissue can be precisely measured. (4) The imaging condition can be controlled; for example, the bioluminescence reaction rate can be modulated by controlling the bioreactor. In addition to those conditions, an integrated optical molecular system, which integrates the structure scanner, the diffuse optical tomography, and the optical tomography imaging, can greatly reduce the structure and optical mismatch. A current popular photon transportation modeling technique in optical tomography is diffusion approximation, which has significant error under unfavorable conditions. Improved approximation methods, which are computationally expensive, with much better accuracy can be applied in optical molecular tomography to improve the photon propagation modeling. With introduction of new computer hardware and improvement of better algorithm, those new approximation methods will gain more attention in this field.

Suggested Readings with Abstracts

Wang G, Li Y, Jiang M. Uniqueness theorems in bioluminescence tomography. Med Phys. 2004;31(8):2289–99.

This paper established the solution uniqueness for BLT under practical constraints despite the ill-posed inverse problem in the general case. Wang et al. first presented the BLT algorithm based on diffusion approximation and then proved that, in the general case, the BLT solution is not unique. The paper posited that in the case of impulse and solid/hollow ball sources, there was unique solution.

Chaudhari AJ, Darvas F, Bading JR, Moats RA, Conti PS, Smith DJ, et al. Hyperspectral and multispectral bioluminescence optical tomography for small animal imaging. Phys Med Biol. 2005;50:5421–41.

This paper presented a multispectral bioluminescence tomography algorithm and demonstrated it using numerical simulation, phantom study, and mouse study. The simulation studies indicated that submillimeter resolution might be attainable given accurate knowledge of the optical properties of the animal. They also included an in vivo study of a mouse with a brain tumor expressing firefly luciferase. Coregistration of the reconstructed 3D bioluminescent image with magnetic resonance images indicated good anatomical localization of the tumor.

Cong WX, Wang G, Kumar D, Liu Y, Jiang M, Wang LV, et al. Practical reconstruction method for bioluminescence tomography. Opt Express. 2005;13(18):6756–71.

In this paper, Cong et al. established a finite element-based method for bioluminescence tomography by using a diffusion approximation. A maximum-likelihood optimization approach coupled with a permissible source region as a priori knowledge for source reconstruction was proposed. The feasibility of the proposed method was demonstrated with a numerical mouse chest simulation and a physical mouse chest phantom with two embedded light sources.

Klose AD, Ntziachristos V, Hielscher AH. The inverse source problem based on the radiative transfer equation in optical molecular imaging. J Comput Phys. 2005;202(1):323–45.

Klose et al. presented the first radiative transport equation-based tomography reconstruction algorithm for fluorescence molecular tomography in highly

scattering biological tissue. An iteratively minimization algorithm was used to find a solution of the inverse source problem.

Cong A, Wang G. Multispectral bioluminescence tomography: methodology and simulation. Int J Biomed Imaging. 2006;ID57614:1–7.

Cong et al. presented a multispectral bioluminescence tomography method from multispectral data measured on external surface. A numerical simulation was conducted on a heterogeneous mouse chest phantom, and the results showed that the multispectral approach significantly improved the accuracy and stability of the BLT reconstruction, even if the data were highly noisy.

Wang G, Cong WX, Durairaj K, Qian X, Shen H, Sinn P, et al. In vivo mouse studies with bioluminescence tomography. Opt Express. 2006;14(17):7801–9.

In this paper, Wang et al. demonstrated the bioluminescence tomography using a modality fusion approach with numerical simulation and depth tissue in vivo mouse experiments. The results showed the feasibility of the methodology for localization and quantification of the bioluminescent activities in vivo with prior knowledge.

Wang G, Shen H, Cong WX, Zhao S, Wei GW. Temperature-modulated bioluminescence tomography. Opt Express. 2006;14(17):7852–71.

In this paper, Wang et al. proposed a temperature-modulated bioluminescence tomography (TBT) for superior BLT reconstruction performance. A focused ultrasound array was used to heat small volumes of interest (VOI), one at a time, to induce a detectable change in the optical signal on the body surface of a mouse. Based on the difference signal, the BLT reconstruction could be stabilized and improved.

Klose AD, Larsen EW. Light transport in biological tissue based on the simplified spherical harmonics equations. J Comput Phys. 2006;220:441–70.

Klose et al. presented an extensive study on a simplified spherical harmonics (SP_N) method for photon transportation in biological tissue with strong forward scattering. The numerical experiment showed that the SP_N model was significantly more accurate than diffusion approximation with the same magnitude of computation cost as diffusion approximation.

Klose AD. Transport-theory-based stochastic image reconstruction of bioluminescent sources. J Opt Soc Am A Opt Image Sci Vis. 2007;24(6):1601–8.

Klose proposed a stochastic search algorithm for bioluminescence tomography. The selection and alteration mechanisms in the random search algorithm were guided by evolutionary principles found in nature, leading to new stochastic samples of source distributions for the next iteration cycle. A least-squares-error solution was obtained after convergence. Numerical experiments demonstrated the feasibility of reconstructing bioluminescent source distributions in tissue-like media.

Ahn S, Chaudhari AJ, Darvas F, Bouman CA, Leahy RM. Fast iterative image reconstruction methods for fully 3D multispectral bioluminescence tomography. Phys Med Biol. 2008;53:3921–42.

This paper investigated fast iterative image reconstruction methods for 3D multispectral bioluminescence tomography using a finite element method. Ahn *et al.* compared a direct calculation approach and an on-the-fly approach and concluded the on-the-fly approach could lead to substantial reductions in computational costs when combined with a rapidly converging iterative algorithm

Wang G, Shen H, Liu Y, Cong A, Cong W, Wang Y, et al. Digital spectral separation methods and systems for bioluminescence imaging. Opt Express. 2008;16(3):1719–32.

A digital spectral separation (DSS) method was presented in this paper for multiview and multispectral bioluminescence imaging. This core component of the system mixed the spectral channels with a spatial shift of one of the spectral channels. Then, the DSS algorithm separated the mixed spectral channels digitally. This approach also permitted the recovery of the dynamic of bioluminescent signal time course, which was useful in studying the kinetics of multiple bioluminescent probes using multispectral bioluminescence tomography (MSBT).

Zhang QZ, Yin L, Tan Y, Yuan Z, Jiang HB. et al. Quantitative bioluminescence tomography guided by diffuse optical tomography. Opt Express. 2008;16(3):1481–6.

In this paper, Zhang et al. presented phantom experimental evidence indicating for the first time that the accuracy of bioluminescence tomography (BLT) could be significantly improved by incorporating prior spatial distribution of optical properties of heterogeneous media obtained from on-site diffuse optical tomography (DOT).

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Bioreactors for Tissue Engineering

10

Scott Maxson, David Orr, and Karen J.L. Burg

10.1 Introduction

A variety of bioreactor designs exist today as a result of previous efforts by engineers and researchers to construct optimal systems for a particular tissue engineering application. The primary purpose of any bioreactor is to provide a sterile cell culture environment that can be tightly controlled. A bioreactor can be as simple as a petri dish and as complex as an automatically controlled, cyclically loaded three-dimensional biochamber. A number of laboratories have designed custom bioreactors that apply mechanical stimuli to cells grown in monolayer or three-dimensional culture conditions. Varying levels of bioreactor complexity exist; typically, the systems incorporate at least one of five common loading regimens, including perfusion fluid flow, hydrostatic pressure, uniaxial displacement, biaxial displacement, or microgravity (Fig. 10.1) [10]. Bioreactors can be used to provide an in vitro environment more similar to the in vivo condition; dynamic culture systems are necessary to provide an appropriate, physiologically relevant environment [1].

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10.2 Aim of the Discipline

One of the major challenges of tissue engineering is the translation of research-scale designs into large-scale production of biologically functional tissues that are reproducible, safe, and cost-effective [21]. These requirements make bioreactors essential in the ex vivo engineering of living tissues. A bioreactor can be described as any apparatus that attempts to mimic and reproduce physiological conditions in order to maintain and encourage cell growth for tissue regeneration [7]. Ideally, a bioreactor should provide an in vitro environment for rapid and orderly tissue development, beginning with isolated cells and, in many cases, three-dimensional scaffolds. While there is not a universally recognized definition for "bioreactor," bioreactors typically are designed to perform one or more of the following functions while maintaining sterile conditions:

- Uniformly seed a concentration of cells onto clinically relevant biomaterial scaffolds
- Enhance mass transfer within cell/scaffold composites
- Control culture conditions such as temperature, pH, oxygen levels, nutrients, metabolites, and regulatory molecules
- Provide physiologically relevant physical signals such as interstitial fluid flow, shear, pressure, compression, and stretch [59]

Seeding scaffolds and creating the appropriate distribution of isolated cells within a scaffold is the first step in establishing a 3D culture. This step is crucial in determining the progression of tissue formation. Bioreactors are able to provide reliable and efficient cell-seeding methods that can produce uniform cell distribution within large scaffolds [37]. Seeding a high

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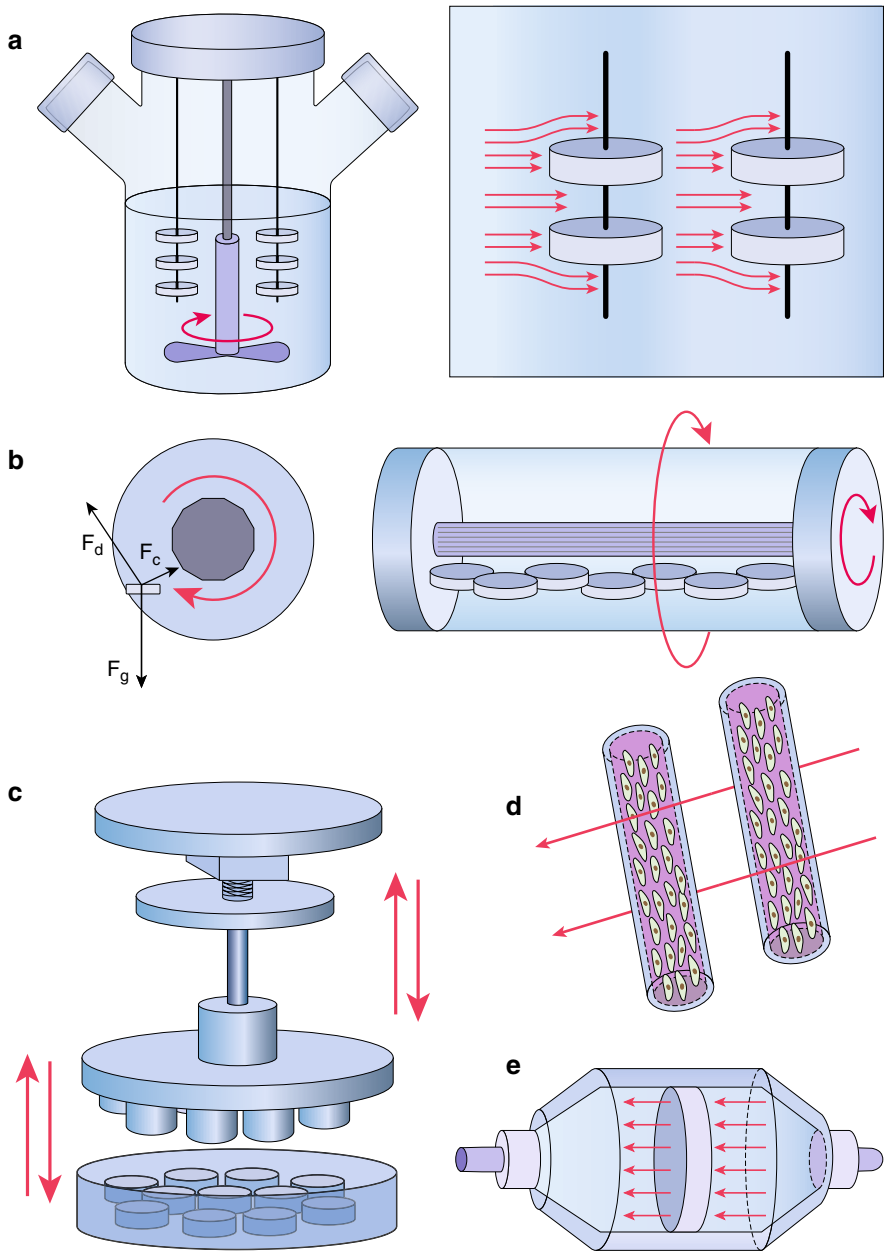

Fig. 10.1 Representative bioreactors for tissue engineering applications: (**a**) spinner flask bioreactors (**b**) rotating-wall vessels (**c**) hollow-fiber bioreactors (**d**) perfusion bioreactors and (**e**) bioreactors that apply controlled mechanical forces [38]

concentration of cells into a scaffold can enhance tissue formation in 3D constructs [31, 60]. Cells should be seeded with the highest possible efficiency in order to limit the biopsy sizes required and reduce the amount of future cell expansion required. Furthermore, the distribution of cells within the scaffold after seeding directly corresponds to the distribution of tissue subsequently formed within engineered tissue [60, 61], suggesting that uniform cell seeding could establish the basis for uniform tissue generation. Static loading (i.e., micropipetting) of cells into a scaffold is by far the most commonly used seeding method; however, this technique is often associated with low seeding efficiencies and nonuniform cell distributions within scaffolds [12, 13], due to the manual- and operator-dependent nature of the process [61].

Perfusing a cell suspension directly through the pores of a 3D scaffold in a bioreactor can enhance the

transport of the cells throughout the entire scaffold volume. Using a direct perfusion bioreactor, cells are seeded into porous biomaterials with higher seeding efficiencies and more uniform cell distributions than by static seeding or a stirred-flask bioreactor method [12, 13, 61, 62]. Perfusion seeding is also attractive because it can easily be integrated into a more complex bioreactor system in which the scaffold is seeded with cells and perfused within a single unit (Fig. 10.2). These bioreactor systems have the potential to streamline the engineering process and to reduce the safety and contamination risks associated with the handling and transferring of constructs between separate bioreactors [63].

After cells are seeded onto 3D scaffolds, bioreactors can be carefully regulated to control cell-culture parameters such as temperature, pH, oxygen level, nutrients, and regulatory molecules during the tissue maturation period. These parameters are important not only to realize controlled, reproducible investigations, but also in routine manufacturing of tissues for clinical applications [21]. In addition, bioreactors are used to modulate mass transfer, which is absolutely essential for both nutrient supply and waste elimination to maintain cell viability within large 3D scaffolds [37]. Mass transport must be regulated to ensure that cell metabolism is kept within a physiological range by providing metabolic substrates and removing toxic degradation products [28]. Section 10.3 provides a more in-depth description of bioreactor designs that accomplish these tasks.

Mechanical stimulation is routinely experienced by cells in vivo and is actually an important modulator of cell physiology [50]. As a result, many bioreactors used for cultivating tissue are designed to mimic the mechanical loading experienced in vivo. A few bioreactor systems incorporate other forms of stimulation, including electrical fields, ultrasound, or centrifugal forces [35, 47, 55]. Numerous studies have shown that mechanical stimulation can have a positive impact on tissue formation [15, 50], particularly in the context of musculoskeletal tissue engineering, cartilage formation, and cardiovascular tissue generation, among others [50]. Evidence shows that mechanical forces improve or accelerate tissue regeneration in vitro [21]. Shear stress induced by fluid flowing across a construct surface and into the cellular porous space is believed to be a very effective mechanical stimulus in activating mechano-transduction signaling. Consequently, fluid flow-induced shear stress is frequently used as a mechanical stimulus [21, 57].

The type of mechanical stimulation that is applied is dependent on the specific tissue that is involved and is chosen with consideration to the environment that the cells would experience in vivo. For example, engineered artificial arteries that experience cyclic stretching or distension show enhanced proliferation and matrix organization by human heart cells (Fig. 10.3) [3, 21].

Cyclic stretch also increases tissue organization and expression of elastin by smooth muscle cells and improves the mechanical properties of tissues generated by skeletal muscle cells [21, 51]. Dynamic deformational loading of chondrocytes stimulates glycosaminoglycan synthesis and enhances the mechanical properties of the resultant engineered cartilage [21, 24]. Translational strain of mesenchymal progenitor cells used for ligament tissue engineering induces cell alignment, formation of orientated collagen fibers, and up-regulation of ligament specific genes. Mechanical compression and hydrostatic pressure can alter gene expression and extra cellular matrix synthesis, thereby facilitating tissue formation in musculoskeletal tissue engineering [21, 39]. These examples show that specific mechanical loading produced by bioreactors can enhance the development of an engineered tissue.

Bioreactors are not only important in the production of clinically useful engineered tissue but also useful in studying the effects of different parameters on cell behavior. Bioreactors provide well-defined culture conditions, thus they are useful for systematic, controlled studies of cellular differentiation and tissue development in response to biochemical and mechanical cues [21]. Theoretically, each parameter can be modified to study its influence on the growth of different tissues and on the final properties of the regenerated construct [7].

10.3 State of the Art

It is essential that bioreactors are designed based on specifications that differ from tissue to tissue [7]. In the human body, cells are continually subjected to mechanical, electrical, and chemical signals that influence their phenotype, morphology, and proliferation

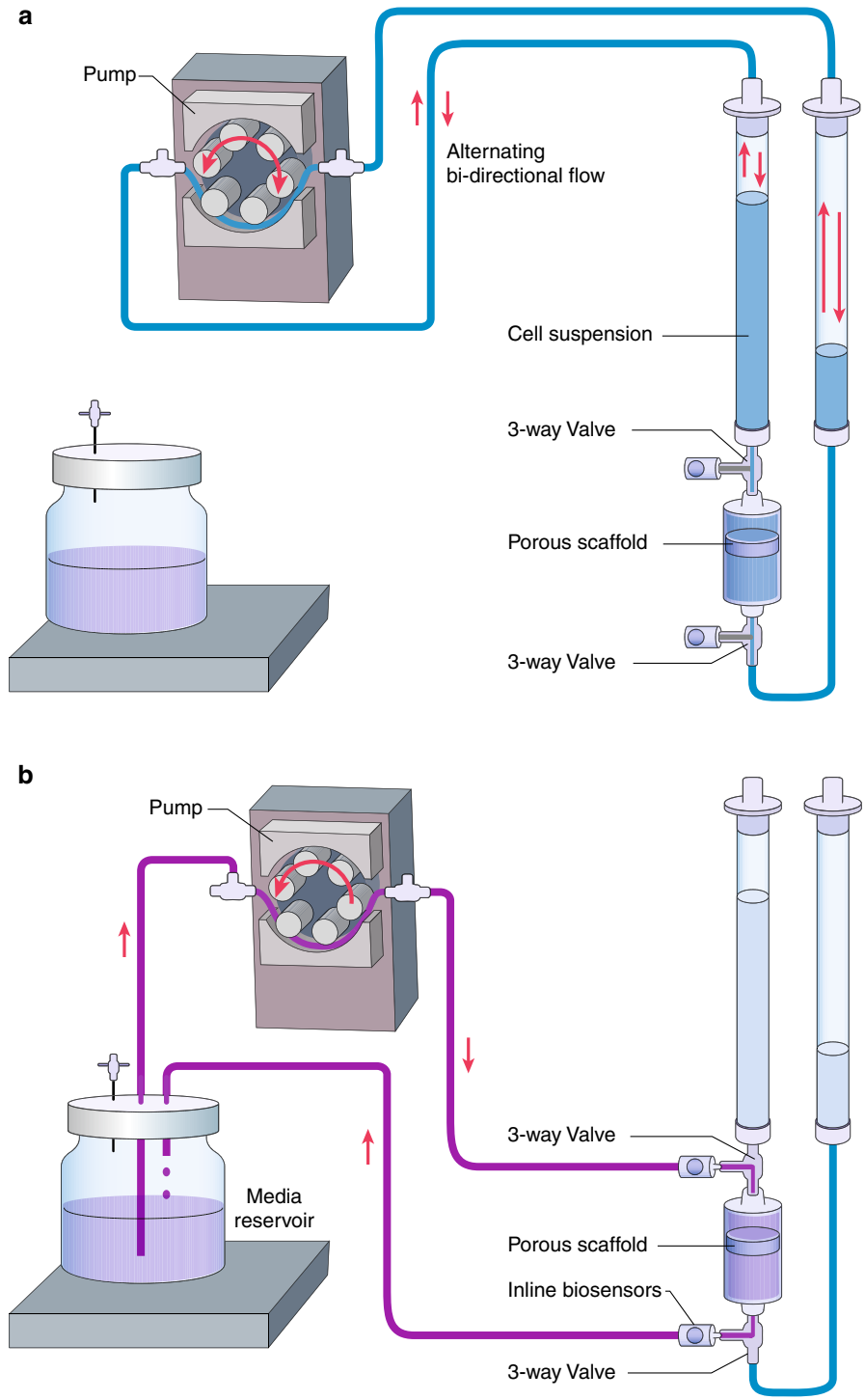

Fig. 10.2 Bioreactor unit for perfusion seeding and prolonged cell culture [63]. (**a**) 3D scaffolds are first seeded with cells using a fluid flow path designed to distribute the cells uniformly throughout the porous scaffold. (**b**) Following the cell-seeding phase, the flow is diverted to an alternative path for prolonged culture [61]

rate. If these signals are inappropriate for the cell type or are absent, cells may not proliferate and form organized tissues and may ultimately die [54]. Accordingly, numerous bioreactor designs incorporate a range of different stimuli. A variety of customized bioreactor designs exist today, for example, as a result of previous efforts by engineers and researchers (Fig. 10.4) to engineer cartilage [55]. Certain aspects of these designs

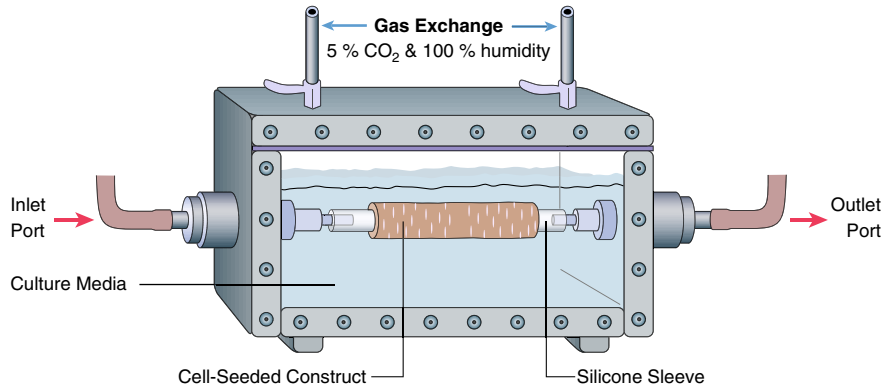

Fig. 10.3 Biomimetic system employed for dynamic conditioning of tubular constructs. Reprinted with permission from the Biomedical Engineering Society [5]

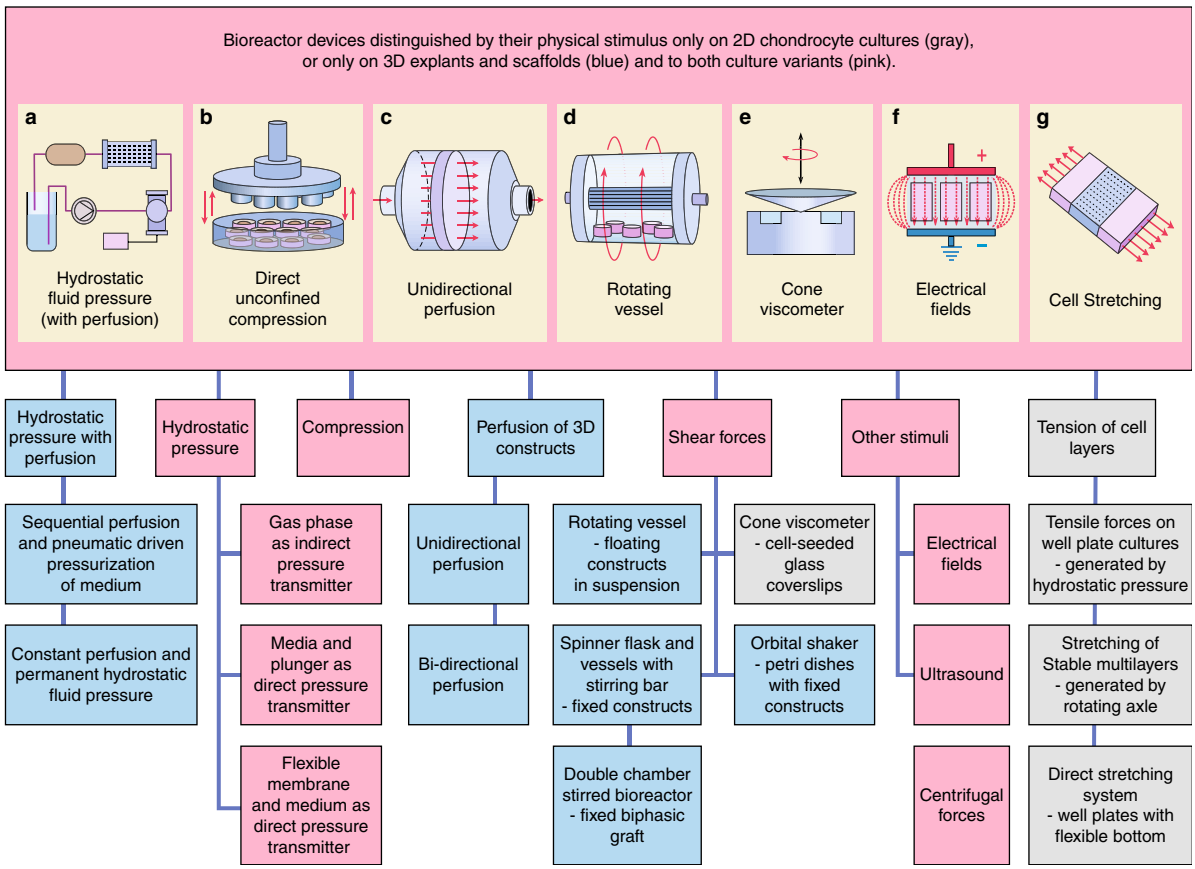

Fig. 10.4 Bioreactor systems commonly used for the cultivation and stimulation of engineered cartilage. An example of a bioreactor is sketched for each physical stimulus [55]

may be integrated to appropriately address criteria for other tissue engineering applications.

Two types of bioreactors are typically used to impart shear forces on tissue-engineered constructs: spinner flasks or rotating vessel reactors (Fig. 10.5). Whether solid or porous scaffolds are used, shear forces affect only those cells growing on the surface [55], i.e., in the case of a solid scaffold, the entire cell population. One such bioreactor design includes the high aspect ratio vessel (HARV) Rotating Chamber, originally designed

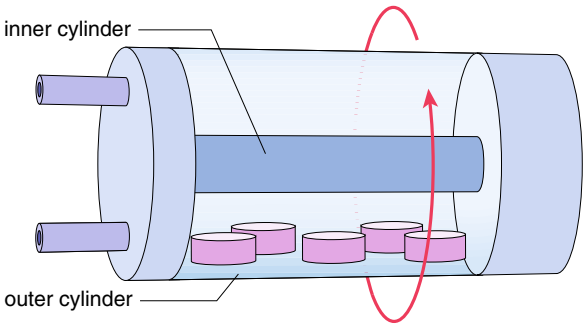

Fig. 10.5 Schematic of a rotating vessel bioreactor. The outer cylinder rotates while the inner cylinder remains still [21]

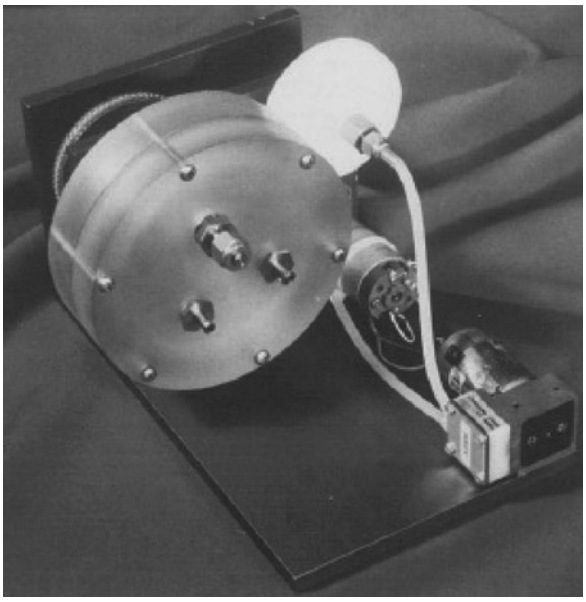

Fig. 10.6 Photograph of a 50-mL high-aspect ratio vessel (HARV) showing the cultivation chamber, sampling ports, 24-DC motor, air pump, and air filter [26]

by a NASA team and currently commercialized by Synthecon, Inc (Fig. 10.6). The HARV system consists of a medium-filled rotating chamber with a variable drive motor to allow adjustable rotation speed. The system can be adjusted to allow the cellular scaffolds exposure to a changing acceleration vector (a tumbling effect) or to provide a continuous perfusion flow. The HARV bioreactor, or a version thereof, has been used with a variety of cell phenotypes and scaffold materials: normal human prostate fibroblasts and prostate cancer (PC3) cells were cultured in HARV reactors to examine three-dimensional cellular and tissue growth [33]; osteoblasts and rat bone marrow stromal cells were seeded on bioactive glass, bioceramic, and composite microspheres to examine 3D bone cell growth on microcarriers [52]; bovine chondrocytes were seeded onto polyglycolide to examine growth under variable metabolic parameters [45]; and human osteoblast-like cells (SaOS-2 line) were seeded onto poly(lactide-co-glycolide) to examine mineralized tissue formation [8].

Another bioreactor system commonly cited in the literature is the perfusion system. The main characteristic of a perfusion system is continuous or intermittent flow of medium through a biochamber containing a cell-biomaterial construct (Fig. 10.7). A perfusion fluid flow system is a specific kind of bioreactor that can also be classified as applying shear force. Shear force is generated by the media being pumped through the scaffold within the bioreactor. The amount of shear that the cells are exposed to is dependent on several variables, including flow rate, port size, and scaffold pore shape [25, 41]. An advantage of using a perfusion flow system is the ability to continuously deliver nutrients and remove waste products. Another advantage of the perfusion system is that functional bioreactor chambers can be readily incorporated in order to expose the cells to additional stimuli to simulate in vivo conditions. Silicone tubing is commonly used throughout the system to provide gas exchange and oxygenation. Previous experiments reported using perfusion systems include the following: foal chondrocytes seeded on nonwoven polyglycolide mesh that were subjected to 500 psi hydrostatic loads to mimic loads found in joints [16]; bovine chondrocytes seeded on polyglycolide that were examined for the effects of fluid flow and media pH on cell proliferation [49]; and vascular cells seeded on a collection of flexible patch materials that were subjected to pulsatile flow to examine cellular conditioning via biomechanical and biodynamic stimuli [56]. Medium flow is commonly provided by a peristaltic pump; however, selected studies have used a respirator pump, diaphragm, and one-way valve configuration to impart the pulsatile flow.

Hydrostatic pressure is often experienced by the cells in vivo, and this condition can also be replicated with bioreactors. One way of creating hydrostatic pressure is to directly connect the gas phase above the culture medium with another pressure chamber. Thus, the entering and exiting gas flow can be controlled to allow the generation of various pressure protocols. The gas phase transmits pressure to the culture medium and the

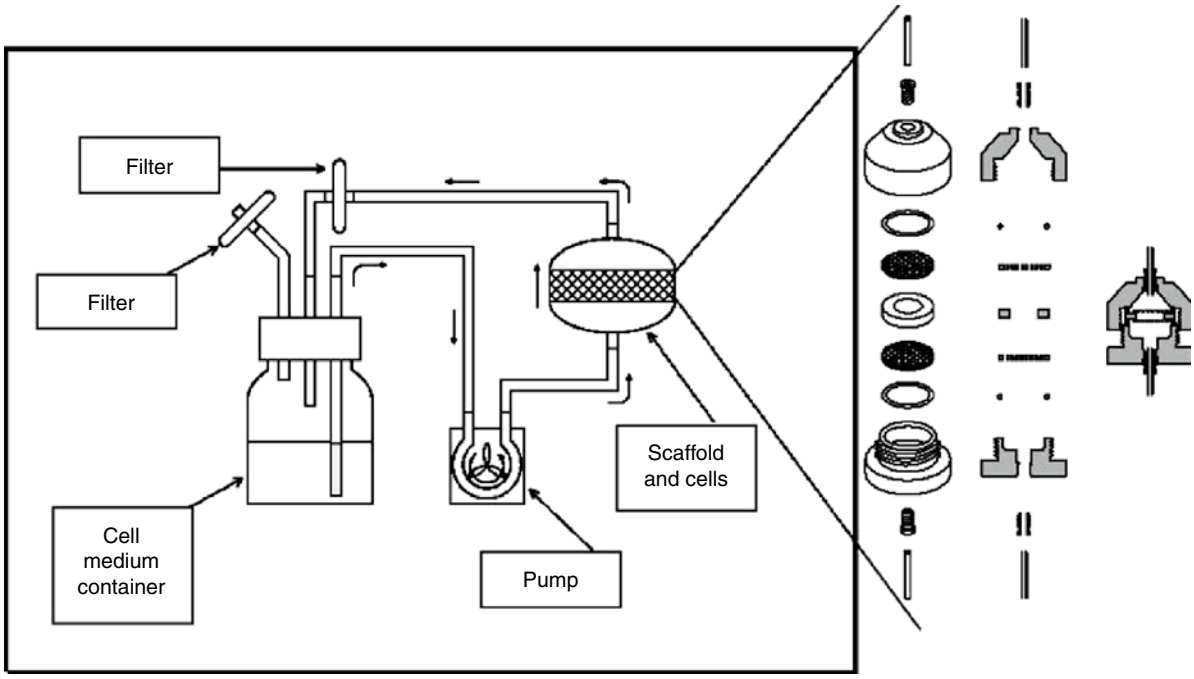

Fig. 10.7 Schematic of a typical perfusion bioreactor [32]

sample within [58]. Other systems put the culture medium directly under pressure without transmission from a gas phase above. The chamber containing the sample, for example, may be connected to a syringe that is capable of creating intermittent pressure protocols. When the syringe moves, it transmits the hydrostatic pressure to the culture volume and sample [30]. A third design to create hydrostatic pressure involves the use of a flexible membrane. The sample in culture medium is sealed with a gas- and fluid-impermeable flexible membrane. A positive pressure is created on the opposite side of the membrane and is transmitted by the flexible membrane to the culture medium and sample. Computer-controlled valves regulate the positive pressure and enable the precise application of various pressure protocols [55].

Other bioreactor systems employ a combination of perfusion fluid flow and hydrostatic pressure (Fig. 10.8). One such design involves perfusing medium through constructs in a glass column that is connected in a closed loop to the medium reservoir, a single-piston cylinder pump, and a back pressure regulator connected to the control device [42]. When the back pressure is released, perfusion flow is active, but when the back pressure is engaged, hydrostatic pressure is created because the pump continues to run. Another design is a vessel in which several constructs are stacked in support cages in culture medium and a peristaltic pump perfuses all the constructs. Valves can be opened or closed at the ends of the vessel, creating an isolated volume of media in the vessel when they are closed. The system has an air-driven cylinder with a piston capable of creating hydrostatic pressure on the culture volume when the valves are closed [17–19]. Both of these bioreactor systems apply either hydrostatic pressure or perfusion fluid flow, but not both at the same time.

A new dynamic bioreactor system was recently designed and built, with which one can assess the cellular response to both perfusion fluid flow and hydrostatic pressure (Fig. 10.9) [46]. This novel biochamber design allows researchers to apply both continuous perfusion flow and cyclic hydrostatic loading in an effort to identify interactions of the two. Preliminary experiments concentrated on refining the bioreactor design as well as defining assays relevant to osteogenic characteristics of bone marrow stromal cells. Static culture studies were performed to monitor the temporal characteristics of select cell-material interactions. Several aspects of these preliminary studies were used to define experimental parameters and endpoints for

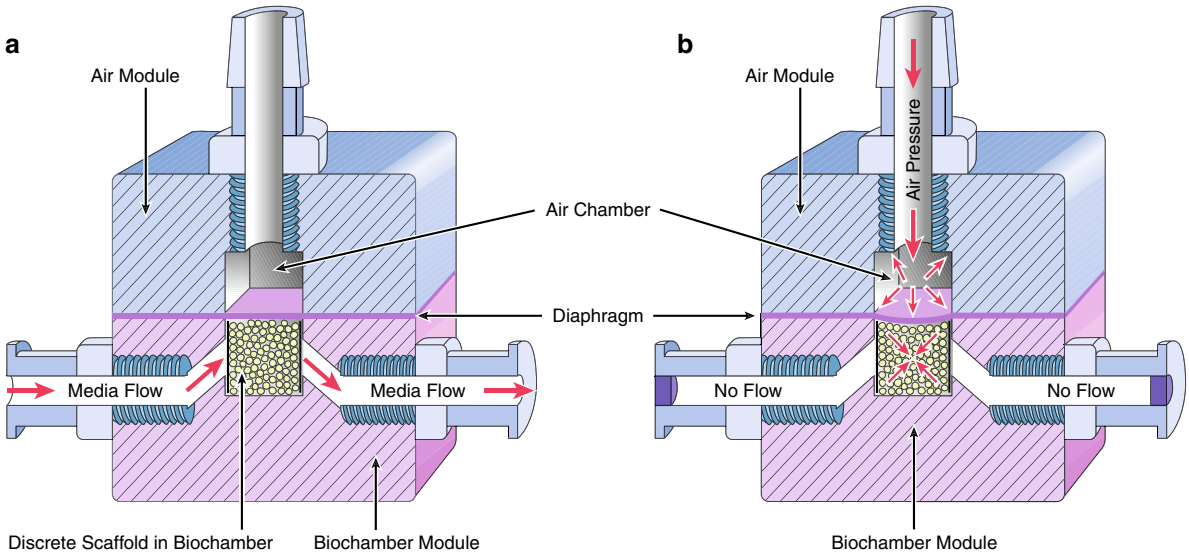

Fig. 10.8 Bioreactor assembly illustrated to demonstrate perfusion flow (**a**) and hydrostatic compression (**b**). Stopcock valves are connected in-line with the medium perfusion tubing on both sides of the bioreactor. The manual valves are opened during perfusion flow and closed during hydrostatic compression to provide a fixed fluid volume within the cell-scaffold chamber [46]

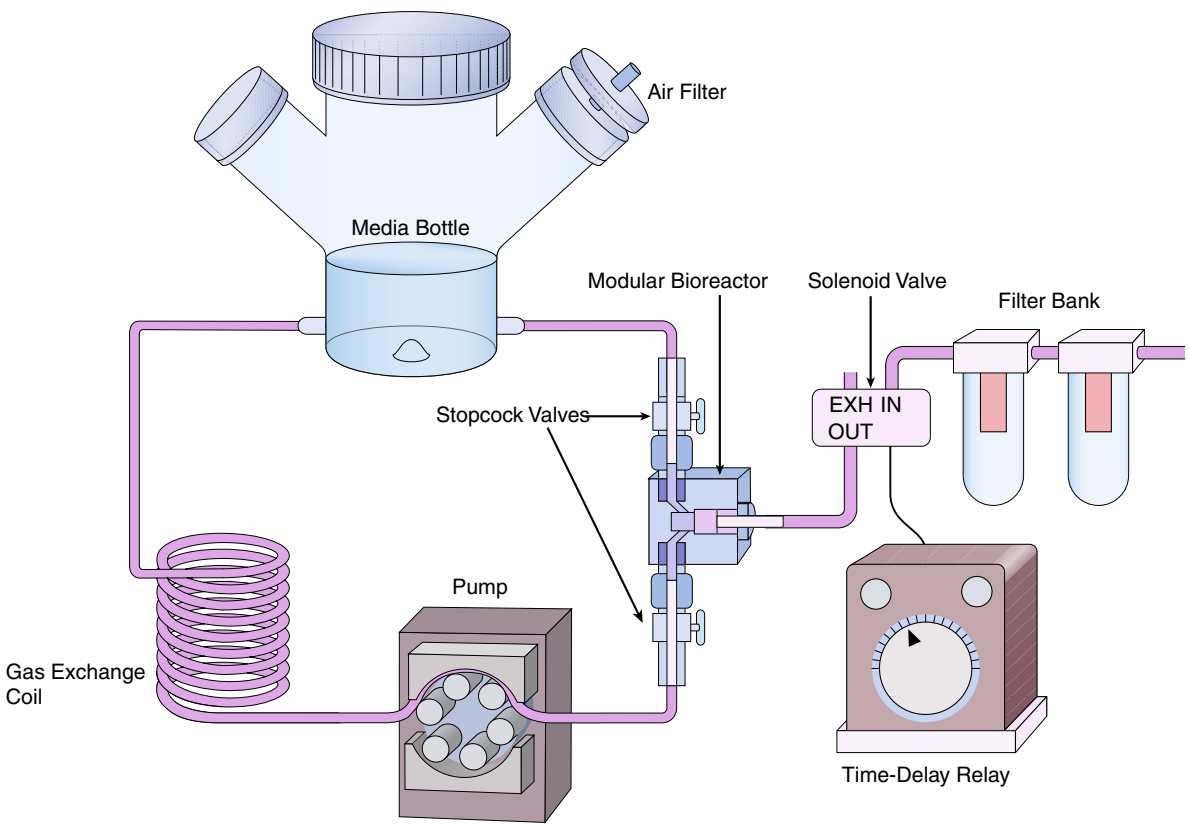

Fig. 10.9 Schematic of bioreactor system incorporating a flow circuit and pressurized air source. The time-delay relay and solenoid valve allow a user to establish cyclic hydrostatic loading patterns [46]

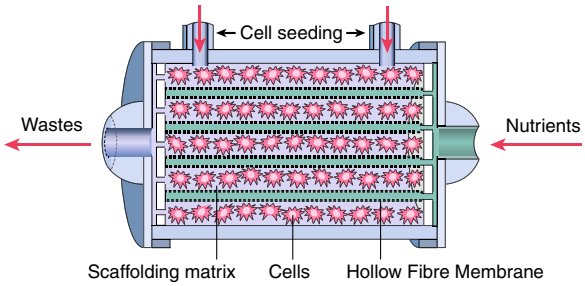

Fig. 10.10 Schematic of a hollow fiber membrane bioreactor [23]

ongoing bioreactor research investigating the interactive effects of perfusion flow and hydrostatic compression. Finally, a modified configuration of the dynamic bioreactor system was assembled to accommodate the coculture of an osteochondral implant simply as a means of revealing the robust nature of the original modular bioreactor design.

An additional bioreactor design reported previously in the literature is the hollow-fiber system (Fig. 10.10). Medium flow is contained within the hollow fibers that extend the length of the bioreactor chamber while cellular constructs reside outside the fibers and within the outermost wall in a static medium-filled volume. The hollow-fiber material has a molecular weight permeability less than or equal to 10 kDa. The fiber permeability allows oxygen and nutrients held in the internal flowing medium to reach the cells on the other side of the fiber wall without subjecting the cells to the flow associated with shear stress.

A multitude of research studies have evaluated cellular response to a variety of other dynamic mechanical stimuli as well. Uniaxial and biaxial stretch, four-point bending, and direct-contact mechanical compression have been applied to in vitro cell culture experiments (Fig. 10.11) [6, 10]. As researchers move forward in the quest for viable three-dimensional tissue-engineered cell constructs, engineers are developing custom bioreactor designs to meet the ongoing needs of the field. Functional bioreactor designs now allow the application of dynamic mechanical stimuli to cellular scaffolds. Progressive designs such as the Rotary Cell Culture System™ (Synthecon, Inc., Houston, TX), Flexercell® Compression Plus™ System (Fig. 10.12) (Flexcell International Corp., Hillsborough, NC), and various perfusion flow systems [4, 44] have moved in vitro cell culture beyond the petri dish. FlexCell International Corporation currently offers commercially available biaxial and uniaxial strain bioreactor systems (BioFlex® and UniFlex™) for testing 2D biomaterial films. Other custom-designed biaxial and uniaxial strain systems have been used in the study of cell applications [6, 27, 66].

The ability to culture viable three-dimensional cellular constructs in vitro that mimic natural tissue has proven very challenging over the years. One of the most difficult of the many problems faced by researchers is that there are multiple dynamic biochemical interactions that take place between and among cells in vivo, many of which have yet to be fully understood. Yet, the in vivo system must be accurately modeled if in vitro development of engineered tissues is to be accomplished. The ideal in vitro system should accurately model the mechanical environment as well as essential cellular interactions found during in vivo development, while providing the necessary physical isolation of the target cells (e.g., those suitable for transplant to a human). Cells intended for implantation may or may not be limited to a single cell type, depending on the application and tissue of interest. Accordingly, improved in vitro coculture methods and systems are always being sought to provide a better understanding of the biochemical interactions between cells, as well as the growth and development of viable three-dimensional engineered tissues.

Many existing coculture systems are simply well-plate products that are static in nature and do not allow for manipulation of the local environment beyond chemical inputs to the system. As such, the development of more dynamic coculture systems has become of interest (Fig. 10.13). However, most of these dynamic systems, similar to the static systems, often provide only a single source of nutrients, growth factors, and other such medium supplements to all of the cell types contained in the system. Moreover, the different cell types that are cocultured in both static and dynamic systems are usually maintained in actual physical contact with one another, preventing the development of an isolated cell population. Figure 10.13 demonstrates a system that allows indirect or direct contact of cells as well as input of multiple growth media to better accommodate coculture applications.

Chang and coworkers [20] developed a double chamber stirring bioreactor to cultivate biphasic osteochondral grafts in a single apparatus (Fig. 10.14). The bioreactor consisted of two tubular-shaped glass chambers, each with a magnetic bar stirrer and ports for

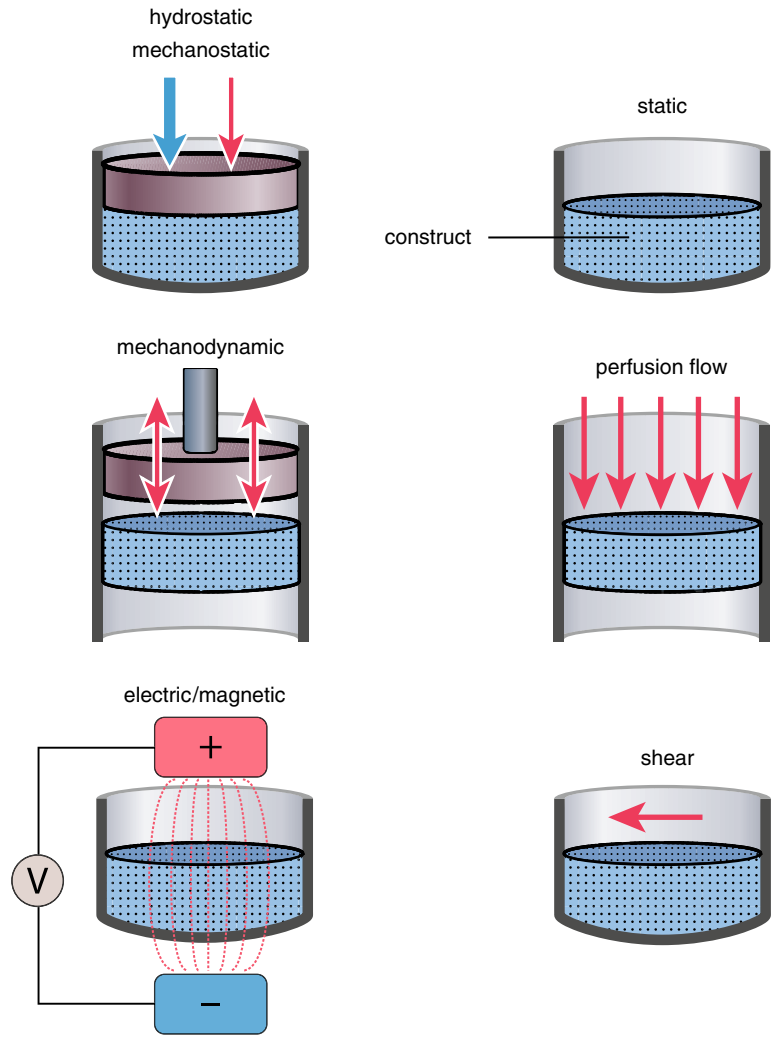

Fig. 10.11 Schematic overview of different stress protocols applicable to scaffold constructs. [29]

media exchange, gas ventilation, and sample aspiration. The chambers were separated by a silicon rubber septum that contained several holes to hold the biphasic composite scaffolds. This design allowed for one side to be cultivated in chondrogenic medium and the other in osteogenic medium, while both were exposed to shear forces caused by the stir bars.

10.4 Clinical Application

One of the largest hurdles for tissue engineering is the transfer from laboratory-scale production to clinical-scale production. Clinical relevance in the United States means producing large numbers of products with the reproducible quality necessary to obtain U.S. Food and Drug Administration approval. In most inert implants, the final attributes do not change and are easily characterized. However, with a living cell product, precise characterization is difficult, so the process of formation needs to be critically controlled [2]. This becomes particularly difficult to realize when scaling up the production from a few to too many, as is required for off-the-shelf availability. For example, certain problems arise with the growth and handling of large volumes of cells, such as maintaining sterility and phenotype [2]. Here, the field of bioreactor design is needed to meet the needs of cell expansion and tissue production, while preserving the vital biological attributes of the product. Closed bioreactor systems are ideal for clinical use as they offer major advantages for manufacturing, since sterility can be assured while maintaining viability of the tissue product.

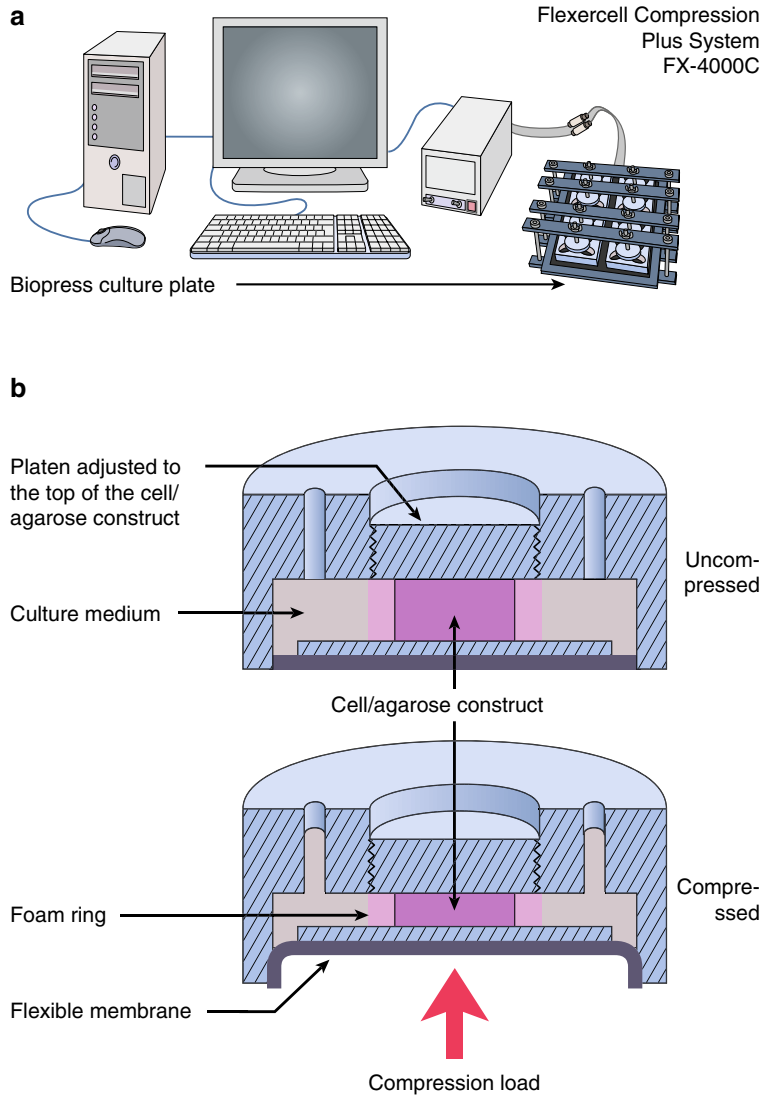

Fig. 10.12 *Compression system* (**a**) FX-4000C™ Flexercell® Compression Plus™ System. Positive pressure compresses samples between a piston and stationary plate. (**b**) Schematic diagram of the culture plate compression chamber in uncompressed or compressed position [9]

Two examples of bioreactors that have been used successfully in clinical practice are used for the manufacture of two tissue-engineered products, Dermagraft® and TransCyte™. Dermagraft® was developed during the 1990s by Advanced Tissue Sciences. The process was acquired in 2002 by Smith and Nephew, who developed it further [36]. The Dermagraft bioreactor is a soft wall bioreactor comprised of a gas permeable bag divided into eight cavities, each containing a knitted poly(glycolide-co-lactide) scaffold (Fig. 10.15). To achieve increased scale-up, twelve bags are connected by an injection-molded manifold, allowing simultaneous seeding and medium change. This modular unit therefore grows 96 pieces of tissue-engineered skin replacement [53]. Multiple such systems can easily be operated as a single lot to give increasing lot sizes, to some thousands of units, providing economies of scale [36]. These bags can be sealed, packaged individually, stored frozen, and delivered to the site, where they are only opened at the time of application, thereby ensuring sterility [53]. Because of the difficulties inherent in packaging a living product under aseptic conditions, the bioreactor was designed, so that such a packaging transfer step would be avoided by making the bioreactor part of the final packaging. In addition to considerations related to the manufacturing process, the design of Dermagraft also considered factors to make the product as easy to use as possible by the customer. All these features were incorporated into the eventual manufacturing process, in addition to the

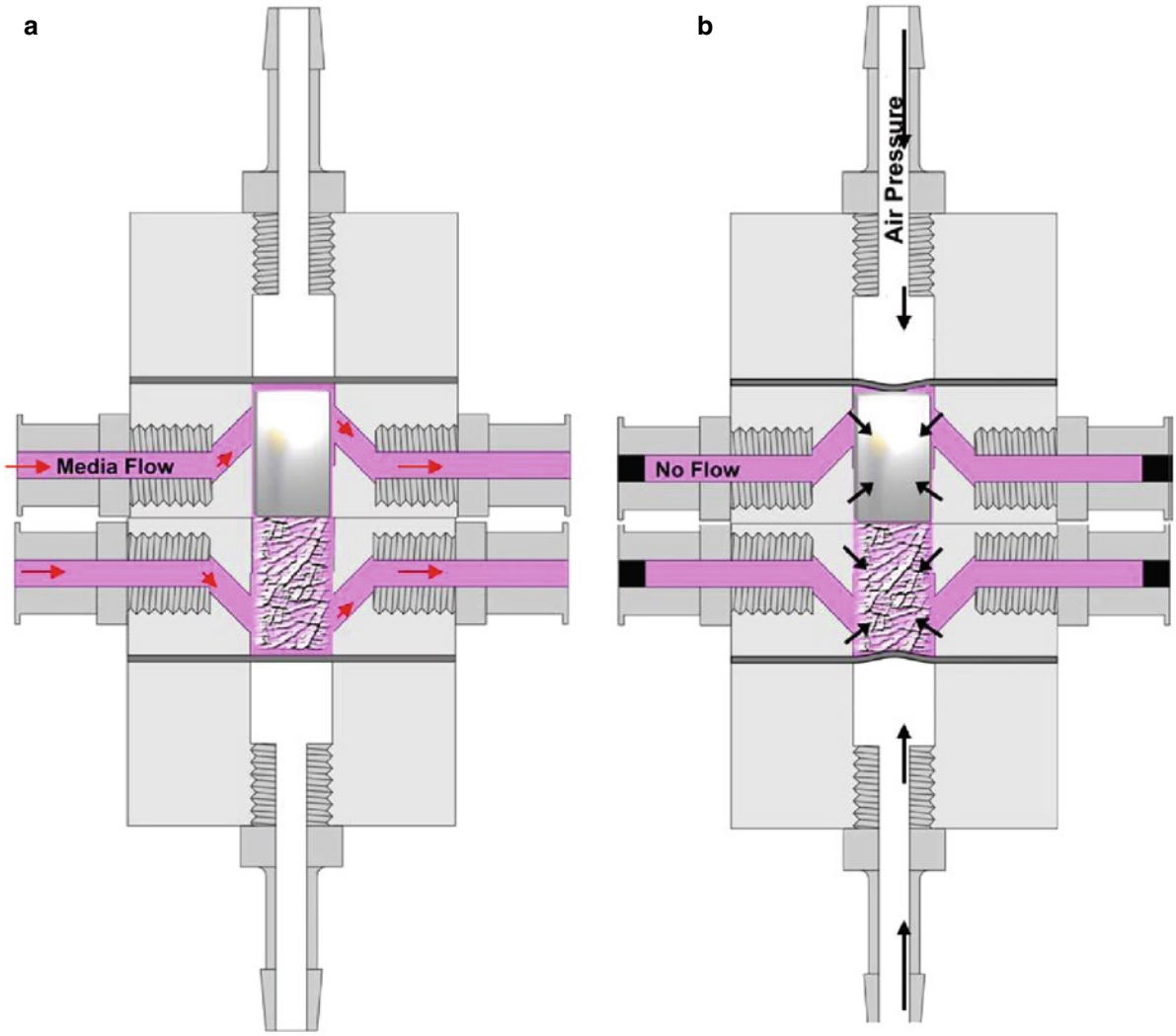

Fig. 10.13 Schematic of dynamic coculture bioreactor that is able to incorporate perfusion flow (**a**) and hydrostatic pressure (**b**). The configuration of this system allows the use of different culture medium for each tissue type

technical considerations such as mass transport, gas exchange, pH control, and medium replenishment [36]. Dermagraft® is indicated for treatment of diabetic foot ulcers.

A similar approach was used in the design of bioreactors for TransCyte™, with some significant modifications [53]. TransCyte™ is cultured in a sealed polycarbonate bioreactor designed as a cassette. Inside the bioreactor are a polymer membrane and a nylon mesh on which newborn human fibroblast cells are cultured under aseptic conditions in vitro. Prior to cell seeding, the nylon mesh is coated with porcine dermal collagen and bonded to the silicone membrane. This membrane provides a transparent synthetic epidermis when the product is applied to a burn. By maintaining fluid flow within the bioreactor system, the cultures are able to form a three-dimensional matrix representative of a human dermis. The bioreactors are designed, so that groups of eight can be placed side by side, with manifold tubing providing the cells and culture media in a uniform manner. At the manufacturing facility, the eight-bioreactor units are stacked within specially designed incubators, to ensure a uniform and consistent environment where the tissue can be grown. At

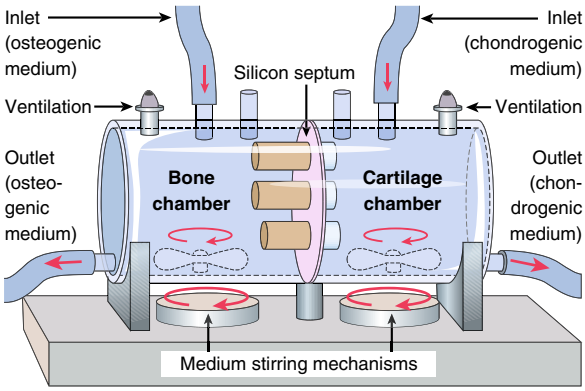

Fig. 10.14 Double-chamber stirred bioreactor for engineering osteochondral grafts. Composite scaffolds traverse a membrane that separates the bioreactor into its two chambers. Each chamber has separate medium recirculation and stirring systems, such that the chondral and bone compartments of the composite can be cultured independently within the respective chamber [61]

the end of the growth process, the cassettes are sealed by closing the inlet and outlet tubing. At this point, the tissues are frozen and can be stored for up to 9 months. These cassettes are also used as the delivery packages for the product, and are only opened at the clinical site, for immediate application. This bioreactor system allows automated cell seeding, media change, in-process monitoring of growth, storage, and delivery and at the same time provides a scaled-up system. The bioreactor cassette, which serves as an aseptic growing chamber and shipping container, withstands temperature changes from the 37°C normal human body temperature to the −70°C of cryogenic storage. A particular challenge for the designers was designing a seal that would perform well with the temperature change, be rugged yet easy to open, and be manufacturable in volume. TransCyte® is indicated

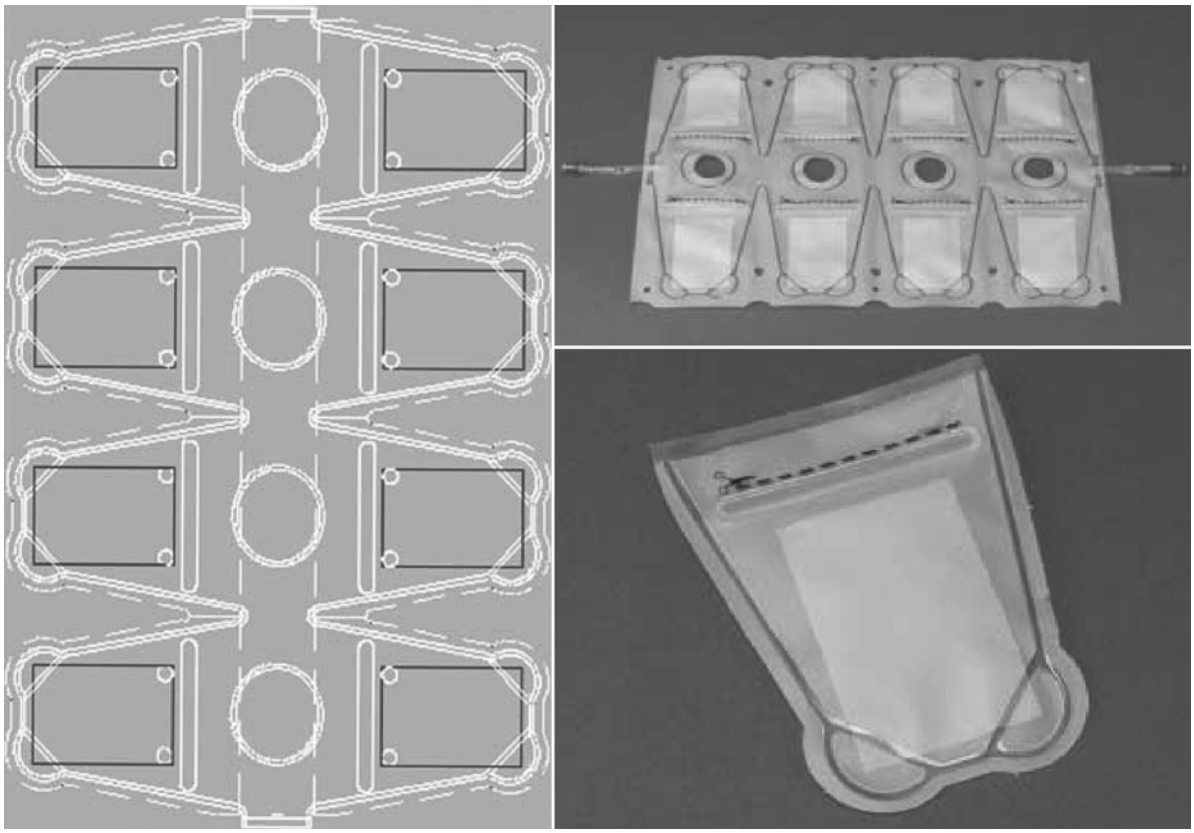

Fig. 10.15 Design of the Dermagraft bioreactor. *Left*-hand panel: *black rectangles* represent knitted poly(lactide-co-glycolide) scaffold. *White lines* are welds and the *white broken lines* cuts. *Right*-hand *upper* panel: complete bioreactor with inlets and outlet. *Right*-hand *lower* panel: following tissue growth, pockets containing individual pieces of Dermagraft are sealed and then cut out of the bioreactor [36]

for treatment of full and deep partial-thickness burns, as well as middermal to indeterminate-depth burn wounds. In 2006, Advanced BioHealing, Inc. acquired the rights to both Dermagraft® and TransCyte® from Smith and Nephew.

10.5 Expert Opinion

Bioreactors are likely very important in the development of clinically viable engineered tissue, but an important question needs to be answered: what are the optimal culturing conditions? Despite numerous proof-of-principle findings that mechanical conditioning can enhance tissue development in vitro [14, 24, 34, 40], it is unknown what conditions, regarding magnitude, frequency, continuous or intermittent, are most stimulatory. In addition, engineered tissues at different stages of development might require different regimes of mechanical conditioning due to the differentiation of mesenchymal stem cells and the increasing accumulation of extracellular matrix [61].

Whether tissue engineering should mimic the conditions during embryogenesis or those in the adult is probably dependent on the cell type of interest [55]. The complex relationship between the numerous factors that bioreactors can control and how they influence the tissue formation are still not fully understood. Several different cues are likely required for the development of functional tissue. To overcome these limitations, further study in the area is needed to increase our fundamental understanding of complex issues that can have an impact on tissue formation in bioreactors. With that in mind, a large amount of work is required to be focused on the fundamental science behind these complex interactions and should not be overlooked while trying to reach the goal of clinically applied products. By providing a comprehensive level of monitoring and control over specific environmental factors in 3D cultures, bioreactors can provide the technological means to perform controlled studies aimed at understanding which specific biological, chemical, or physical parameter plays which function in engineering a defined tissue. This fundamental interdisciplinary research will provide the basis for identifying environmental and operating conditions required for the generation of specific tissues [37].

10.6 Five-Year Perspective

One of the major challenges to bring a tissue-engineered product into routine clinical use is to translate research-scale production models into clinically applicable manufacturing designs that are reproducible, clinically effective, and economically acceptable while complying with good manufacturing practice (GMP) requirements [53]. Therefore, the application of cell-based tissue engineering approaches in the routine clinical practice critically depends on the development of innovative bioreactor systems [61]. In the future, bioreactor systems will have to be tightly integrated into the entire tissue development scheme, starting with a patient's tissue biopsy or marrow aspirate and ending with implantation. A "smart" bioreactor system of the future should isolate cells, allow proliferation, distribute cells on a scaffold, and differentiate specific cell types, thereby performing all the different processing phases within a single closed and automated system (Fig. 10.16) [61]. Future bioreactors should also allow easy and sterile delivery of the engineered tissue to the surgical room [50]. In many cases on the research scale, the bioreactor is only used for tissue formation. However, for a comprehensive approach from biopsy to the implantation of the tissue, the entire procedure should be coordinated to decrease the number of steps, risk of contamination, and labor costs among others. This is particularly important with respect to the manufacture of engineered tissue constructs for clinical applications, in which GMP requirements must be met [43, 53].

This model is exemplified by the concept of the on-site hospital-based Autologous Clinical Tissue Engineering System (ACTES™; Millenium Biologix, Kingston, Ontario, Canada). As a fully automated closed bioreactor system, ACTES™ is intended for a bedside production of biphasic osteochondral transplants. The bioreactor is designed to digest a patient's cartilage biopsy after a disposable biopsy chamber with the cartilage tissue biopsy is inserted. After the system has isolated the chondrocytes, they are then expanded in a culture chamber inside the apparatus and then transported to a production chamber. After the cells are expanded, the chondrocytes are seeded and cultured onto the surface of an osteoconductive porous scaffold, thereby generating an osteochondral graft within a single closed bioreactor system [55]. One or more so-called Cartigrafts™ can be cultivated

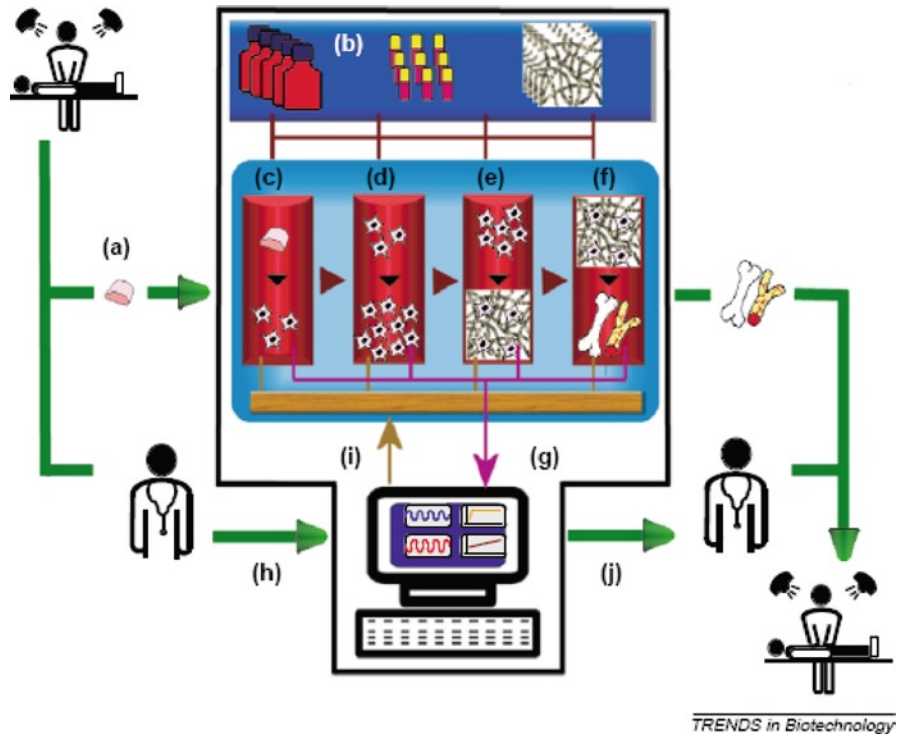

Fig. 10.16 Vision for a closed-system bioreactor for the automated production of tissue-engineered grafts. (**a**) The surgeon would take a biopsy from the patient and introduce it into the bioreactor located on-site at the hospital. (**b**) All reagents (e.g., culture medium, medium supplements, and scaffolds) would be stored in compartments under appropriate conditions (i.e., temperature, humidity). The bioreactor system could then (**c**) automatically isolate the cells, (**d**) expand the cells, (**e**) seed the cells onto a scaffold, and (**f**) culture the construct until a suitably developed graft is produced. (**g**) Environmental culture parameters and tissue development would be monitored and inputs fed into a microprocessor unit for analysis. In conjunction with data derived from clinical records of the patient (**h**), the inputs would be used to control culture parameters at predefined optimum levels automatically (**i**) and provide the surgical team with data on the development of the tissue, enabling timely planning of the implantation (**j**) Figure reprinted with permission [38]

within this production vessel. The grafts are cell-based biphasic, osteochondral transplants where the expanded cartilage cells are attached to one or more osteoconductive, synthetic bone scaffolds and are then available for surgical implantation.

An enormous technological advantage of this closed culture concept is in the complete elimination of the costly manufacturing facilities. This important feature enables hospitals and clinics to carry out autologous tissue engineering for their own patients, eliminating the logistical issues of transferring specimens between locations, and likely reducing the cost of engineered grafts [55]. Altogether, when efficiently designed for low-cost operation, novel bioreactor systems like ACTES™ could facilitate the spreading of novel and powerful therapeutic approaches that would otherwise remain confined within the context of academic studies or be too expensive for widespread use [61].

For the manufacturing of engineered tissues to be operated successfully in an automated fashion, bioreactors will need to include low-cost state-of-the-art systems for monitoring and controlling the physicochemical culture parameters. As with more traditional bioreactors, culture parameters such as pH, oxygen, and glucose will have to be maintained within a defined range to ensure reproducibility and standardization. However, more nontraditional technologies, such as noninvasive fluorescence-based or enzyme-based biosensors, will be required due to the unique constraints imposed by a tissue engineering bioreactor system [61]. The resulting bioreactor systems will provide an economically viable approach to the automated manufacture of functional engineered tissue, possibly

bringing cell-based tissue engineering approaches into reality and making them clinically accessible on a larger scale.

In addition to generating tissue for clinical use, bioreactors will also be crucial to engineering complex tissue for answering basic scientific questions about disease processes [11, 48], e.g., assessing how a specific drug is metabolized by the liver (using engineered liver), or how stem cells behave in the presence of stromal cell signals [65]. Of direct clinical relevance, such 3D bioreactor systems will lend to vaccine and therapy development as well as provide relevant, dynamic 3D tissue environments for cytotoxicity studies, i.e., bioreactors will be crucial as tissue test systems that will minimize animal and human testing.

10.7 Limitations/Critical View

To date, the goals and expectations of bioreactor development have been fulfilled only to some extent, as bioreactor design in tissue engineering is very complex and still at an early stage of development [63]. Despite being promising for the manufacture of implantable tissue substitutes, most engineered tissues cultivated in the bioreactors exhibit inferior biological functions and mechanical properties compared to the native tissues [22]. One bottleneck is the complex interplay between various factors influencing the tissue formation. For example, although dynamic compression is known to enhance the mechanical properties of engineered cartilage and cardiac tissues, the optimal magnitude of loading and frequency has yet to be defined. Moreover, the development of functional tissue equivalents may require multiple mechanical cues (e.g., compression, deformational loading and shear), but currently most bioreactors fail to provide all the necessary stimuli [21]. To overcome these limitations, further study in the area is needed to increase our fundamental understanding of complex issues that can have an impact on tissue formation in bioreactors. On the one hand, devices are required to have a well-described microenvironment for fundamental studies. On the other hand, transition from a laboratory scale to an industrial scale will require a high adaptability of specialized bioreactors in a standardized production process. This transition in bioreactor design is necessary in order for tissue engineering to fulfill the high expectations that are placed upon the field [63]. This is not to say, however, that small-scale research-oriented bioreactors do not play an essential role in the development of tissue engineering.

Another major limitation in understanding bioreactor performance to grow clinically relevant tissue lies in our inability to obtain direct information on conditions within the reactor and their effect on the cells [64]. It is very difficult to find inexpensive commercial biosensors that can be used to monitor the growth of tissue-engineered constructs [37]. This results in a limitation in the noninvasive monitoring of the growth and differentiation processes of the cell population in closed production systems. For the automated manufacturing of tissues, bioreactors will need to include state-of-the-art systems for monitoring and controlling the physicochemical culture parameters. Typical environmental factors (e.g., temperature, pH, and oxygen) will have to be maintained at defined levels to ensure reproducibility and standardization [38]. Because the development of engineered tissues might progress at varying rates for different cell batches, additional parameters to be monitored would be cell number, phenotype and metabolism, or specific tissue mechanical properties [38].

A possible answer to this problem is to monitor the development of the tissue through the incorporation of advanced technical tools for online observation of the structural properties of the tissue such as video microscopy, magnetic resonance imaging, and microcomputerized tomography. All collected inputs could be analyzed by a microprocessor unit and fed back to the bioreactor system to optimize the control of culture parameters at predefined levels [38]. However, these advances in bioreactor technology are still yet to be realized and are a limiting factor.

Finally, an important principle in the design of bioreactors is to minimize the number of aseptic operations, as each such operation is an opportunity for contamination. With conventional tissue culture techniques, there are on average 7680 aseptic operations throughout the entire process [36]. The Dermagraft® and TransCyte® bioreactors require only nine aseptic operations for a 384-unit lot size. Nonetheless, even nine aseptic operations are too many, and the ideal is to perform the whole growth process, from expansion to final product, with only a single aseptic operation, the surgical implantation [36].

10.8 Conclusion/Summary

The introduction of the bioreactor to tissue engineering has advanced the science of tissue engineering immensely. The major functions of bioreactors include supplying metabolites, such as oxygen and nutrients, removing waste products, maintaining temperature and pH, and applying the appropriate mechanical stresses to stimulate the formation of the ECM. By producing and maintaining pseudo-physiological conditions specific to and essential for cell activities, bioreactors can lead to the culture of well-differentiated 3D tissues that can be clinically useful [7]. Dermagraft® and TransCyte® are two examples of how a simple bioreactor design can advance tissue engineering from the research laboratory into clinical practice. In the future, bioreactors will be used in the entire process from biopsy to the implantation of the tissue, thereby decreasing the number of steps, risk of contamination, and labor costs. Currently, bioreactors are limited by the amount of monitoring and feedback that is possible, as well as our knowledge of the complex interplay between various factors influencing the tissue formation. Ultimately, bioreactor is an essential tool needed for the success of tissue engineering.

Suggested Readings

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Part II

Tissue Engineering of Organs

Issues in Bioartificial Liver Support Therapy for Acute Liver Failure

11

John F. Patzer II and Jörg C. Gerlach

11.1 Introduction

Acute liver failure (ALF) is a medically intransigent problem. Diverse etiologies, which include viral infection, overdose of acetaminophen or many other drugs, mushroom poisoning, hereditary metabolic or autoimmune diseases, complications of pregnancy, and "cryptogenic" causes, can lead to ALF [39]. Acute-on-chronic liver failure (AoCLF) in patients with chronic liver disease can be precipitated by metabolic stress due to drugs or toxins, intestinal bleeding, or infections, among other factors [72].

Early symptoms and signs of ALF include alterations in mental status, abnormal blood clotting function, jaundice, and/or kidney failure [92]. One of the most dramatic features of ALF is development of hepatic encephalopathy and coma, which is associated with brain swelling and herniation, multisystem organ failure, and opportunistic infection [28, 56]. ALF is associated with high mortality rates because best medical care is palliative in nature and may not correct the metabolic derangements and organ failures that occur with ALF, limiting the ability of the liver spontaneously [4, 27, 116, 134]. Medical aspects of ALF and AoCLF liver diseases are the subject of many review articles [39, 72, 73, 92, 134].

Improvements in best medical care have led to improvements in overall survival, from approximately 20% in the 1970s to approximately 60% during the past decade [8]. Survival, however, is etiology dependent and ranges from ~25% for drug-induced ALF, hepatitis B, and cryptogenic cases to ~60% for acetaminophen overdose, hepatitis A, and ischemia [73]. Liver transplantation (LTx), with 1-year survival rates of 60–80%, remains the therapy of choice for ALF patients [43, 77]. Availability of organs for LTx has historically been and remains problematic: 6,530 patients out of 17,298 on the wait list received a liver transplant (38%) in the United States in 2006–2007 (www.ustransplant.org, 2008). Etiologies and transplant rates vary from country to country [88, 103] but reflect similar trends. In many countries, the high social costs of transplantation with the associated lifetime immunosuppressive therapy requirements also pose serious ethical questions on the eligibility criteria for LTx and often further limit the number of LTx procedures [37].

Because liver tissue has the ability to regenerate and heal [75], restoration of essential liver functions by means of auxiliary methods may improve the prognosis of many ALF patients, alleviating the need for LTx [8, 10, 73]. Recognition of the regenerative capacity of the liver has led to the development of innovative treatments for ALF that include split-LTx, extracorporeal nonbiological detoxification by artificial liver (AL) support, extracorporeal bioartificial liver (BAL) support (cell-based systems), and in vivo tissue or cell transplantation [37].

Split liver transplants, which typically involve transplantation of a portion of the liver from a live donor, have grown from 62 in 1996 to 321 in 2005 in the United States [117] and have partially alleviated the problem. Success of split LTx has been reported to be nearly the same as LTx, but the procedure has potential risks for the donor and is not widely performed,

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and surgery may be complicated by anatomical variations [40]. Xenotransplantation of livers obtained from pigs faces enormous challenges before being available to help address the donor organ shortage.

Hepatotoxicity, liver cirrhosis, and viral hepatitis, as well as a pediatric population with congenital liver diseases, have all contributed to a significant number of adult patients with failing liver. Despite decades of research and clinical evaluation, no clinically acceptable or reliable liver support system or therapy is available. The shortage of donor organs means that LTx cannot be an option for all who would benefit. The imperative for regenerative medicine is exploration of strategies that can sustain patients without LTx, or, if absolutely necessary, maintain patients until a suitable donor organ becomes available for LTx.

11.2 Aim of the Discipline

Creating a functional BAL is not simply a matter of perfusing hepatocytes in a bioreactor. Because a BAL must recapitulate, in some sense, the operational function of the liver, understanding the structure of the liver, i.e., the spatial relations of cells to one another, cell-cell contacting and signaling, and the flow regimes in the liver, is important to proper design of a BAL.

Liver structure is fundamental to accomplishing its many tasks: oxidative *detoxification*; intermediate *metabolism*; toxin and waste *excretion* through the bile; protein and macromolecule *synthesis*; and *modulation* of immune and hormonal systems. On the macroscopic scale, 75–80% of the liver's blood flow enters through the portal vein, which drains the splanchnic bed and presents the liver with the first pass results of ingestion: nutrients, vitamins, metals, toxins, and drugs. The portal vein blood is oxygen depleted, P_{O_2} ~40 mmHg, as a result of supplying the oxygen needs of the splanchnic bed. The hepatic artery delivers the remaining 20–25% of the blood flow at P_{O_2} ~100 mmHg. Combined blood flow to the liver is on the order of 1 mL/min (g/liver). Blood flow exits the liver through the hepatic vein. The liver also generates bile, which is secreted through the biliary system.

A phenomenological description is often used to represent the next level of liver tissue organization: a tessellating system of hexagonal constructs, one unit of which is depicted in Fig. 11.1. Portal triad (PT) units, consisting of portal vein, hepatic artery, and bile duct running normal to the diagram sit at the hexagonal vertexes. The PT vessels are interconnected by smaller vessels running along the sides of the hexagon. The central venule (CV) is located at the center of the hexagon. Blood flows from the PT units toward the CV through sinusoidal channels (Figs. 11.1 and 11.2). The hexagonal structure is further subdivided into three functional zones: the periportal zone (PP) near the PT; the centrilobular zone (CL) near the CV; and the midlobular zone (ML) between the periportal and CL. The zones roughly correspond to oxygen and nutrient gradients, with the PP being richest in both and CL poorest. Bile flows in the opposite direction of blood through bile canniculi to the bile duct system.

The functional unit of the liver is the sinusoid. As schematically depicted in Fig. 11.2, the sinusoid is a space where blood percolates from the distributing venules and arterioles of the PT to the CV. The

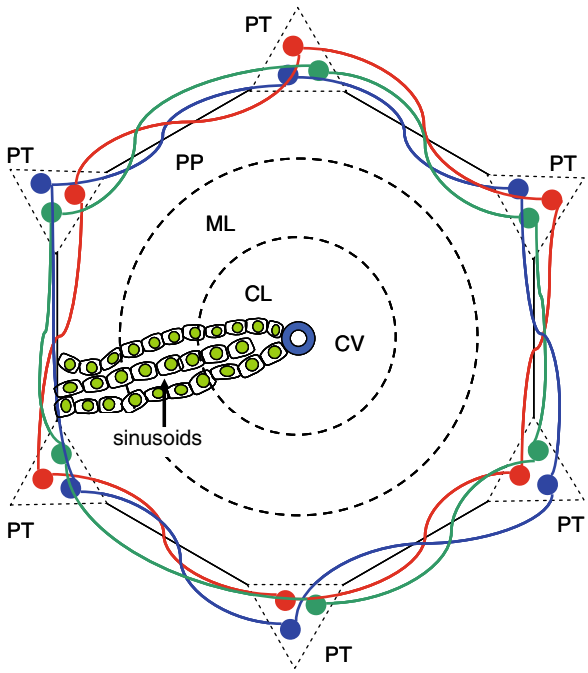

Fig. 11.1 Hexagonal lobular depiction of native liver structure consisting of portal triad (PT) and central venule (CV). Arterial (*red*) and venous (*blue*) blood vessels in the PTs distribute blood to smaller vessels that define the periphery of the hexagon. Bile (*green*), collected from the lobule, flows countercurrent to the blood path. Blood percolation from the periphery of the hexagonal structure toward the CV through the sinusoids divides the structure into three general regions with decreasing oxygen content: the periportal region (PP), the midlobular region (ML), and the centrilobular region (CL)

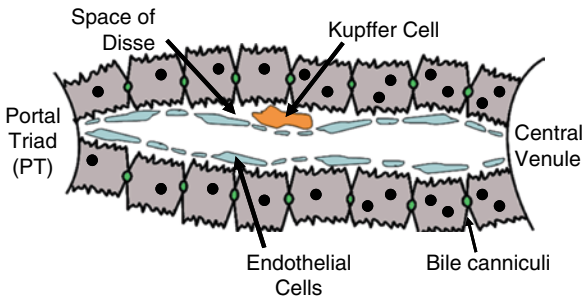

Fig. 11.2 Schematic of liver sinusoid. The sinusoid, surrounded by cords of hepatocytes, drains from the PT to the CV. Endothelial cells, characterized by fenestrations (*holes*) line the hepatocyte cords. The space of Disse lies between the endothelial cells and hepatocytes. Stellate and Kupffer cells are found in the space of Disse. Hepatocytes are polar with epithelial faces exhibiting microvilli to enhance species exchange with the blood and hepatocyte-hepatocyte tight junctions that create bile canniculi. Hepatocytes become multinucleated moving from the PT toward the central venule along the hepatocyte cord

sinusoidal space is delineated by cords or beams of hepatocytes surrounded by sinusoidal endothelial cells. Unlike most endothelial cells lining blood vessels, sinusoidal endothelial cells are characterized by fenestrations (pores) that enhance permeation of noncellular blood components to the hepatocytes. The fenestrations are not static in size; they respond to local signals and change diameter as needed [12]. A small gap, referred to as the space of Disse, separates the endothelial cells from the hepatocytes. Stellate (Ito) and occasional macrophage (Kupffer) cells are found in the space of Disse. The stellate cells extend processes that wrap around the sinusoids, with microprojections through the endothelial cell fenestrations that can generate contractile forces on the sinusoid [33]. Hepatocytes, which are the metabolically active cell in the liver, form the core of the structure. Hepatocytes are polar cells [49]: cell surfaces that face the space of Disse have microvilli that increase effective surface area for uptake from the space of Disse and express different surface receptors than cell surfaces between hepatocytes. Also, hepatocyte-hepatocyte interfaces have tight junctions that form bile canniculi that are used to drain bile to the bile duct. Hepatocyte cords are typically arranged so that every hepatocyte has at least one surface fronting on the space of Disse.

Hepatocyte polarity localizes specific tasks to specific domains [49]. For example, proteins involved in trafficking of metabolites from the bloodstream are found in the basal domain, and proteins specific to bile-acid elimination are found in the apical domain. Similarly, detoxification enzymes are regionally localized in the hepatocyte. Gluconuridation and sulfation enzymes are located in the cytosol. Oxidative detoxification enzymes, the cytochrome P450 system (named for the typical peak enzyme absorption wavelength), are localized at mitochondrial interfaces where they can take advantage of the electron transfer chains in oxygen metabolism. The metabolic and detoxification activities also change spatially along the sinusoid in a phenomenon termed "liver zonation" that is believed to be regulated by gradients in oxygen tension, hormones, and extracellular matrix composition [35, 57, 58].

The sinusoid is an amazing biological structure designed for enhanced permeation of plasma constituents through the fenestrations of the endothelial cells with minimal diffusion distances. The combination reduces diffusional resistances to biochemical transformations in which species must reach the interior of a hepatocyte for conversion. Evidence exists for gradients of hepatocyte metabolic activity (e.g., carbohydrate metabolism) and detoxification activity (e.g., CYP450 enzyme function) along the length of the sinusoid [58]. Toxins and drugs have also been shown to regional specificity along the sinusoid [76]. Existence of such gradient effects suggests, in turn, differential gene expression along the sinusoid that is referred to as "liver zonation" is thought to be regulated by gradients of oxygen, hormone, and ECM composition [57, 61].

The origin, role, and the anatomical niche of liver-specific adult stem cells remain unresolved [3, 13, 31, 123, 124, 132], but increasing agreement posits that at least one important population resides in the canals of Hering. The canals of Hering are cytokeratin CK7 and CK19 positive cells that form a repetitive structure connected to a bile duct and are typically located in the periportal area of the liver lobuli between bile ducts and the parenchymal bile canaliculi.

Because oxygen is an important nutrient in BAL operation that appears to modulate hepatocyte viability and function [57, 61] and is consumed at a reasonably high metabolic rate, researchers have focused considerable effort in understanding and enhancing oxygen transport throughout the cell mass in the cell compartment. Although 100 μm has been claimed as maximal permissible diffusion distance for oxygen transport in a BAL [16], the actual situation is more complex and requires careful attention to the combination of

convective and diffusive oxygen transport as well as oxygen uptake rate in the BAL [45, 46, 96]. That said, diffusion distances can place a severe limitation on the cell mass that can be expected to be supported in systems employing membranes.

11.2.1 Membranes and Compartmentalization

Bioreactors are reacting systems that involve transport of nutrients and oxygen from the perfusing medium to the cells, where metabolism produces waste materials and biological products that need to be transported back to the perfusing media for removal from the bioreactor. Early three-dimensional BAL designs adapted hollow-fiber membrane compartmentalization from hemodialysis to separate the cell mass from the perfusing media. The hollow-fiber membranes have perm-selective properties that reject high molecular weight molecules (>100–250 kDa, depending upon the system). As previously noted, the use of hollow-fiber membranes introduces a diffusive mass transport limitation. Membrane optimization for both nutrient delivery and metabolic product removal is problematic. Controversy exists as to whether such designs can provide sufficient oxygen to hepatocytes. Substantial gradients along the axis of the bioreactor may have an impact on overall hepatocyte function since hepatocytes are typically exposed to low gradients in normal liver.

Determination of whether traditional hollow-fiber designs can provide sufficient clinical efficacy awaits proper design of a clinical trial with well-defined therapeutic endpoints. In the meantime, BAL bioreactor design has been moving toward compartmentalization that segregates delivery of nutrients, delivery of oxygen, removal of wastes, and housing of cells into their own compartments [89, 115]. Xu et al. [140] suggest that four-compartment bioreactors may be necessary to enable integral oxygenation and distributed mass exchange with low gradients typical of the liver.

While membranes have been traditionally considered inert materials with desirable mass transport properties that are used to separate regions of the BAL, membranes are actually active components in the bioreactor. Membranes adsorb proteins, a process that will alter the mass transport properties of the membranes over time. Transient adsorption of toxins on membranes can alter bioreactor performance. Membranes can act as a cell attachment surface, which can alter both the mass transport and metabolic properties of the BAL. Legallais et al. [74] provide a review of many of these issues. The reported results are qualitative and ambiguous but point to the need to consider membranes and membrane properties carefully in design of a BAL bioreactor.

11.2.2 Hepatic Cell Source

Appropriate cell sources for extracorporeal liver support have been controversial [4, 38, 85, 122]. Since a clinically proven BAL would have an annual demand in the thousands of procedures, practical considerations about cell source include the following: ready availability; biochemical functional similar to native human liver; and minimal risk of immunogenic response or infection to the patient. Adult human hepatocytes are the gold standard with respect to biochemical function and minimization of immunogenic risk. Unfortunately, they are not available in the quantities required for general BAL use.

The majority of reported clinical evaluations of BAL support systems have used primary porcine hepatocytes because of ready availability. Porcine hepatocytes exhibit many of the same biochemical functions and activity as primary human hepatocytes. However, concern over the potential of transmission of xenoviruses has created controversy even though no such transmission has been detected [138]. Potential immunogenic response, whether by the patient in response to the porcine hepatocytes or by the porcine hepatocytes in response to the patient, is also of concern. Most of the systems used in clinical evaluations thus far have employed membrane barriers that minimize the likelihood of cell-mediated damage in either direction.

Human hepatocyte cell lines are readily available and can be easily expanded to create the number of cells needed for a clinical-scale BAL [29, 47, 82, 106]. However, these cell lines typically display altered function from primary hepatocytes and frequently, when function is present, at a reduced level. As with porcine cells, care must be taken to prevent potential delivery of a cell from the line back to the patient.

Stem cells, unspecialized cells that perpetuate themselves through self-renewal and generate

specialized cells through differentiation [108], may be the ideal cell source for cell-based therapies developed to treat debilitating and life-threatening diseases [110]. While the potential of stem cells is quite promising, realization of that potential lies in the future. Focus on liver progenitor cells with a view to developing liver stem cell expansion systems is warranted.

11.2.3 Tissue Engineering with Hepatocytes

Tissue engineering directed toward recapitulation of the complex structure and cellular interactions in the native liver is a daunting task. One problem is that, other than isolated claims in the literature [11], clonal expansion of primary cells that maintain a fully differentiated hepatocyte phenotype has proven elusive. The result is that functional liver tissue engineering has relied on the pioneering collagenase digestion techniques of Seglen [118–120] to isolate individual, mature, primary cells from liver tissue on a large scale.

11.2.3.1 Monolayer Culture

Maintenance of differentiated hepatocyte function is, at a minimum, dependent upon the hepatocyte being attached to or anchored to a surface. While the cells can form a limited number of cell-cell junctions in comparison with native liver architecture, the flattening disrupts the cytoskeleton and leads to loss of cell polarity with concomitant loss of liver-specific functions [5]. Improved maintenance of polarity, increased density of cell-cell junctions, and possible formation of bile canniculi have been observed in sandwich monolayer systems resulting in longer-term maintenance of cell viability and liver-specific function [20, 41, 70, 84].

11.2.3.2 Spheroid Suspension Culture

Simple collagenase digestion of the liver produces a cell mixture containing approximately 95% hepatocytes and 5% nonparenchymal cells including endothelial cells, Kupffer cells, and lymphocytes. Further purification by density gradient separation can increase the purity of the hepatocyte fraction to nearly 100% (with concomitant reduction in yield). However, the nonparenchymal fraction is necessary if one seeks to reproduce functional liver tissue through tissue engineering. Indeed, the appropriate cell mixture under appropriate culture conditions can result in formation of characteristic and reproducible three-dimensional tissue architecture composed of a superficial layer of biliary epithelial cells, an intermediate layer of connective tissue and hepatocytes, and a basal layer of endothelial cells [62, 80, 104, 105].

Reports as early as 1985 [69] indicated that cells from primary collagenase digestion could spontaneously reorganize to form spheroids of approximately 70–250 μm diameter with endothelial cells enclosing a core of hepatocytes under the right conditions. Such spheroids were found to have high liver-specific activities in comparison with monolayer cultures and to maintain liver-specific activity for longer periods of time [71, 113, 133]. Further research has shown considerable structural similarity between the spheroids and the native liver tissue [1, 6, 44, 65]. Spheroid surfaces appear to be smooth under scanning electron microscopy with barely detectable cell-cell borders. Hepatocytes in the spheroid interior establish more cell-cell contacts than typical (or even possible) in monolayer cultures and deposit extracellular matrix in spaces between groups of cells. Furthermore, spheroid hepatocytes maintain structural polarity, show junctions, and form functional bile canaliculi.

Despite improved methods for spheroid production that include choice of substrata surface material [51, 52, 60, 101, 102] and culture rotation or agitation [93, 129, 139, 141], spheroids still lack an important feature of native liver tissue: individual cell proximity to a sinusoid that continuously and convectively replenishes nutrients and oxygen. Transport of nutrients and oxygen into the interior of a spheroid is by diffusion alone. Spheroids greater than about 100 μm diameter may suffer from anoxic conditions in the interior. Encapsulated spheroids are at even greater risk for anoxia and nutrient limitation because the encapsulating gel represents an additional diffusional resistance for transport.

11.2.3.3 Three-Dimensional Culture

Several research groups have shown that three-dimensional culture of the collagenase digestion mix of parenchymal and nonparenchymal cells results in self-assembly into native liver-like structures in vitro [34, 36, 51, 52, 54, 112]. The inoculated cells are observed to from multicellular aggregates reminiscent of hepatocyte acini with endothelial cells on the exterior of hepatocytes, bile canaliculi formation, and neo space of Disse. The three-dimensional systems have several qualities that are appealing for a BAL to support patients with ALF: the systems are easily perfused; the systems evidence maintenance of hepatocyte-specific functions over weeks; and the hepatocytes in the systems maintain tight junctions and form bile canaliculi.

11.3 State of the Art

BAL bioreactor design faces several challenges that must collectively be satisfied in order to create a clinically viable system: (1) adequate cell seeding/cell density; (2) adequate transport of oxygen, nutrients, and toxins to the cells; (3) adequate transport of metabolic products from the cells; and (4) maintenance of sufficient hepatocyte-specific function over the time needed to meet a desired therapeutic goal. These challenges are not mutually exclusive; collectively, they must meet the constraint that the BAL delivers the desired, and as yet unspecified, therapeutic dose. Adequate cell seeding/cell density impacts the maintenance of hepatocyte-specific function: hepatocytes without appropriate cell-cell contact and resultant polarity rapidly dedifferentiate and loose function. Adequate transport, in turn, depends upon cell seeding/cell density: maintenance of adequate transport is easier in lower-density systems, but lower-density systems might not be adequate to maintain function or deliver the desired therapeutic dose. Several excellent reviews of BAL approaches and variations on the approaches that discuss these interactions are available [17, 37, 63, 95, 136].

11.3.1 Perfusion

BAL systems have used extracorporeal perfusion to provide therapeutic treatment. Blood flow rates to the BAL are limited by physiological tolerance of the patient for the treatment. Since liver failure patients tend to be hemodynamically unstable, clinical blood flow rates have typically been in the range of 50–300 mL/min (compared with normal liver perfusion of 1,500 mL/min). BAL devices need to achieve a therapeutic effect at perfusion rates 3–20% that of the normal liver.

A still unresolved question in extracorporeal BAL bioreactor design is whether perfusion by whole blood or plasma is preferable [99]. Plasma can be easily and continuously separated from the blood by continuous centrifugation or a plasmapheresis membrane module. In either whole blood or plasma perfusion, plasma is the carrier for soluble and protein-bound solutes into the bioreactor and liver-specific proteins and soluble factors such as clotting factors from the bioreactor. In whole blood, the red blood cells also act as efficient oxygen and carbon dioxide transporters. In both cases, in long-term extracorporeal support, even with anticoagulant supplementation (e.g., heparin or citrate), proteins in the plasma (at least those of the complement cascade) may adhere to membrane surfaces in the plasma filter and/or the bioreactor, resulting in fouling and crippling of mass exchange and separation properties. Whole blood perfusion carries the additional risk that activation of the coagulation cascade may lead to platelet cell aggregation and blood clots that totally obstruct bioreactor perfusion. At the same nominal blood draw rate, plasma-perfused bioreactors will treat approximately 20–50%, depending upon the plasma separation procedure, of the total plasma in the blood stream as a whole blood-perfused bioreactor.

Thus, bioreactor perfusion with either blood or plasma has both advantages and disadvantages relative to the other. Regardless of perfusion, comparison of therapeutic dose between whole blood and plasma-perfused systems should be in terms of the Damkohler number, Da.

11.3.2 Mass Transport

Bioreactors are reacting systems that involve transport of nutrients and oxygen from the perfusing medium to the cells, where metabolism produces waste materials and biological products that need to be transported back to the perfusing media for

removal from the bioreactor. Ensuring proper transport of essential nutrients (oxygen, sugars, amino acids, etc.) to the cells and waste metabolites (CO_2, lactate, etc.) and liver-specific metabolic products (clotting factors, growth factors, etc.) away from the cells in a BAL is essential for proper bioreactor performance.

Convective transport is more efficient than diffusive transport. However, direct convective transport to the cells requires plasma perfusion rather than whole blood perfusion because of immunological and clotting issues. Even direct, convective perfusion with plasma has immunological considerations if a cell source other than primary human hepatocytes is used. Because of problems associated with direct plasma perfusion, the majority of BAL bioreactors to date have used perm-selective membranes to segregate various compartments in the BAL bioreactor from direct contact with the perfusing stream. Hence, the primary mode of mass transport in a BAL is diffusion: from (and to) the perfusing medium; across the perm-selective membrane; and to (and from) the cell mass.

Diffusive transport is, however, complicated by (1) the presence of a perm-selective membrane, (2) the relatively high cell density believed necessary to obtain adequate hepatocyte-specific function, (3) typically low concentration gradients of nutrients, and (4) sensitivity of liver cells to waste metabolites [7, 15, 16, 32, 111]. In non-BAL applications, poor oxygen and glucose supply has been correlated with necrotic regions in tumors and in dense cell aggregates [87, 130, 131, 137]. Nonuniform spatial distributions of nutrients, metabolic wastes, and products impact diffusion and may have important effects on hepatocyte phenotype and survival, on BAL performance, and on the therapeutic efficacy of the treatment as a whole.

Diffusive transport is further complicated by the use of protein, e.g., type I collagen, or polysaccharide, e.g., alginate, gels as an extracellular matrix to provide hepatocytes with three-dimensional scaffolding in some BAL approaches. Matrix gels have been shown to enhance attachment, promote polarization, and maintain differentiation of primary hepatocytes. Drawbacks to the use of gels include lowered oxygen diffusivity relative to plasma or media and a likely increase in hydraulic resistance in the cell compartment that can hinder the occurrence of (convective) Starling flow through the cell mass [14].

11.4 Clinical Application

11.4.1 Medical Therapies

As noted, significant advances have been made over the last 50 years in understanding the pathophysiology behind clinical features of ALF and AoCLF: encephalopathy, cerebral edema, hemorrhage, electrolyte and metabolic disturbances, renal failure, cardiovascular instability, and increased risk of infection [39, 72, 73, 92, 134]. Cerebral edema, multiorgan failure, and sepsis are associated with significant mortality.

Medical treatment for ALF is directed toward bridging the patient to either a spontaneous recovery of liver function or LTx. The bridging period is characterized by intensive care, mechanical and pharmacologic support for major organ failures (mechanical ventilation, vasopressors, hemodialysis), correction of metabolic derangements (hypoglycemia, acidosis, coagulopathy), and interventions to prevent or slow the development of brain swelling (edema) and herniation. Interventions for brain edema may include sophisticated diagnostic techniques to measure cerebral blood flow, activity, and pressure. Commonly practiced therapeutic interventions include hyperventilation, induced hypothermia, barbiturate coma, and plasmapheresis [18, 91]. Although brain edema occurs only rarely in patients with AoCLF, such patients also require multiorgan support [21]. Several overviews on medical therapies are available [28, 56, 73, 116]. Conventional medical therapies, while improving, have limited impact in changing patient outcome because native liver regeneration may be too slow in ALF or may not occur at all, as in AoCLF.

11.4.2 Extracorporeal Liver Support

Conventional medical therapies for ALF and AoCLF are typically directed toward mitigation of individual symptoms rather than correction of the underlying causes for the symptoms. Development of extracorporeal liver support systems has been directed toward an adjunct to conventional therapy that seeks to either prolong the "window" for successful bridge to LTx or allow spontaneous regeneration of the diseased liver. Extracorporeal systems are directed toward partially restoring hepatic function through removal of

endogenous toxins and/or normalizing the regulation of factors associated with cerebral and systemic hemodynamic dysfunction via blood metabolism. Two distinctly different approaches have been developed: detoxification devices and BALs.

11.4.2.1 Detoxification (Artificial Liver) Devices

Detoxification devices, sometimes referred to as AL, are directed toward the removal of toxins that cannot be removed by conventional dialysis because the toxins are bound to protein carriers, such as albumin, in the blood stream. With the exception of plasmapheresis, all detoxification approaches rely upon use of a secondary binder to promote removal of the toxin from the blood stream [97, 98, 100]. Plasmapheresis seeks to detoxify by removing a fraction of the patient's plasma and replacing it with fresh plasma. Plasmapheresis has been largely abandoned due to lack of clinical efficacy.

Three detoxification approaches have reached clinical evaluation. The Biologic-DT is similar to conventional dialysis except that the dialysate contains a finely divided activated carbon that acts as a toxin scavenger [2, 50]. The Molecular Adsorbent Recirculating System (MARS) [83, 125] uses a primary dialysis cartridge to dialyze blood against a recirculating albumin-containing dialysate. Both protein-bound and water-soluble species pass from the blood to the albumin-containing dialysate. A secondary dialysis cartridge is then used to dialyze the albumin-containing dialysate against conventional dialysate to remove water-soluble species picked up from the blood in the primary cartridge. Finally, the albumin-containing dialysate is then passed through columns with granulated solid adsorbents to remove toxins from the albumin before being returned to the primary dialysis cartridge. Prometheus [66, 109] uses a plasma filter to directly pass a fraction of the patient's plasma through granulated solid adsorbent columns for toxin removal. Three case studies of "single pass albumin dialysis" [19, 67, 121], also known as "bound solute dialysis" [97, 98, 100], have been reported. Single pass albumin dialysis is essentially dialysis with albumin added to the dialysate.

Clinical evaluations of detoxification systems have found significant removal of blood stream toxins for each of the systems. However, the impact of toxin removal on clinically significant outcomes, such as patient survival, need for intubation, use of vasopressors, and nontransient improvement in hepatic encephalopathy, is not clear.

11.4.2.2 Metabolic, Cell-Based (Bioartificial Liver) Devices

The liver is a complex organ that performs several functions, including the following: oxidative *detoxification* of species produced in the body and xenobiotics (drugs and toxins) from ingestion; intermediate *metabolism* (carbohydrate, protein, and fat) and supply of nutrients to the body; toxin and "waste" *excretion* through the bile; protein and macromolecule *synthesis*; and *modulation* of immune and hormonal systems. In recognition of the multiple liver functions, BAL devices have been developed in order to provide more support to ALF and AoCLF patients through use of viable hepatic cells than is provided by artificial detoxification approaches alone.

The first generation of BALs to reach clinical evaluation was based upon the use of hollow-fiber cartridges to house hepatocytes in the extraluminal space between the hollow fibers. The HepatAssist [23] passes plasma through a bioreactor cartridge containing cryopreserved primary porcine hepatocytes that have been seeded onto collagen-coated dextran beads prior to placement in the bioreactor. The ELAD [29, 82] passes plasma through a cartridge containing the C3A derivative of the HepG2 human hepatocyte cell line that was seeded and grown to confluence within the extraluminal space. The MELS [89, 114] passes plasma through a unique tripartite arrangement of hollow fibers that houses primary human hepatocytes embedded in a collagen matrix in the extraluminal space. The BLSS [78, 79] passes whole blood through a cartridge housing a high-density culture of primary porcine hepatocytes. The radial flow bioreactor [86] and the AMC-BAL [135] perfuse plasma directly over and around cultured, primary porcine hepatocytes.

Several reviews comparing and contrasting the various approaches in clinical practice are available [26, 37, 63, 95, 136]. Although preliminary investigations have shown trends toward normalization of blood chemistries, hemodynamic stability, respiratory status, coagulopathy, and encephalopathy, the impact on survival and clinically significant outcomes remains unclear.

11.4.3 Cell Transplantation

Cell-based therapies involving transplantation of regenerative cells to replace damaged tissue are being actively pursued for heart disease [126], diabetes [59], Parkinson's disease [53], and muscular dystrophy [22], as well as liver disease [25, 127, 128]. Hepatic cell transplantation methods generally seed hepatocytes into the portal vein of the liver. The hepatocytes are then transported with the blood into the liver sinusoids where they are captured because of their large size relative to the sinusoidal blood vessels. Although limited availability of human hepatocytes has hampered clinical evaluation, several small-scale investigations have been conducted [9, 42, 68, 107, 128]. Additional single-subject case reports of hepatocyte transplantation for various etiologies are available [30, 48, 64, 90]. While such results are promising, as with extracorporeal liver support in general, the studies are small, uncontrolled, and inconclusive. As with BAL, availability of a suitable cell source for wide-scale clinical application is problematic.

11.5 Expert Opinion

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Because oxygen is an important nutrient in BAL operation that appears to modulate hepatocyte viability and function [57, 61] and is consumed at a reasonably high metabolic rate, researchers have focused considerable effort in understanding and enhancing oxygen transport throughout the cell mass in the cell compartment. Although 100 μm has been claimed as maximal permissible diffusion distance for oxygen transport in a BAL [16], the actual situation is more complex and requires careful attention to the combination of convective and diffusive oxygen transport as well as oxygen uptake rate in the BAL [45, 46, 96]. That said, diffusion distances can place a severe limitation on the cell mass that can be expected to be supported in systems employing membranes.

11.5.3 Membranes and Compartmentalization

Bioreactors are reacting systems that involve transport of nutrients and oxygen from the perfusing medium to the cells, where metabolism produces waste materials and biological products that need to be transported back to the perfusing media for removal from the bioreactor. Early three-dimensional BAL designs adapted hollow-fiber membrane compartmentalization from hemodialysis to separate the cell mass from the perfusing media. The hollow-fiber membranes have perm-selective properties that reject high molecular weight molecules (>100–250 kDa, depending upon the system). As previously noted, the use of hollow-fiber membranes introduces a diffusive mass transport limitation. Membrane optimization for both nutrient delivery and metabolic product removal is problematic. Controversy exists as to whether such designs can provide sufficient oxygen to hepatocytes. Substantial gradients along the axis of the bioreactor may have an impact on overall hepatocyte function since hepatocytes are typically exposed to low gradients in normal liver.

Determination of whether traditional hollow-fiber designs can provide sufficient clinical efficacy awaits proper design of a clinical trial with well-defined

therapeutic endpoints. In the meantime, BAL bioreactor design has been moving toward compartmentalization that segregates delivery of nutrients, delivery of oxygen, removal of wastes, and housing of cells into their own compartments [89, 115]. Xu et al. [140] suggest that four-compartment bioreactors may be necessary to enable integral oxygenation and distributed mass exchange with low gradients typical of the liver.

While membranes have been traditionally considered inert materials with desirable mass transport properties that are used to separate regions of the BAL, membranes are actually active components in the bioreactor. Membranes adsorb proteins, a process that will alter the mass transport properties of the membranes over time. Transient adsorption of toxins on membranes can alter bioreactor performance. Membranes can act as a cell attachment surface, which can alter both the mass transport and metabolic properties of the BAL. Legallais et al. [74] provide a review of many of these issues. The reported results are qualitative and ambiguous but point to the need to consider membranes and membrane properties carefully in design of a BAL bioreactor.

11.5.4 Hepatic Cell Source

Appropriate cell sources for extracorporeal liver support have been controversial [4, 38, 85, 122]. Since a clinically proven BAL would have an annual demand in the thousands of procedures, practical considerations about cell source include the following: ready availability; biochemical functional similar to native human liver; and minimal risk of immunogenic response or infection to the patient. Adult human hepatocytes are the gold-standard with respect to biochemical function and minimization of immunogenic risk. Unfortunately, they are not available in the quantities required for general BAL use.

The majority of reported clinical evaluations of BAL support systems have used primary porcine hepatocytes because of ready availability. Porcine hepatocytes exhibit many of the same biochemical functions and activity as primary human hepatocytes. However, concern over the potential of transmission of xenoviruses has created controversy even though no such transmission has been detected [138]. Potential immunogenic response, whether by the patient in response to the porcine hepatocytes or by the porcine hepatocytes in response to the patient, is also of concern. Most of the systems used in clinical evaluations thus far have employed membrane barriers that minimize the likelihood of cell-mediated damage in either direction.

Human hepatocyte cell lines are readily available and can be easily expanded to create the number of cells needed for a clinical-scale BAL [29, 47, 82, 106]. However, these cell lines typically display altered function from primary hepatocytes and frequently, when function is present, at a reduced level. As with porcine cells, care must be taken to prevent potential delivery of a cell from the line back to the patient.

Stem cells, unspecialized cells that perpetuate themselves through self-renewal and generate specialized cells through differentiation [108], may be the ideal cell source for cell-based therapies developed to treat debilitating and life-threatening diseases [110]. While the potential of stem cells is quite promising, realization of that potential lies in the future. Focus on liver progenitor cells with a view to developing liver stem cell expansion systems is warranted.

11.6 Five-Year Perspective

Therapeutic goals for systems designed to support patients with liver failure will become better defined as more systems are placed into clinical evaluation. This in turn will define the therapeutic, pharmacokinetic dose, i.e., Da, that functional support systems must have to achieve clinical acceptance. Given that, the clinical efficacy of both AL (detoxification) and BAL (cell-based metabolic support) will be verified in the next 10 years. However, the clinical timing and indication will be different for the two systems: detoxification approaches will be employed earlier in the disease progression and, most likely, for AoCLF while BAL support will be used to support ALF patients and will be used when the disease continues to progress despite best medical therapy and detoxification.

Research will focus on hepatic cell source in order to address ready availability, activity and function, and relatively low cost. Identification of human stem cell-derived hepatic cells to address immunologic concerns and species differences in metabolism as well as understanding and controlling the proliferation and

differentiation of liver progenitor cells to functional hepatocytes will be important topics.

As new cell sources become available, new bioreactor designs will emerge that provide a better cell culture environment than present-day designs. The new designs will rely heavily on pharmacological and engineering (mathematical) modeling of the entire patient/bioreactor perfusion system rather than just the bioreactor alone.

11.7 Limitations/Critical View

Development of efficacious BALs for the treatment of ALF remains one of the most elusive biomedical engineering endeavors. One reason for this is that hepatocytes have proven one of the most difficult mammalian cells to maintain in culture with an active but stable phenotype. The ability to maintain functional hepatocytes in long-term, three-dimensional culture systems is relatively recent and is still not fully understood. A second reason is lack of agreement on the expected outcome from BAL intervention, e.g., reversal of coma, bridge to transplant, or recovery from liver failure. A third reason is that the "engineering" (pharmacokinetic) requirements for a BAL are not well defined. While a general consensus exists that mass exchange between blood and hepatic cells is a limiting feature in current BAL designs, quantification of the mass exchange requirement is lacking.

Unlike other artificial organ technologies, such as heart or lung, where the "engineering" need is fairly well understood or identifiable, engineering requirements for a BAL are yet to be defined – partly because the necessary therapeutic requirements remain a matter of speculation, i.e., how much liver function must be provided over what period of time to achieve the therapeutic goal, presumably recovery from liver failure. To put this in perspective, an artificial heart requires volumetric output equivalent to the heart either for life or until a replacement organ becomes available. Renal dialysis, on the other hand, can be provided three times a week for as little as 3 h per session to achieve comparable elimination by the kidney over the same time frame. Such specifications are not generally agreed upon for therapeutic result from a BAL.

Absent a defined therapeutic goal, we can still make progress in developing engineering design equations

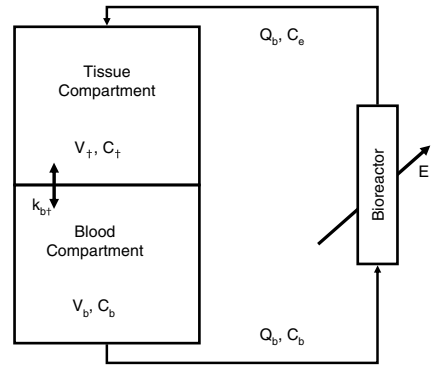

Fig. 11.3 Two-compartment representation of extracorporeal extraction device

suitable for characterizing BAL performance [99]. A simplified, generic flow diagram for a BAL is depicted in Fig. 11.3. The patient is treated as a two-compartment reservoir consisting of a well-mixed tissue (intracellular fluid) compartment with volume V_t (mL) and concentration C_t (mmol/mL) and a well-mixed blood (extracellular fluid and blood) compartment with volume V_b (mL) and concentration C_b (mmol/mL). Mass transfer between the tissue and blood compartments is assumed to be isotropic, i.e., not dependent on the direction of transfer, to occur by diffusion only, and to be characterized by the intercompartmental mass transfer parameter, k_{bt} (mL/min). Blood flows to the BAL at flow rate Q_b (mL/min). Although depicted as if the BAL were an extracorporeal device, this scenario applies equally well to an implanted BAL that is directly perfused by arterial flow. Blood enters the BAL at concentration C_b and exits at concentration C_e (mmol/mL) to the well-mixed blood compartment.

The BAL bioreactor is modeled as a first-order conversion/extraction unit, similar to a dialyzer in renal dialysis. This is justified through the observation that reacting species must move through several resistances in series that will make the BAL a mass-transport-limited reactor and likely reduce reactive species concentrations to the linear, first-order region in Michaelis-Menton kinetics. The relationship between in-flow and out-flow concentrations, C_b and C_e, respectively, for a first-order extractor is

$$C_e = C_b(1-E), \tag{11.1}$$

where

$$E = 1-\exp(-k_{brx}/Q_b) \tag{11.2}$$

Table 11.1 Dimensionless parameters in two-compartment extraction model

Parameter	Definition	Meaning	Range
κ	k_{bt}/Q_b	Internal reequilibration rate/BAL perfusion rate ratio	2.0–8.0
V_{tb}	V_t/V_b	Tissue/blood volume ratio	2.0
E	$1-\exp(-Da)$	BAL extraction parameter	
Da	k_{brx}/Q_b	Damkohler number: extraction rate/throughput rate ratio	0.1–2.0

From [24]: $k_{bt} \sim 800$ mL/min; $V_t \sim 28{,}000$ mL; $V_b \sim 14{,}000$ mL

is the bioreactor conversion/extraction parameter. k_{brx} (mL/min) is a lumped first-order parameter reflecting the combined effects of species diffusion through resistances and conversion at the active site within an hepatocyte (each species will have its own k_{brx}). k_{brx} should not be confused with the reaction rate constant for actual biochemical conversion at the reaction site within a hepatocyte. The ratio of k_{brx} to Q_b forms a dimensionless parameter called the Da, which represents the rate of removal of a species by reaction to the rate of supply of that species to the bioreactor. The relationship between clearance, CL (mL/min), a term borrowed from renal dialysis therapy that represents the volume of blood that is totally "cleared" of species in a single pass through the bioreactor, and the bioreactor extraction is

$$CL = Q_b E. \tag{11.3}$$

Thus, the maximum clearance of a species is equal to the perfusate flow rate (when $E=1$). Since E will generally be less than 1, clearances will generally be less than the perfusate flow rate.

Using the scalings

$$\{C_t, C_b, C_e, t\} = \{C^*, C^*, C^*, (V_b / Q_b)\}, \tag{11.4}$$

where t is time and C^* is a reference concentration (usually taken to be the initial blood concentration at start of therapeutic intervention), a material balance produces the nondimensional, ordinary differential equations (ODEs)

$$\frac{d\tilde{C}_t}{d\tilde{t}} = \left(\frac{\kappa}{V_{tb}}\right)(\tilde{C}_b - \tilde{C}_t), \tag{11.5a}$$

$$\frac{d\tilde{C}_b}{d\tilde{t}} = k(\tilde{C}_t - \tilde{C}_b) - E\tilde{C}_b, \tag{11.5b}$$

i.e., $$\tilde{C}_t = \tilde{C}_t^0; \quad \tilde{C}_b = \tilde{C}_b^0 \quad \text{at } \tilde{t} = 0. \tag{11.5c}$$

The tilde over-bar, ~, denotes the nondimensional, scaled variable, which is equivalent to the dimensional variable divided by its scaling factor. The dimensionless parameters associated with BAL performance are provided in Table 11.1.

Each species of interest will have its own set of associated values for the dimensionless parameters in Eqs. (11.5a–c). The possible ranges for the dimensionless parameters provided in Table 11.1 are restricted due to physiological constraints imposed by the patient. For example, blood draw rates for renal dialysis therapy are in the range of 200–500 mL/min. Blood draw rates from liver failure patients, who are generally more hemodynamically unstable than renal patients, will probably be restricted to the range of 100–300 mL/min.

The parameter that characterizes the bioreactor biochemical conversion activity, the Da, is not seen in the equations as shown because it is contained in the dimensionless extraction ratio, E, as represented in Eq. (11.2). The Da, which is the ratio of the extraction rate to the throughput rate in the bioreactor, is the only measure of bioreactor activity in the model. Given that the blood flow rate from a liver failure patient is rather restricted, higher Da generally corresponds to more active bioreactors while low Da corresponds to less active bioreactors.

The effects of varying Da on blood concentration, $\tilde{C}_b$, vs. time, $\tilde{t}$, are depicted in Fig. 11.4 at constant $\kappa=5$. As might be expected, the rate of reduction in blood concentration increases with increasing Da up to the limiting curve represented by Da $\rightarrow \infty$, which is equivalent to an extraction ratio of 1 and overall clearance, CL, equal to Q_b per Eq. (11.3). Clearly, a point of diminishing returns on the rate of reduction in blood concentration with respect to increasing bioreactor activity is reached when the Da exceeds two.

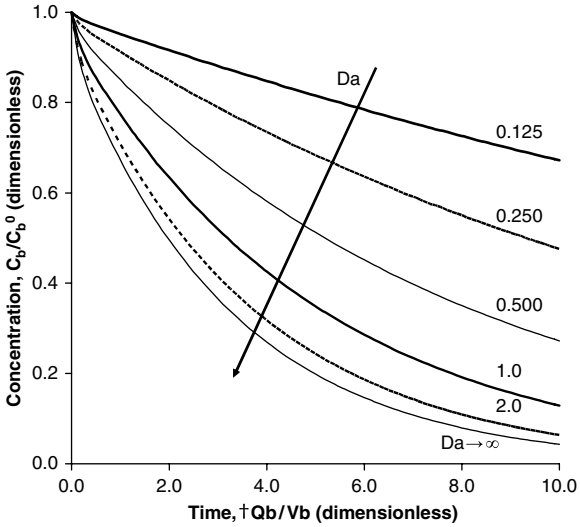

Fig. 11.4 Dimensionless blood concentration vs. dimensionless perfusion time as a function of Damkohler number, Da, at constant $\kappa = 5$

At constant Q_b, the *dimensional* perfusion time scales with the dimensionless perfusion time in Fig. 11.3, and each curve directly represents the effect of bioreactor activity on blood concentration reduction. Conversely, if one has bioreactors of equivalent and constant activity operating at different blood flow rate, the *dimensional* perfusion time does not scale with the dimensionless perfusion time. In essence, reporting fractional reductions in concentration as a function of time without reference to the perfusion rate, as is commonly done, does not permit comparison in functional activity between different bioreactor designs.

The preceding analysis points to the need to characterize BAL reactors in terms of the intrinsic kinetics as described by $Da = k_{brx}/Q_b$, or, equivalently, in terms of the clearance, $CL = Q_b E$. Either characterization, provided Q_b is specified, will allow meaningful comparison of different bioreactor designs. Iwata and coworkers [54, 55, 94] have also called for reporting BAL bioreactor activities in terms of the clearance and perfusion rate. Their reported clearance values [54], $CL^{NH_3} \sim 10$ mL/min and $CL^{lido} \sim 20$ mL/min for ammonia and lidocaine, respectively, coupled with a reactor perfusion rate of 200 mL/min, lead to estimates of $k_{brx}^{NH_3} \sim 10$ mL/min and $k_{brx}^{lido} \sim 21$ mL/min for ammonia and lidocaine, respectively. The nominal Das for their reactor and perfusion rate are thus $Da^{NH_3} = 0.05$ and $Da^{lido} = 0.11$, respectively. For perspective, the human liver, with an approximate mass of 1,500 g and blood flow rate $Q_b \sim 1$ mL/min (g/liver) [81] has a lidocaine clearance, $CL^{lido} \sim 950$ mL/min [81]. This translates to $Da^{lido} \sim 1$ (i.e., the liver is "set" to clear xenobiotics such as lidocaine at a rate commensurate with flow to the liver) and $k^{lido} \sim 1{,}500$ mL/min.

A very real issue in BAL design and development lies in determining what constitutes a reasonable therapeutic dose of BAL support for a patient with ALF. The preceding analysis, which is easily extended to systems that use plasma perfusion rather than whole blood [99], suggests that realistic quantification of therapeutic dose is possible given knowledge of the bioreactor activity as measured by the Da. The analysis further suggests that unlimited increases in system performance with increasing bioreactor activity are not reasonable. In fact, creating BALs with Da approaching one, i.e., Da comparable to native liver, is problematical when the architecture of the native liver, which tends to minimize mass transport resistances to xenobiotic removal, is considered. Determination of required therapeutic dose, i.e., Da, to achieve the desired clinical outcome requires clinical evaluation of systems with varying Da.

The analysis provided here indicates that arguments over the appropriate mass of cells required for a clinically functional BAL are moot. The issue is not how many cells are needed but, rather, how to most effectively use cells to maximize Da and obtain an, as yet unspecified, desirable clinical outcome.

11.8 Conclusions/Summary

ALF is a devastating diagnosis with low survival rates. LTx is the therapy of choice but is limited by the scarce availability of donor organs. In well-characterized cases where the liver tissue has the capability to regenerate and heal, the prognosis of liver failure patients may improve if essential liver functions are restored during liver failure by means of extracorporeal temporary liver support. BAL systems are in development that use hepatic cells to provide patients with liver-specific metabolic function necessary to relieve some of the symptoms and to promote liver-tissue regeneration. The most promising BAL treatments are based on three-dimensional culture of hepatic tissue constructs with mature liver cells in disposable continuous-flow

bioreactors. The hepatic cells may derive from expansion and subsequent differentiation of hepatic progenitor cells. Clinical evaluations of the proposed BALs show that they are safe but have not clearly proven the efficacy of treatment as compared to LTx. Ambiguous clinical results, the loss of cellular activity during treatment, and the possible presence of a necrotic core in the cell compartment of many bioreactors suggest that improvement in transport of nutrients to and metabolic wastes and products from the cells in the bioreactor is critical for the development of therapeutically effective BALs. Strategies proposed to improve mass transport in bioreactors and liver support systems are reviewed. Future BAL designs will rely heavily on pharmacological and engineering (mathematical) modeling of the entire patient/bioreactor perfusion system rather than just the bioreactor alone.

Suggested Readings

Gerlach JC, Zeilinger K, Patzer JF II. Bioartificial liver: why, what, whither. Regenerative Med. 2008;3:575–95.
Comprehensive review of the state of the art in bioartificial liver development and clinical applications.
Iwata H, Ueda Y. Pharmacokinetic considerations in development of a bioartificial liver. Clin Pharmacokinet. 2004;43: 211–25.
Patzer JF II, Campbell B, Miller R. Plasma versus whole blood perfusion in a bioartificial liver assist device. ASAIO J. 2002;48:226–33.
Comprehensive developments on pharmokinetic considerations in bioartificial liver application. Both articles elucidate the need to couple descriptions of bioreactor performance with clinical requirements in order to properly design efficacious bioreactors.
Selden C, Hodgson H. Cellular therapies for liver replacement. Transpl Immunol. 2004;12:273–88.
Gerlach JC. Prospects of the use of hepatic cells for extracorporeal liver support. Acta Gastroenterol Belg. 2005;68: 358–68.
Discussion of choice of cell source for bioartificial liver support and the pros and cons associated with the various options.
Stange J, Hassanein TI, Mehta R, Mitzner SR, Bartlett RH. The molecular adsorbents recycling system as a liver support system based on albumin dialysis: a summary of preclinical investigations, prospective, randomized, controlled clinical trial, and clinical experience from 19 centers. Artif Organs. 2002;26:103–10.
Kramer L, Bauer E, Schenk P, Steininger R, Vigl M, Mallek R. Successful treatment of refractory cerebral oedema in ecstasy/cocaine-induced fulminant hepatic failure using a new high-efficacy liver detoxification device (FPSA-Prometheus). Wien Klin Wochenschr. 2003;115:599–603.
Descriptions of the MARS albumin dialysis system and Prometheus approach to detoxification in support of patients with liver failure.
Catapano G, Gerlach JC. Bioreactors for liver tissue engineering. In: Ashammakhi N, Reis R, Chiellini E, editors. Topics in tissue engineering (ExpertIssues); 2007.
Comprehensive description of issues in bioartificial liver bioreactor design.

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Central Nervous System

12

T. Führmann, J. Gerardo-Nava, and G.A. Brook

Abbreviations

aFGF	Acidic fibroblast growth factor
BBB	Basso, Beattie, Bresnahan Locomotor Rating Scale
BDNF	Brain-derived neurotrophic factor
C	Cervical
C/PCL	Collagen type I/Poly caprolactone
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
CSPG	Chondrotin sulphate proteoglycan
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
ECM	Extracellular matrix
EDC	1-Ethyl-3(3-dimethylaminopropyl) carbodiimide
EPO	Erythropoietin
FN	Fibronectin
GABA	Gamma-aminobutyric acid
GDNF	Glial cell derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GTP	Guanosine Triphosphate
HA	Hyaluronic acid
HAMC	Hyaluronan-methylcellulose
kGy	Kilo grey
L	Lumbar
MAG	Myelin-associated glycoprotein
MgSO4	Magnesium sulphate
MSC	Mesenchymal stromal cells
mTOR	Mammalian target of rapamycin
NF	Neurofilament
NGF	Nerve growth factor
NgR	Nogo receptor
NT-3	Neurotrophin 3
OEC	Olfactory ensheathing cells
OmgP	Oligodendrocyte myelin glycoprotein
PAN/PVC	Poly(-acrylonitrile-co-vinilchloride)
PCL	Poly caprolactone
PDLA	Poly-D-lactic acid
PEG	Poly-ethylene glycol
PELA-PDLLA	poly(ethylene oxide)-block poly(D,L-lactic acid)
PGA	Poly-glycolic acid
PHB	Poly-β-hydroxybutyrate
PHEMA	Poly-hyrdoxyethyl methacrylate
PHEMA-MMA	Poly(2-hydroxyethyl methacrylate-co-methyl methacrylate
PHPMA	Poly[*N*-(2-hydroxypropyl)methacrylamide]
PirB	Paired immunoglobulin-like receptor B
PLA-b-PEG-b-PLA	Poly(lactic acid)-b-poly (ethylene glycol)-b-(poly lactic acid)
PLGA	Poly-L-glycolic acid
PLLA	Poly-L-lactic acid
PNS	Peripheral nervous system
PSN	Propriospinal neurons
PTEN	Phosphatase and tensin homolog
PVC	Poly-vinylcholride
RhoA	Ras homologue A
SCI	Spinal cord injury
T	Thoracic

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12.1 Introduction

This chapter focuses on the development of biomaterials and tissue engineering strategies intended to promote axon regeneration following traumatic injury, in particular, of the CNS following spinal cord injury (SCI). Human SCI is a condition for which no widely accepted treatment strategies are available apart from the surgical stabilisation of damaged vertebrae followed by intensive rehabilitation. The complexity of the CNS and its response to injury dictates that the latest advances in bioengineering and macromolecular chemistry be implemented in the design of scaffolds intended to promote neuroprotection and functional repair. The reader will be presented with a brief overview of some aspects of the pathophysiology involved in traumatic SCI. The chapter will then highlight some aspects of device design as well as a range of biomaterials, derived from natural and synthetic polymers, which have been developed for applications in traumatic SCI. Issues related to prosthetic devices for electrical stimulation (e.g. cochlear or retinal implants) will not be covered in this chapter.

12.2 Aim of the Discipline

Over recent decades, enormous progress has been made in obtaining a fundamental understanding of the pathophysiology of neurological diseases and disorders, including traumatic SCI. Numerous cellular and molecular mechanisms have been identified as targets for intervention strategies aimed at promoting functional tissue repair and these have shifted the long-standing pessimistic outlook of clinicians and scientists to one of optimism and great hope. Furthermore, recent advances in material sciences have brought closer the notion that biomaterials may contribute to tissue engineering strategies that could compliment future multifactorial approaches in promoting functional tissue repair following SCI. The goal of the use of biomaterials in tissue engineering and regenerative medicine is to replace or restore the anatomic structure and function of damaged or missing tissues following any injury or disease process by combining the topographical cues of biomaterials with cells or bioactive molecules. The development and design of biocompatible polymers for CNS tissue engineering has resulted in the appreciation that … "This approach requires in-growth and close contact with regenerating neuronal structures and integration of host tissue within and around the implant" [187]. Providing an axon growth-permissive environment for damaged neural tissue is essential for the regeneration of the injured nervous system. In order to prepare an environment that promotes orientated axon regeneration, the physical, chemical, and biochemical characteristics of the implant must be engineered to ensure the presentation of guidance cues that will facilitate axon re-growth across the lesion site and allow the navigation of growth cones back into host tissue, crossing the implant-host interface and extending towards functionally relevant distant targets. Before presenting some of the latest developments in biomaterials and tissue engineering related to CNS repair, the pathophysiology of SCI will be briefly described to allow an appreciation of the challenges facing the medical and scientific communities aiming to promote the repair of such complex tissues.

12.3 State of the Art

12.3.1 Pathophysiology of Traumatic Spinal Cord Injury

The devastating consequences, permanent functional loss and a poor prognosis after traumatic SCI have been well known for thousands of years. The first medical documentation of SCI was recorded on Egyptian papyrus, dating back to approximately 1500 BC and reported: *If you examine a man with a neck injury ….. and find he is without sensation in both arms and legs, and unable to move them, and he is incontinent of urine… it is due to the breaking of the spinal cord by dislocation of a cervical vertebra. This is a condition which cannot be treated* (taken from The Edwin Smith Surgical Papyrus, JH Breasted (ed.) The University of Chicago Press, 1930; cited by Raisman [133]). The first indications of the cellular basis for the poor outcome after SCI were determined by Ramon y Cajal and his students in the early twentieth century using silver impregnated tissue sections of experimental spinal cord lesions. Ramon y Cajal reported that *In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree* [135].

Traumatic human SCI is widely acknowledged to be a highly heterogeneous disorder and the characteristics of the lesion site are known to depend on a number of parameters including the type of injury, severity and time after injury. Nonetheless, four main types of injury have been identified [20, 21]:

1. Compression injury
2. Contusion injury
3. Laceration injury
4. Solid core lesions

This has resulted in the development of a number of experimental models of SCI, which mimic (to a greater or lesser degree) the pathophysiology seen in a range of different human spinal cord injuries. These models have also been used to investigate the cascade of cellular and molecular events that are initiated by the primary insult, as well as for the development and evaluation of a range of biomaterials designed to promote tissue repair e.g. [162]. The permanent motor, sensory and autonomic disturbances caused by severe SCI are the result of the immediate destruction of numerous descending and ascending nerve fibre pathways and the concomitant disruption of the highly organised cytoarchitecture of the spinal cord white matter. These primary degenerative events also include the mechanical destruction of grey matter at the point of injury and are followed by a cascade of secondary degenerative events including bleeding, ischaemia, free-radical production, oedema, excitotoxicity, inflammation, programmed cell death, scarring and cystic cavitation; all of which contribute to the augmentation of tissue loss [20, 82, 146] (see Schematic illustration in Fig. 12.1).

The dynamics of the secondary degenerative events may take place over a time scale of hours, days, weeks and even months after the injury. It is

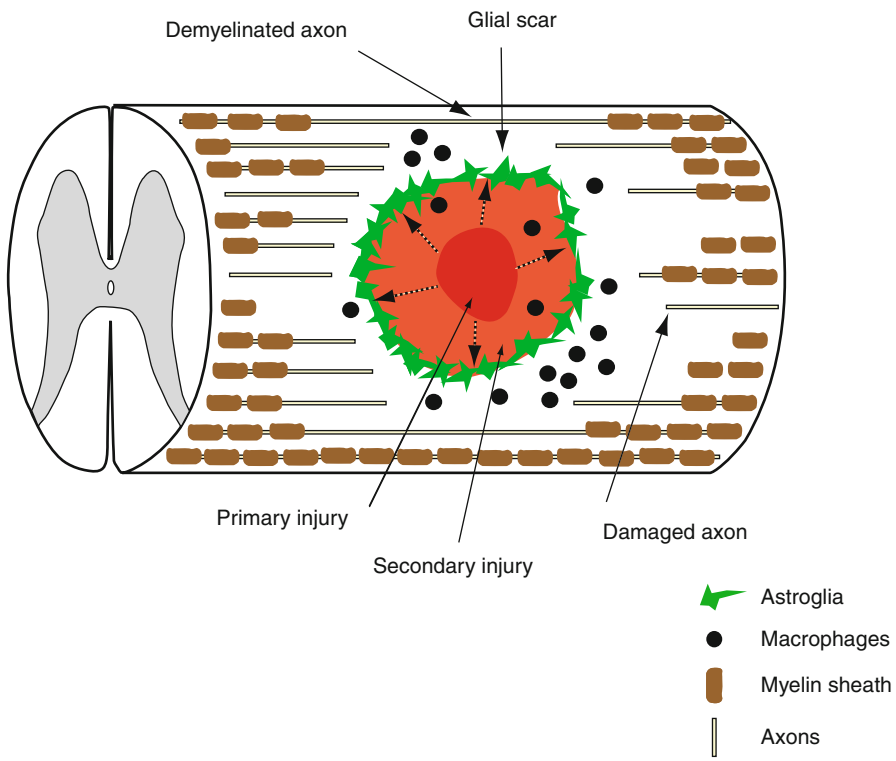

Fig. 12.1 Schematic diagram of the pathological events initiated by compression- or contusion-type traumatic injury to the spinal cord. The injury causes rapid degeneration of the neural cell bodies and processes within the spinal cord grey matter at the lesion epicentre, accompanied by local bleeding. The ensuing ischaemia, free-radical production, excitotoxicity, inflammation, oedema and programmed cell death all contribute to secondary degeneration which increases the size of the lesion over time. Reactive astrocytes express elevated levels of CSPGs and contribute to scar formation. Oligodendrocytic necrosis and apoptosis result in myelin debris being released at and around the lesion site and some degree of demyelination of spared axons. Mild- and moderate compression and contusion injuries often demonstrate some sparing of axons at the outermost aspect of the white matter

now widely acknowledged that the lack of any functionally significant axon regeneration in the lesioned CNS is due to an imbalance of local axon growth-promoting and growth-inhibitory mechanisms, including the relatively poor expression of neurotrophic factors and guidance cues at the lesion site, as well as the presence of potent molecular and physical barriers to axon growth, for reviews see [41, 70, 75, 151].

Critical experiments of Aguayo and colleagues in the 1980s clearly demonstrated that lesioned CNS axons were capable of mounting a significant regenerative response when presented with the growth-permissive environment of a grafted peripheral nerve, many regenerating axons growing for long distances within the graft. However, relatively few axons were able to re-cross the PNS–CNS interface to re-enter the CNS [31, 137, 174]. The biomaterials presented in this chapter have been designed to import growth factors, cells and drugs into the lesion site within a structured framework or scaffold, effectively resetting the balance between growth-promoting and growth-inhibitory cues in favour of tissue repair.

Investigations into the mechanisms underpinning the poor sprouting and regenerative growth of CNS axons resulted in the eventual identification of two environmental factors that are widely believed to play major roles in preventing axon regeneration: CNS myelin and the formation of the glial scar.

12.3.1.1 Myelin-Associated Inhibitors

The primary and secondary degenerative events caused by traumatic injury release, as debris, a number of potent myelin-associated axon growth-inhibitory molecules into the lesion site and the surrounding tissues. The first of these molecules to be identified was NI-35/250 (recently cloned and renamed NOGO-A) [26, 56, 132]. This membrane-bound glycoprotein constitutes only a few percent of the total protein content of mature central myelin, but is an extremely potent inhibitor of axon growth. It induces the rapid, Nogo receptor (NgR)-mediated collapse of axonal growth cones by the activation of the small GTP-ase RhoA [49, 50, 114]. The development of a monoclonal antibody (IN-1) which blocks the inhibitory activity of NOGO-A has proved to be extremely useful in promoting the regeneration of axotomised corticospinal axons, as well as substantial sprouting and re-organisation of non-lesioned axonal projections [5, 15, 165, 197]. Other potent myelin-associated inhibitors are myelin-associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp), both of which also act via NgR and share the same intracellular signalling pathway [36, 175, 176]. NOGO-A, MAG and OMgp have also been found to act via a supplementary receptor system, via paired immunoglobin-like receptor B (PirB) [1]. Recent investigations have clearly demonstrated that the myelin-associated axon growth-inhibitory molecules stabilise the existing connections within the mature CNS and suppress plasticity. In this context, the use of anti-NOGO-A antibodies or blocking peptides has been shown to promote functional recovery by supporting some degree of regeneration by lesioned corticospinal axons as well as compensatory (or collateral) sprouting [95]; see also recent review by Gonzenbach and Schwab [55].

12.3.1.2 Astroglial Scarring and Axon Growth-Repulsive Molecules

The cell types involved in CNS scarring include astroglia, microglia, macrophages and leptomeningeal fibroblasts. Hyperplasia and interdigitation of reactive astrocytic processes during scar formation, combined with the invasion of meningeal fibroblasts and inflammatory cells results in the increased expression of a family of extracellular matrix (ECM) molecules, collectively termed the chondroitin sulphate proteoglycans (CSPG) [37, 51, 112]. The generation of a tightly packed network of overlapping reactive astrocytic processes and the deposition of new basal lamina form a physical and molecular barrier that is important for the protection of the adjacent, relatively intact nervous tissue from subsequent or ongoing damage [136, 155]. The rapid expression of the highly sulphated proteoglycans in and around the lesion during the early stages of tissue damage and scarring plays a major role in inhibiting axon growth across the scar [41, 42, 51, 151]. The infusion of the enzyme chondroitinase ABC results in the digestion of the inhibitory sulphated glycosaminoglycan side chains of the CSPGs and promotes enhanced axon regeneration and plasticity of long-distance projecting sensory axons and the return of some degree of motor and sensory function [14, 51, 107].

12.3.1.3 Other Axon Growth-Inhibitory Molecules

In addition to the myelin and CSPG-related inhibitory molecules, a number of other extrinsic and intrinsic factors have been shown to exert significant influence over the balance between axon growth-inhibitory and promoting mechanisms, including the up-regulation of ECM-related molecules such as tenascins, ephrins and semaphorins, [42, 57, 125] as well as the intrinsic neuronal molecules phosphatase and tensin homolog (PTEN), a negative regulator of the mammalian target of rapamycin (mTOR) pathway [124].

12.3.1.4 Spontaneous Axon Sprouting and Regeneration

Although no spontaneous functional regeneration of the long-distance projecting axons takes place after SCI, it has become clear that the processes of axonal sprouting and cellular re-organisation that occur after SCI are more extensive and organised than had previously been appreciated. Experimental models of SCI (i.e. compression- and lacerating-type injuries) have revealed that the ECM framework deposited during early bleeding in the lesion site supports the migration of different populations of cells, i.e. polymorphonuclear neutrophils, followed by endothelial cells, meningeal fibroblasts, macrophages, activated microglia and Schwann cells [146]. The presence of migrating Schwann cells appears to tip the balance between growth-promotion vs. growth-inhibition towards the support of axon regeneration, predominantly by populations of local intrinsic spinal cord GABAergic and glycinergic interneurons [7, 16, 17, 61]. All of the axonal re-growth taking place after compression- and laceration-type injuries is associated with a framework of overlapping Schwann cell bodies and processes which present the important growth-promoting cell adhesion molecule, L1 [17] (see schematic illustration in Fig. 12.2). The framework is remarkable in that, in many places, it demonstrates a high degree of orientation, in parallel with the longitudinal axis of the spinal cord. Similar observations of orientated Schwann cells and axons have been reported to form following human spinal cord lesions [178].

An even more striking, plasticity-mediated, return of function has been demonstrated following partial thoracic (hemisection-type) injuries [6]. Lesioned lumbar projecting corticospinal axons were found to sprout and innervate short- and long-distance projecting propriospinal neurons (PSN). With time, contacts with the short-distance projecting PSN were lost, while those with the long-distance projecting PSN were maintained. The long-distance projecting PSN extended synaptic contacts to include de-afferented lumbar motoneurons. The spontaneously sprouting corticospinal axons thus established contacts which effectively by-passed the lesion site [6]. A better understanding of the regulation and restrictions of these spontaneous events may lead to the identification and manipulation of novel targets which enhance and maintain endogenous tissue repair processes.

Clearly, the optimal design of biomaterials for promoting tissue repair across spinal cord lesions will need to support and enhance the endogenous tissue repair mechanisms. Since many populations of CNS neurons and their local circuits, both rostral and caudal to the lesion site, remain intact following SCI (e.g. the central pattern generator for stepping movements) [44], substantial effort has been spent trying to promote functional repair by re-connecting severed nerve fibres to their original targets. These endeavours have been encouraged by the fact that only a small percentage (1–10%) of the original nerve fibre projection is needed for the maintenance of useful motor or sensory function [43, 97]. Over recent years, a range of increasingly sophisticated natural and synthetic polymer nerve guides and hydrogels have been developed in an attempt to bridge spinal cord lesions, supporting optimal graft-host integration to allow axon regeneration across both the interfaces (i.e. rostral and caudal) of the implant-host transitional zone. Such biomaterials have been engineered to present physical or haptotactic guidance cues with key cellular and molecular components.

12.3.2 Design of Implantable Biomaterials for SCI

12.3.2.1 Hollow Conduits

One of the simplest physical guides for nerve repair is the hollow conduit or nerve guide which is intended to span the gap created by experimental SCI. Such

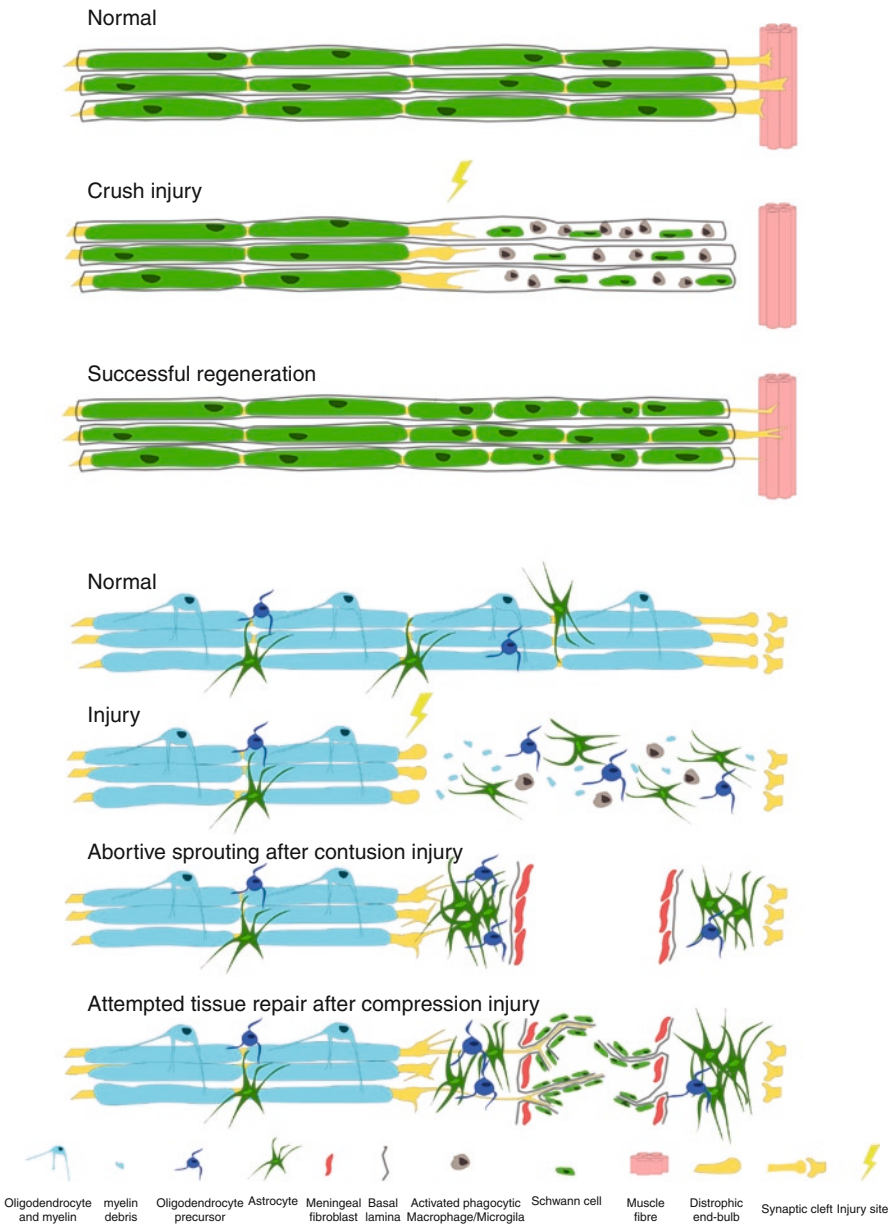

Fig. 12.2 Schematic diagram illustrating the differences in tissue responses between lesioned nerve fibres in the PNS and those in the CNS white matter tracts. The upper three panels represent PNS injury and the lower four panels represent CNS injury. In the upper top three panels, injury causes local destruction of axons and Schwann cells. Wallerian degeneration of the distal stump involves axon fragmentation and Schwann cell proliferation within the basal lamina tubes. Sprouting axons regenerate along the Schwann cell-filled basal lamina tubes (bands of Büngner), eventually reaching their targets and re-establishing neuromuscular junctions. In the lower four panels, injury also causes the local destruction of axons and glia, followed by a slower Wallerian-type degeneration. In contusion-type injuries, the rapid migration of leptomeningeal fibroblasts into the lesion site and their interactions with reactive astrocytes causes the deposition of basal lamina at the scarring interface, the interior of the lesion usually progressing to cystic cavitation. In compression-type injuries, the migration of Schwann cells (from damaged spinal nerve roots) and leptomeningeal cells causes the establishment of a framework within the lesion that is capable of supporting axon regeneration, largely from local spinal cord interneurons. In all cases of spinal cord injury, the lesioned long-distance projecting axons do not regenerate to their original targets

cylindrical conduits needed to be flexible, sterile and permeable to gaseous exchange, as well as demonstrating properties that do not lead to irritation or mechanical damage of adjacent spinal cord tissues. The advantage presented by such simple conduits includes the ability to load additional elements into the hollow centre, including axon growth-promoting glia, bioactive molecules, drugs and orientated microstructures [30, 52, 143, 189, 190, 192], Fig. 12.3, adapted from [74].

The choice of the cell types to be loaded into the hollow conduits have included peripheral glia (e.g. Schwann cells, olfactory ensheathing cells) as well as neural progenitor cells [23, 127, 134, 189–191, 198].

An issue of considerable importance for the use of hollow conduits (or any three-dimensional biomaterial-based scaffold) in the repair of SCI is that most compression- and contusion-type injuries demonstrate some (limited) degree of recovery over the first months post insult, e.g. [42]. Much of this functional recovery is due to the conduction of impulses along spared axons that occupy the outermost rim of the spinal cord white matter. The preservation of these axons and the modalities that they subserve is of utmost importance to the maintenance of any residual function of the SCI patient. Any intervention strategy (including the implantation of biomaterials) should aim to cause minimal disruption or damage to the survival and function of these spared axons.

12.3.2.2 Hydrogels

Hydrogels are cross-linked networks of insoluble hydrophilic polymers that swell in aqueous solutions [171]. The elastic modulus of hydrogels may be very similar to the host CNS tissues they will be implanted into and their gel-like consistency affords them the advantage of readily filling the hollow conduits or of being able to adopt to irregular lesion conformations, making them particularly useful for soft tissue repair [3, 171, 182]. The high water content and loose 3D network of hydrogels facilitates cell adhesion and proliferation, supported by the ready exchange of nutrients and metabolites between the implant and the host [64, 93, 180]. Hydrogels can also be designed for the encapsulation and timed release of bioactive molecules such as growth factors or DNA [78]. The local gelation of hydrogels after injection into the CNS lesions may be induced by mechanisms such as ionic cross-linking [67] or temperature changes [98].

12.3.2.3 Topographical Cues and Patterning

Defining the mechanisms by which substrate topography and chemical composition influence the cell form and function has become a subject of increasing interest in the field of biomaterials and tissue engineering

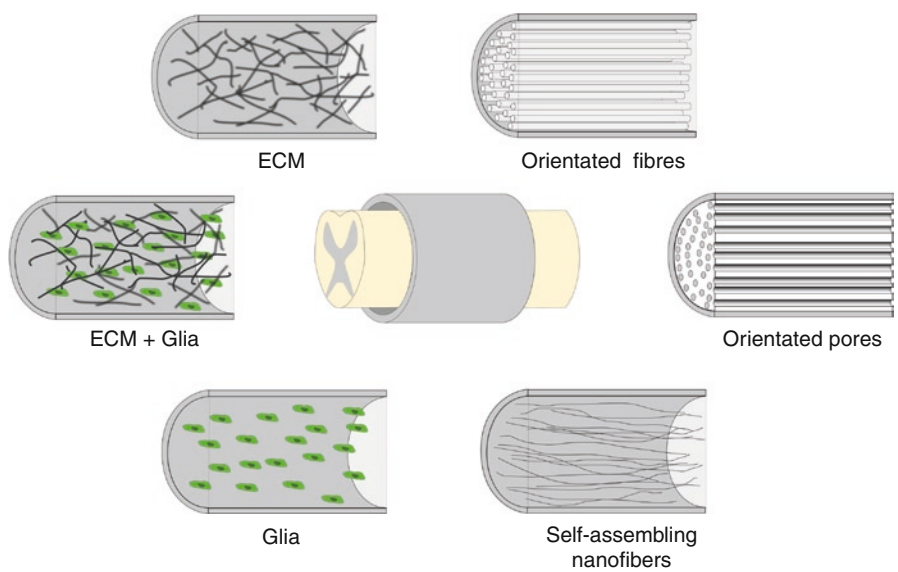

Fig. 12.3 Schematic representation of the versatility of hollow conduit design for spinal cord injury. The transacted spinal cord can be bridged by the implantation of hollow conduits loaded with a range of axon growth-promoting cells, molecules or drugs including: ECM, peripheral glia, ECM and peripheral glia, self-assembling nanofibres, clusters of orientated electrospun nano- or extruded microfibres, as well as scaffolds containing an orientated microporous structure

[34, 152]. Although the crucial experiments performed by Ingber and colleagues employed non-neural cells, the striking influence of substrate topography on cell shape and function has become clear. Using contact micro-printing to generate "islands" and "tracks" of cell-adhesive ECM surrounded by non-adhesive regions, hepatocyte cell spreading and proliferation could be controlled to the extent that the cells seeded onto small islands underwent programmed cell death. Furthermore, culturing capillary endothelial cells on the tracks of ECM of varying width markedly influenced cell differentiation; cells grown on 20 μm-wide tracks underwent only a moderate degree of spreading but cooperated with each other, differentiating to form tubular (capillary-like) structures. Such cooperation and differentiation could not be observed when the cells were grown on 30 μm-wide tracks [24, 25, 34, 152]. The principles highlighted by Ingber and colleagues have spurred investigations into the effect of substrate topography on a range of cell types including neural cells of the PNS and CNS.

It has become clear that mechanical cues can assist in the guidance of growing axons. A promising aspect to patterned substrate research has been the demonstration that aligned ECM, as well as micron scale topography of fibres or grooves have profound effects on the guidance and orientation of regenerating cells and their processes [9, 39, 99, 113, 179]. This is of particular interest for the development of biomaterial-based scaffolds capable of promoting the functional repair of traumatically injured nerves of the PNS or white matter tracts of the CNS [99, 194, 195]. For example, in vitro studies have demonstrated the influence of fibre diameter on the orientation of process outgrowth. The greater surface curvature of small diameter fibres prompted an increasing tendency for neurite outgrowth to follow the longitudinal axis of the fibres; small calibre fibres (5–30 μm diameter) promoted much greater orientated process growth than large calibre fibres (up to 500 μm diameter). This phenomenon is due to the topological effect of the fibre on neurite growth [153, 154]. The orientation and microstructure of pores within sponges has also been demonstrated to exert a directional influence on the processes of neurons and glia. Sponges with diameters of approximately 50 μm are capable of promoting the orientated migration of a range of glia including Schwann cells, olfactory ensheathing cells and astrocytes. Furthermore, directional axonal growth from dorsal root ganglia (DRG) and neuronally differentiated human SH-SY5Y neuroblastoma has been demonstrated [12, 13, 111].

12.3.2.4 Nanofibres

Recent advances in nanotechnology and renewed focus on electrospinning has led to the innovation and potential advantage of engineering biomaterials as orientated fibres with sub-micron diameters, thus presenting directional axon growth-promoting cues with a significantly enhanced surface to mass ratio. Our group has demonstrated a novel method for electrospinning highly orientated fibres (up to several cm in length) that can be transferred and stabilised at either high or low density on flat surfaces, allowing the interactions between single cells and single fibres to be observed in vitro over a number of days [144]. The ability of fibres with diameters in the sub-micron range to affect the behaviour of non-neural cells [109, 159] as well as neural cells of the PNS and CNS has recently been demonstrated using in vitro assays [27, 53, 144]. Cell adhesion, survival and proliferation were well supported, but morphology and alignment were strikingly altered by orientated nanofibres. Orientated fibres promoted longer axonal extension than non-orientated fibres [27, 28], but fibre composition was also found to play a role; functionalisation of polycaprolactone (PCL) fibre surfaces with type I collagen (C/PCL) increased cell adherence, proliferation and process extension of PNS-related cells [144]; however, neural progenitor-derived astroglia showed different interactions with adhesion and process length being unaffected by collagen-I functionalisation of the fibres. Astrocytic migration was the only parameter to be enhanced by the presence of collagen on the surface of the nanofibres [53]. Thus, it is clear that nanofibres may play a significant role in future scaffold designs intended to promote CNS axon regeneration.

12.3.2.5 General Considerations for Implantable Biomaterials for CNS Applications

A number of general issues need to be borne in mind when considering the choice of biomaterial and the design of scaffolds intended to promote the

repair of the traumatically injured spinal cord, including:

1. Biocompatibility, minimising adverse tissue reactions in vivo
2. Topographic cues for orientated tissue integration
3. Cytocompatibility, promoting optimal cell adhesion, migration and axon outgrowth
4. Physical properties (elasticity, strength, deformability) that approach those of the host tissue
5. Biodegradability with the production of non-toxic by-products

Although degradability has been identified as an ideal property for a biomaterial intended for CNS repair, non-degradable materials have also been investigated. The following section describes the use of both degradable and non-degradable natural and synthetic polymers.

12.3.3 Biomaterials Developed for SCI: Natural Polymers

12.3.3.1 Collagen

Collagen is one of the major ECM proteins, accounting for up to 30% of the total protein in the human body. Collagens are among the most popular materials in bioengineering, being applicable to both soft- and hard tissue (e.g. bone) reconstruction [138]. In comparison to many synthetic polymers, biomaterials made from collagen offer several advantages, including biocompatibility, non-toxic degradation products and the induction of minimal foreign body responses [141, 142]. In vitro studies have shown that collagen promotes neural cell attachment as well as neurite outgrowth [22, 166]; it is amenable to the controlled manipulation of its shape, microstructure and stability and has already been fabricated in the form of tubular guidance channels for peripheral nervous system (PNS) repair e.g. [77] and CNS repair, e.g [100]. Bridging the transected spinal cord with collagen gels has induced vascularisation of the implant and resulted in the re-growth of small numbers of myelinated axons from the host spinal cord, even when combined with neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) [32, 71, 72, 81, 106]. However, care must be taken when using collagen in the form of a gel because, if used at too high a concentration, it may act as a physical barrier to axon regeneration [108]. Furthermore, the use of collagen alone as a matrix-filling for hollow guidance conduits that were implanted into spinal cord transection injuries was found to promote the regeneration of a relatively small number of brain stem neurons [170]. However, the use of thin sheets of polymerised collagen as supports for highly purified Schwann cells that could be rolled into orientated tube-like structures and implanted into dorsal spinal cord resection injuries has resulted in the regeneration of numerous myelinated and non-myelinated host axons [123].

Apart from the choice of material, the orientation and microstructuring of the scaffold will have an enormous influence on its suitability as a bridging device. Recently, extruded collagen microfibres (20–30 μm diameter) have been used to promote a partial functional recovery in experimental models of complete spinal cord transection injuries [195]. Instead of the surface of collagen fibres providing the orientational cue, collagen scaffolds or sponges with parallel oriented micropores have been engineered by a patented directional freezing process [145]. Video microscopy of the synchronous, orientated growth of ice-crystals used to form uniformly orientated pores within a collagen solution can be seen at the following web site: http://www.optimaix.com/html/product.html. The rate of degradation of the freeze-dried collagen-based scaffolds can be modified by chemical cross-linking, e.g. using 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) [121] and the scaffolds can be sterilised with 25 kGy gamma irradiation prior to use. Such orientated microporous collagen scaffolds have been found to be cytocompatible with a range of PNS and CNS-related cell types. Investigations of the cell-substrate interactions of neuronal and non-neuronal cell types, including dorsal root ganglion neurons, Schwann cells, fibroblasts, olfactory nerve ensheathing cells (OEC) and astrocytes, have demonstrated excellent adhesion and process extension as well as orientated migration following the longitudinal axis of the microporous channels [12, 13, 111]. Implantation of OEC seeded scaffolds into mid-cervical resection injuries of the adult rat spinal cord have resulted in substantial orientated growth by axonal and vascular elements as well as improvements in behavioural tasks [110].

Recent advances using collagen scaffolds have involved the fusion of growth factors (e.g. BDNF) to orientated collagen fibres via a linker polypeptide

(possessing a collagen binding domain). The orientated collagen fibres supported PC12 cell survival and longitudinally directed axon regeneration from DRG in vitro. Implantation of the BDNF-linked collagen fibres into mid-low thoracic spinal cord hemisections promoted host axonal regeneration and improvement of locomotor function [62].

12.3.3.2 Fibronectin

Fibronectin (FN) is an ECM glycoprotein involved in a number of cell and tissue events, including blood clotting, cell adhesion and migration [193]. Adherence and axon extension by CNS neurons over FN substrates have been shown to be dependent on the interactions with the RGD (arginine, glycine, aspartate) peptide sequences of this ECM molecule [140]. Mats of fibronectin have demonstrated excellent cytocompatibility with Schwann cells and astrocytes in vitro [128]; however, their use to promote repair after SCI has proved rather disappointing. It was reported that orientated regeneration of some populations of host axons, including calcitonin gene-related peptide (CGRP)-positive and substance P-positive sensory axons, GABAergic, cholinergic and noradrenergic axons, was supported by grafts of naïve (untreated) FN mats [87]. However, axons generally failed to re-enter the host tissues, even when combined with a range of neurotrophins, e.g. nerve growth factor (NGF), BDNF or NT-3, or with the co-application of anti-transforming growth factor-β antibodies that were intended to interfere with the scarring process [88, 89].

12.3.3.3 Fibrin

Fibrin is a fibrous protein involved in blood clotting, where it is polymerised to form a "mesh" which, in combination with the platelets, acts as a haemostatic clot at the site of blood vessel injury. Fibrin is generated by the action of thrombin on the soluble plasma glycoprotein, fibrinogen, and is cross-linked by factor XIV to form a clot. It has proved possible to manipulate the organisation of the fibrin matrix in a controlled manner by applying high-strength magnetic fields (4.7 and 9.4 T) during fibril polymerisation [39]. Fibre diameter and alignment exerted significant effects on neurite outgrowth from dorsal root ganglion explants in vitro: fibrin fibres with diameters of 460 nm provided better guidance and support for neurites than those with diameters of approximately 150 nm; furthermore, aligned fibres supported longer growth than randomly orientated fibres [39]. The inclusion of fibrin matrix into hollow synthetic conduits has been particularly successful in promoting the regeneration of axons from reticulospinal neurons with concomitant improvement of locomotor function (BBB open field rating) following implantation into complete spinal cord transections [170]. Although fibrin has been used for the controlled release of the neurotrophin NT-3 into complete spinal cord transections and has been reported to induce a qualitatively stronger regenerative response by neurofilament (NF)-positive axons at 9 days post implantation [163], the longer-term effects (8 weeks) on axonal growth were not significantly different from those induced by fibrin alone [170]. Furthermore, the ability of the NT-3 releasing fibrin matrix to support axon regeneration from brain stem neurons was (for reasons unknown) substantially inferior to that supported by fibrin alone [170]. Recently, the implantation of pre-polymerised fibrin scaffolds into T9 dorsal spinal cord hemisections has been performed at the sub-acute stage (2 weeks) after injury – reflecting a time scale that would be more realistic for the future treatment of spinal cord-injured patients [79]. Implanted scaffolds reduced GFAP-positive astrocytic scarring and supported host axon regeneration into the scaffold that was often associated with NG2-positive cellular structures (probably newly formed blood vessels), but no significant improvement of function could be detected [79].

12.3.3.4 Alginate

Alginate is a polyanionic co-polymer predominantly derived from the cell walls of brown algae (in particular, seaweed). Implantation of alginate into complete spinal cord transection injuries of young and adult rats has been shown to reduce the expression of growth-inhibitory CSPGs and to promote the regeneration of descending and ascending populations of host axons with the recovery of some degree of motor-evoked and somatosensory function [84, 160, 161]. Furthermore, anterograde tracing techniques have demonstrated regenerating axons leaving the implant to integrate

with de-afferented spinal cord tissue [161, 168]. Alginate has also been reported to act as a cell carrier capable of preventing the rejection of non-autologous fibroblasts as well as supporting the survival and eventual migration of neural progenitors into adjacent host spinal tissues [131, 188]. The incorporation of unidirectional capillaries (or pores) into alginate hydrogels has demonstrated their ability to promote orientated axonal growth in in vitro organ slice preparations as well as following implantation into defined dorsal column lesions of the adult rat spinal cord [131]. However, it was not possible to determine whether the orientated axon regeneration resulted in improved function, because behavioural studies were not performed in the latter investigation.

12.3.3.5 Agarose

Agarose is a polysaccharide extracted from seaweed that will form gels on cooling. Agarose gels modified with the peptide CDPGYIGSR resulted in improved DRG neurite growth compared to unmodified agarose [11], which were, themselves, capable of supporting some degree of DRG axon growth in vitro [196]. The immobilisation of other functional bioactive molecules (e.g. GRGD peptide sequence) into uniformly orientated 200 μm diameter channels within agarose hydrogels has been achieved using photolithographic techniques. Such modified agarose hydrogels were observed to support the attachment and growth of DRG axons which preferentially followed the trajectory of the functionalised channels in vitro [104]. As an alternative method, freeze-drying has been developed to generate agarose hydrogels with uniaxial linear pores (125 μm diameter) that could be loaded with axon growth-promoting molecules (e.g. growth factors) [156]. Implantation of BDNF-loaded (or BDNF/mesenchymal stromal cells (MSC)-loaded) scaffolds into cervical (level C3) dorsal column lesions resulted in orientated blood vessel and Schwann cell migration into the scaffold as well as directional axon regeneration. However, possible implant-mediated functional recovery has not been assessed using this biomaterial. Such agarose scaffolds elicited only minimal host inflammatory reactions and were not encapsulated by reactive tissues, however, little or no neurite growth was detected in the scaffolds [78, 157, 158].

12.3.3.6 Chitosan

Chitosan is prepared from chitin, the second most abundant polysaccharide in nature (after cellulose), and is the main component of crustacean- and insect-exoskeletons, and of cell walls of some bacteria and fungi [85, 116]. The non-toxicity, biocompatibility and biodegradability of chitosan has resulted in its use as a conduit, the lumen of which could be filled with axon growth-promoting molecules e.g. type I collagen, [96], segments of peripheral nerve [117] or neural progenitors [118, 198]). Loaded or non-loaded chitosan conduits have been implanted into spinal cord lesions and the beneficial effects of the implants compared. All the loaded conduits supported substantial host axon regeneration, whereas relatively few regenerating axons could be identified in the empty chitosan conduits. Although some improvement of motor function was reported as early as 2 weeks after implantation into partial spinal cord lesions, it is most likely that such rapid recovery be due to implant-induced tissue sparing rather than the axon regeneration that was claimed by the authors [96]. In contrast, implantation of chitosan-based conduits into complete spinal cord transections resulted in no motor improvement [117, 118].

12.3.3.7 Poly-β-hydroxybutyrate (PHB)

Poly-β-hydroxybutyrate (PHB) can be obtained from micro-organisms in which it exists as energy storage granules [38]. Fibres of PHB coated with alginate and fibronectin (with or without Schwann cells seeding) have been implanted in spinal cord lesions. Although the PHB-containing conduits integrated well with the host tissues and supported the survival of axotomised rubrospinal neurons, relatively little axonal regeneration took place [119]. More recently, ECM-coated (i.e. fibronectin, laminin and collagen type IV) PHB has been demonstrated to support Schwann cell adhesion, proliferation and survival, and the implantation of such conduits into adult rat cervical spinal cord hemisections promoted the regeneration of populations of descending and ascending axons (e.g. serotonergic and CGRP-positive sensory axons). However, no functional analyses have been performed with such implants [120].

12.3.3.8 Hyaluronic Acid

Hyaluronic acid (HA) is a naturally occurring polysaccharide and has an important role in peripheral nerve repair [177]. Hyaluronan-based conduits have been reported to be biodegradable, non-cytotoxic, and biocompatible [2]. When used in combination with low-power laser radiation treatment, implanted HA maintained the survival of donor embryonic spinal cord tissue, induced host axon regeneration and a moderate recovery of somatosensory evoked potentials following complete spinal cord transection injuries [139]. Similarly, a HA-poly-D-lysine co-polymer hydrogel has been reported to support neural cell growth and differentiation and integrate well with host astroglia after implantation into CNS lesions [167]. More recently, a hyaluronan-methylcellulose (HAMC) composite gel has been developed as a biodegradable, non cell-adhesive hydrogel that can be injected locally to CNS injuries for the controlled release of bioactive molecules [60]. Injection of HAMC into the intrathecal space of normal and compression-injured adult rat spinal cords had no detrimental effects in normal tissues and even caused slight improvements to injured animals, probably due to the anti-oxidant and anti-inflammatory effects of HAMC. Accordingly, lesion volumes were smaller, fewer inflammatory cells were found at the injury site, and locomotion and coordination showed improved trends after HAMC injection [60]. The efficacy of intrathecal injection of HAMC as a delivery mechanism for the neuroprotective agent, erythropoietin (EPO), was compared with direct intrathecal bolus injection- and intraperitoneal injection of EPO [83]. The HAMC hydrogel route of EPO delivery provided the greatest neuroprotective benefit to rats receiving a mild–moderate compression injury, raising the possibility that such a system might form the basis for the efficacious local delivery of neuroprotective agents to patients suffering SCI.

12.3.4 Synthetic Polymers

Biomaterials made of synthetic polymers have some advantages over natural polymers, in particular, of reduced impurities, pathogens or contaminant and lower batch-to-batch variability, as well as consistent and reproducible mechanical and physical properties. Some of the synthetic polymers that have been applied to SCI research are presented below.

12.3.4.1 Poly(-Hydroxy Acids)

Poly(-hydroxy acids) such as poly-glycolic acid (PGA), poly-L-lactic acid (PLLA), poly-D-lactic acid (PDLA) and PCL are biocompatible polymers that are degraded by hydrolysis and have mechanical characteristics that can be readily controlled. Poly(-hydroxy acids) are already widely used in the medical industry (e.g. for sutures). Guidance conduits made from a range of poly(-hydroxy acids) have proved useful in promoting axon regeneration and functional recovery of the PNS [30]. Their usefulness in promoting CNS axon regeneration after SCI has also been demonstrated and the composition of the conduits have been modified to improve their properties. For example, Schwann cell-containing PDLA conduits implanted into complete rat spinal cord transection injuries underwent rapid degredative hydrolysis and collapsed or fragmented over a number of months, the ensuing change in conduit geometry having detrimental effects of axon regeneration and tissue repair [122]. To modify the degradation rate, other polymers including PCL, poly(D,L-lactic acid-co-glycolic acid) (PLGA), or a mixture of the two have been assessed. Neural stem cell-seeded PLGA conduits have been reported to promote tissue repair and functional recovery after implantation into rat spinal cord hemisection injuries [164]. Attempts to improve conduit microstructure have included co-polymers of poly(ethylene oxide)-block poly(D,L-lactic acid) (PELA-PDLLA) which were moulded into rods and bound in groups using acidic fibroblast growth factor (aFGF) containing fibrin glue. The conduit (with or without BDNF supplement) promoted directional host blood vessel, Schwann cell and axon regeneration following implantation into complete spinal cord transection injuries, but no functional improvement was reported [105, 126].

A two-component polymer scaffold composed of PLGA and a block co-polymer of PLGA-poly-lysine has been engineered such that the porous inner portion emulated spinal cord grey matter and its longitudinally orientated porous outer portion emulated the spinal cord white matter. The inner layer of the scaffold was seeded with neural precursor cells prior to implantation into hemisection injuries of the rat spinal cord.

Interestingly, the implant reduced astroglial scar formation and promoted substantial host axon regeneration (including descending corticospinal axons) that was associated with a long-term functional improvement [164]. Others have generated parallel channels within PLGA of approximately 500 μm, which supported Schwann cell and mesenchymal stromal cell attachment and proliferation in vitro, and induced only minor macrophage responses at the implant-host interfaces and no glial responses in the adjacent spinal cord tissue, demonstrating the biocompatibility of such biomaterials [63]. Such scaffolds have also been developed for combined tissue engineering and gene therapy applications. The non-viral local delivery of DNA to lesioned host tissues has been achieved using lipoplexes bound to fibronectin-coated PLGA implants. The walls between the channels served as a reservoir for the material (DNA) being delivered. It was reported that local transgene expression of a marker protein was detectable for up to 3 weeks following implantation, suggesting that such combined tissue engineering/gene therapy strategies may be of use in the treatment of SCI [33].

12.3.4.2 Poly-(Acrylonitrile-Co-Vinylchloride)

Poly-(acrylonitrile-co-vinylchloride) (PAN/PVC) is a structurally stable, biocompatible and non-toxic copolymer [150], which has been used to form conduits for the repair of SCI. Empty (or Matrigel™ containing) PAN/PVC conduits provided little regenerative support, e.g. [19, 191]; however, conduits loaded with axon growth-promoting peripheral glia (e.g. Schwann cells or olfactory ensheathing glia) and different combinations of growth factors (e.g. BDNF, NT-3 or glia-derived nerve growth factor (GDNF)) have induced significant axon regeneration and remyelination [4, 46, 76, 80, 134, 191, 192]. Increasing the complexity of the combinatorial approach to repair SCI has included the implantation of Schwann cell-seeded PAN/PVC conduits into complete spinal cord transection injuries, accompanied by OEC implantation into the rostral and caudal spinal cord stumps and the infusion of the enzyme, chondroitinase ABC, at the implant-host tissue interface where it is capable of digesting and reducing the local axon growth-inhibitory effects of CSPG [46, 48]. Such strategies have promoted axon regeneration from spinal cord PSN as well as from populations of brain stem neurons (including serotonergic fibres from the Raphe nucleus, vestibulospinal and reticulospinal neurons), all of which play a role in stepping and locomotion [46, 173]. Interestingly, this combined strategy has also improved the bladder function of spinal cord-injured rats [47].

12.3.4.3 Poly(2-Hydroxyethyl Methacrylate)

Poly(2-hydroxyethyl methacrylate) (PHEMA) is a non-biodegradable, biocompatible and non-toxic polymer. PHEMA-based conduits have a similar elastic modulus to spinal cord tissues and provide long-term stability for tissue regeneration [29, 45, 73]. The implantation of PHEMA scaffolds into the lesion cavity of rat spinal cord contusion injuries promoted an enhanced degree of host axon regeneration [54]. Interestingly, the implantation of empty *poly (2-hydroxyethyl methacrylate-co-methyl methacrylate) (PHEMA/PHEMA-MMA)* conduits into complete spinal cord transection injuries was reported to support significant host axon regeneration from reticulospinal, vestibulospinal and Raphe nuclei in the brain stem, some of which traversed the lesion (at T8) and were reported to extend as far as T13/L1. Although other biomaterial-implantation studies had reported axon regeneration into biomaterial-based scaffolds, this study was significant in that it was the first to demonstrate such extensive axonal growth into unfilled synthetic hydrogel conduits [169]. However, partial collapse of the conduits was observed by 2 months after implantation, prompting modifications involving coil-reinforcement. Attempts to enhance the extent of axon regeneration by the inclusion of different combinations of growth factors (e.g. aFGF) and ECM components (e.g. fibrin) proved unsuccessful, because animals developed signs of syringomyelia, suggesting that conduit design required further development [115]. Attempts to systematically assess the regenerative potential of adding ECM (fibrin, collagen, Matrigel™ or methylcellulose) and/or growth factors (aFGF or NT-3) to the original simple design of the PHEMA/PHEMA-MMA conduits indicated that the inclusion of fibrin into the conduit enhanced the number of brain stem (reticulospinal) neurons growing into and beyond the lesion. However, inclusion of aFGF with fibrin had no apparent effect on the number of regenerating neurons while inclusion of NT-3, in contrast, caused a

precipitous drop in the number of regenerating brain stem neurons in comparison to the unfilled, control channels. The inclusion of collagen into the PHEMA/PHEMA-MMA conduit induced minor increases in the number of brain stem (reticulospinal and vestibulospinal) neurons above that seen in empty conduits; however, inclusion of aFGF with the collagen markedly increased the number of regenerating neurons from both brain stem nuclei, while inclusion of NT-3 (similar to fibrin plus NT-3) caused a massive decrease in numbers. The reason for the detrimental effects of NT-3 remain unclear [170].

Manipulation of the electric charge carried by the HEMA hydrogel by polymerisation with positively or negatively charged co-polymers demonstrated that positively charged co-polymers imparted a beneficial effect by improving cell adhesion (as tested with bone marrow stromal cells)[94, 130]. Furthermore, implantation of positively charged hydrogel was recently reported to promote greater ECM deposition and axonal growth than uncharged hydrogels following implantation into experimental spinal cord lesions [66]. Although in vitro studies demonstrated that the deposition of collagen and laminin onto the surface of the hydrogels increased the adhesion and growth of mesenchymal stromal cells and astrocytes [18], in vivo implantation studies demonstrated little or no host astrocytic growth into the hydrogels [66]. The delayed implantation (7 days after complete spinal cord transection) of the positively charged hydrogels was also found to induce greater ECM deposition, in-growth of blood vessels, Schwann cells and axons, as well as reducing cystic cavitation in comparison to the acute implantation of such hydrogels, or to the non-implanted controls [65].

12.3.4.4 Polyethylene Glycol

Polyethylene glycol (PEG) is a hydrophilic polymer that has low protein and cell adhesion properties. Application of PEG solution in a number of experimental models of SCI has resulted in the re-establishment of anatomical continuity, reduced lesion volume and improved functional recovery [10, 92, 147–149]. It has been suggested that the neuroprotective properties of PEG may be due to its ability to re-seal damaged cell plasma membranes, anti-oxidative and anti-apoptotic effects following SCI [102, 103]. PEG has also been used to modify surfaces, making them non-adhesive to cells and protein for a number of weeks, and have thus been used in a number of cell-substrate patterning experiments [53, 90, 144]. Biodegradable PEG-based hydrogels containing poly(lactic acid)-b-poly(ethylene glycol)-b-(poly lactic acid) (PLA-b-PEG-b-PLA) and the growth factor NT-3 have been developed in which gelation was induced via blue light photopolymerization. Such PEG-based gels implanted into spinal cord hemisection injuries promoted axon regeneration and some degree of functional recovery [129]. PEG has also been used as a formulation for the targeted delivery of the neuroprotective agent, magnesium sulphate, to both compression-type SCI [35] and more recently to contusion-type SCI [91]. Optimising the administration of $MgSO_4$/PEG with a dosing time window starting up to 2 h after injury followed by up to 6 separate infusions provided the optimal neuroprotection with the lesion volume reduced by approximately 50% in comparison to saline-injected control animals [91].

12.3.4.5 Poly[N-(2-Hydroxypropyl) Methacrylamide

Poly[N-(2-hydroxypropyl)methacrylamide] (PHPMA or Neurogel™) is a synthetic polymer that forms a hydrogel that has proven useful for spinal cord repair as well as PHEMA [181]. Implantation of NeuroGel™ (with or without growth factors) into the transected cat spinal cord promoted neo-vascularisation, infiltration by glial cells and axonal regeneration and myelination of the descending and ascending populations of axons. The axon growth traversed the implant-host interfaces which demonstrated reduced astroglial scarring. The tissue repair supported by such implants was associated with some degree of functional improvement [101, 184–186]. Furthermore, implantation into chronic rat SCI (3 months after the initial insult) also reduced lesion cavity size, promoted host blood vessel and Schwann cell migration and axon regeneration, and improved functional recovery [183].

12.3.4.6 Self-Assembling Nanofibre Scaffolds

A recent development in the field of biomaterials is the molecular self-assembly of structures for use in biomedicine, in particular for the repair of the injured CNS. The capacity of nanofibre scaffolds to self-assemble

into a framework capable of supporting functional tissue repair following implantation has been investigated following experimental brain- and SCI.

The constituents of these biomaterial scaffolds are self-complementary amphiphilic oligopeptides that have regular repeating units of positively charged residues (lysine or arginine) and negatively charged residues (aspartate or glutamate) separated by hydrophobic residues (alanine or leucine). The assembly of ionic self-complementary synthetic peptides from the alternating positive and negative L-amino acids is initiated by the presence of physiologically relevant concentrations of salt solutions, as in the extracellular or cerebrospinal fluid [69].

Three-dimensional, self-assembling peptide nanofibre scaffolds have already proven successful in reducing local astrocytic scarring and inflammation, as well as effectively "knitting" the two edges of a lesion together following relatively thin, penetrating injuries of the CNS. When introduced into living animals, these self-assembling scaffolds did not induce a measurable immune or inflammatory response by the host tissues [68] and generated an axon growth-permissive environment that was capable of supporting some degree of functional tissue repair following lesions of the optic tract or cerebral cortex [40, 59]. Furthermore, self-assembling nanofibre scaffolds have been demonstrated to reduce apoptosis and astrocytosis, as well as supporting axon regeneration and a moderate degree of functional recovery in models of SCI [58, 172]. It is possible that the future use of orientated nanofibres may be able to improve the efficacy of the axon regeneration across large lesions. The design of such self-assembling nanofibre scaffolds presents a number of characteristics that are advantageous for CNS repair:

1. The peptides can be injected into- and fill the cavitating, irregular-shaped lesion sites that typically form following SCI.
2. This approach causes minimal damage to the surrounding rim of intact spinal cord tissue that is usually present in most contusion or compression-type cases of SCI.
3. The nanofibre scaffold forms a hydrogel with physical properties that are unlikely to provoke any additional damage to the host tissues.
4. The scaffold is biodegradable, breaking down to natural amino acids.
5. The scaffold is also immunologically inert, avoiding potentially damaging issues of enhanced inflammation and rejection. These characteristics make the self-assembling nanofibre scaffolds very promising biomaterials for bridging SCI.

12.4 Clinical Application

The application of biomaterials to promote functional axon regeneration in clinical cases of SCI still remains a goal for the future development of the discipline. Current clinical tests for biomaterials related to SCI tend to be limited to the use of materials or devices intended for spinal column stabilisation [86]. The discipline is not at a point where a material has been tested and its interactions with the lesioned host tissues sufficiently understood and controlled to be able to contemplate the clinical trials. Furthermore, any beneficial effect brought about by a particular biomaterial in small laboratory animals (with well-defined and highly reproducible spinal cord lesions) does not guarantee that a similar effect would be seen in humans. An unavoidable concern in the issue of translating advances in "bridging" spinal cord lesions in the laboratory to similar applications in the clinic is that of size. Most experimental spinal cord lesions used for bridging experiments induce gaps of several mm in length. Severe SCI in humans could, however, result in lesions of up to several cm in length. It would be a major advance in the field if regenerating axons were able to grow such distances and reach the distal, partially de-afferented spinal cord tissue. Currently, the implantation of relatively simple "first generation" bioengineered scaffolds has resulted in the support to some degree, albeit limited, of functional repair in experimental animals. Any functional improvement that can be promoted in patients with SCI would be a welcome development. Even small improvements, such as lowering the apparent segmental level of the functional deficit by a single spinal cord level could lead to the acquisition of the control of finger movements in patients with cervical injuries, potentially bringing about a substantial increase in the quality of life.

An issue of considerable clinical importance is that most compression- and contusion-type spinal cord injuries are followed by some (limited) degree of recovery over the first months post-injury. As mentioned earlier,

much of this recovery of function is due to the conduction of impulses along spared axons that occupy the outermost rim of the white matter. The preservation of these axons and the modalities that they serve is of utmost importance to the maintenance of residual function in the SCI patient. Any intervention strategy (including the implantation of biomaterials) should aim to cause minimal disruption or damage to the survival and function of these spared axons. It is therefore likely that certain biomaterial designs will be of particular interest in the repair of different types of spinal cord injuries; for example, the minimally traumatic approach of injecting hydrogels may be more appropriate for contusion- or compression-type injuries, while the implantation of pre-formed, orientated, scaffolds may be more appropriate in resection or laceration-type injuries.

12.5 Expert Opinion

Investigations into the use of biomaterials for the repair of the lesioned PNS have the advantage of the widely accepted "gold standard" autograft for the repair of peripheral nerves. Numerous clinical studies have demonstrated that the best degree of functional peripheral nerve axon regeneration is promoted by similarly sized segments of "donor" peripheral nerve, harvested from the patient themselves e.g. [8]. This has provided the investigators with a benchmark, facilitating the development of implantable biomaterials that could mimic the properties of the autograft. The performance of the bioengineered scaffolds could be compared with that of the autograft. No such gold standard exists for SCI research, resulting in the need to assess the regenerative properties of a wide range of natural and synthetic polymers. As has already been seen with "first generation" biomaterials, future generations of biomaterials will probably result in similar degrees of functional improvement. However, it will be difficult to draw any detailed conclusions about the relative merits of the different materials because of the disparate investigative techniques employed by different researchers. Only direct comparisons of different materials within the same group or alternatively, two research groups adopting identical investigative procedures would provide accurate comparative data. Such approaches for the identification of the most promising biomaterial for further development have, so far, only rarely been adopted.

Although current in vivo investigations often assess the consequences of biomaterial implantation on motor (and sometimes sensory) function, relatively few groups include studies into the development of pathological pain e.g. allodynia. It is possible that implanted biomaterials may modify the connectivity or function of sensory neurons. Future investigations of potentially interesting biomaterials would clearly benefit from the inclusion of studies aimed at addressing this issue.

12.6 Five-Year Perspective

The striking reparative effects of injecting self-assembling nanofibre hydrogels into experimental CNS lesions suggests that modification of the composition of such gels will lead to even more interesting observations. Future investigations which promote the effective tailoring of functional peptide sequences to control specific cell-substrate interactions will represent a major step forward in the discipline. However, it is unlikely that such modifications will be restricted to self-assembling nanofibres; the coupling of a range of functional molecules (e.g. growth factors, functional peptide sequences from cell adhesion molecules or ECM, or enzymes capable of degrading the inhibitory influences in and around the lesion site) to both natural and synthetic polymer scaffolds will provide valuable information about the implant-mediated consequences of modifying the environment of the lesion site.

It is encouraging to see that the in vivo assessment of biomaterials (alone or as part of a combination strategy) is being conducted more frequently following delayed implantation. The identification of the effective window of opportunity for any particular intervention strategy represents the adoption of an experimental approach that more realistically reflects the likely delays that would take place before any SCI patient would be in a position to be treated with biomaterials. It is anticipated that the next few years will bring even more systematic studies into the effects of both acute and delayed implantation of biomaterials with interest focussing on, as yet, poorly understood host responses including the effects on the inflammatory response and neo-vascularisation. Although the beneficial effects of biomaterials should ideally be determined in small

rodents, larger animals and eventually non-human primates before entering clinical trials, it would not be surprising to witness the initiation of limited clinical safety trials for promising strategies involving hydrogels within the next 5 years.

12.7 Limitations/Critical View

As indicated earlier, SCI affects both the ascending and descending populations of axons. These nerve fibre tracts within the spinal cord have their own particular stereotypical distribution within the white matter and their own growth requirements, being preferentially responsive to certain growth factors, cell adhesion molecules and ECM molecules. Optimal tissue repair would ideally involve the successful regeneration of a number of these different nerve fibre populations, requiring that the biomaterial (or scaffold) be able to present compartmentalised growth-promoting substrates (each of which being specifically tailored to a particular population of axons). The design of "intelligent" biomaterials that are capable of the controlled release of growth factors (or other axon growth modulating molecules) requires a clear definition of the timing of such a release. Bearing this in mind, the current state of the art for the design of biomaterials with axon growth-promoting properties is still rather crude. However, this will no doubt change in the near future. The ultimate goal of promoting some degree of functional recovery by axon regeneration remains a medium- to-long-term goal for research in the field of neurotraumatology; however, improved function by enhancing tissue sparing and compensatory plasticity is more likely to be achieved in the short- to-medium term.

12.8 Conclusion/Summary

The development of effective bioengineering strategies for the repair of SCI has presented a number of complex challenges. Nonetheless, it has become clear that numerous synthetic and natural biomaterials are capable of integrating with the lesioned host spinal cord and promoting some degree of axon regeneration. However, the properties of implantable biomaterials that influence the biomaterials' ability to interact with the lesioned host tissues in a controlled and specific manner are, at this time, rather limited. The future development of implantable devices that will be able to target and modify a number of the key pathophysiological events that arise after SCI will require increasingly intense collaborations between clinicians and scientists from a wide range of disciplines. Although no single biomaterial has yet been identified as the ideal substrate for prompting functional axonal regeneration, the application of self-assembling nanofibres has presented itself as a particularly interesting and versatile development. The field of biomaterials research for CNS applications has clearly grown beyond its infancy and is developing into an area of multi-disciplinary research. This is likely, in the future, to deliver real benefits for regenerative medicine.

Suggested Readings with Abstracts

Dike LE, Chen CS, Mrksich M, Tien J, Whitesides GM, Ingber DE. Geometric control of switching between growth, apoptosis, and differentiation during angiogenesis using micropatterned substrates. In Vitro Cell Dev Biol Anim. 1999; 35:441–48.

Past studies using micropatterned substrates coated with adhesive islands of ECM revealed that capillary endothelial cells can be geometrically switched between growth and apoptosis. Endothelial cells cultured on single islands larger than 1,500 μm^2 spread and progressed through the cell cycle, whereas cells restricted to areas less than 500 μm^2 failed to extend and underwent apoptosis. The present study addressed whether island geometries that constrained cell spreading to intermediate degrees, neither supporting cell growth nor inducing apoptosis, cause cells to differentiate. Endothelial cells cultured on substrates micropatterned with 10-μm-wide lines of fibronectin formed extensive cell–cell contacts and spread to approximately 1,000 μm^2. Within 72 h, cells shut off both growth and apoptosis programmes and underwent differentiation, resulting in the formation of capillary tube-like structures containing a central lumen. Accumulation of ECM tendrils containing fibronectin and laminin beneath cells and the reorganisation of the platelet endothelial cell adhesion molecule-positive cell–cell junctions along the lengths of the tubes preceded the formation of these structures.

Cells cultured on wider (30 μm) lines also formed cell–cell contacts and aligned their actin cytoskeleton, but these cells spread to larger areas (2,200 μm^2), proliferated, and did not form tubes. The use of micropatterned substrates revealed that altering the geometry of cell spreading can switch endothelial cells among the three major genetic programmes that govern angiogenesis-growth, apoptosis and differentiation. The system presented here provides a well-defined adhesive environment to further investigate the steps involved in angiogenesis.

Xu XM, Guenard V, Kleitman N, Aebischer P, Bunge MB. A combination of BDNF and NT-3 promotes supraspinal axonal regeneration into Schwann cell grafts in adult rat thoracic spinal cord. Exp Neurol. 1995;134:261–72.

We previously demonstrated that Schwann cells (SCs) in semipermeable guidance channels promote axonal regeneration in adult rat spinal cord transected at the mid-thoracic level. Propriospinal but not supraspinal axons grew into these channels. Here, we tested the ability of the exogenous brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) to promote axonal regeneration in this novel model. The two neurotrophins were delivered simultaneously into the channel by an Alzet minipump at a rate of 12 μg/day for each neurotrophin for 14 of 30 days tested; phosphate-buffered saline, the vehicle solution, was used as a control. Significantly, more myelinated nerve fibres were present in SC/neurotrophin grafts than in SC/vehicle grafts (1523 ± 292 vs. 882 ± 287). In the graft, at least 5 mm from the rostral cord-graft interface, some nerve fibres were immunoreactive for serotonin, a neurotransmitter specific to raphe-derived axons in rat spinal cord. Fast blue retrograde tracing from SC/neurotrophin grafts revealed labelled neurons in ten nuclei of the brain stem, 67% of these being in the lateral and spinal vestibular nuclei. The mean number of labelled brain stem neurons in the SC/neurotrophin group (92; $n=3$) contrasted with the mean in the SC/vehicle group (6; $n=4$). Our results clearly demonstrate that BDNF and NT-3 infusion enhanced propriospinal axonal regeneration and, more significantly, promoted the axonal regeneration of specific distant populations of brain stem neurons into grafts at the mid-thoracic level in adult rat spinal cord.

Teng YD, Lavik EB, Qu X, Park KI, Ourednik J, Zurakowski D, Langer R, Snyder EY. Functional recovery following traumatic spinal cord injury mediated by a unique polymer scaffold seeded with neural stem cells. PNAS. 2002;99:3024–29.

For better direct repair following spinal cord injury (SCI), we designed an implant modelled after the intact spinal cord consisting of a multi-component polymer scaffold seeded with neural stem cells. Implantation of the scaffold-neural stem cells unit into an adult rat hemisection model of SCI promoted long-term improvement in function (persistent for 1 year in some animals) relative to a lesion-control group. At 70 days post-injury, animals implanted with scaffold-plus-cells exhibited coordinated, weight-bearing hindlimb stepping. Histology and immunocytochemical analysis suggested that this recovery might be attributable partly to a reduction in tissue loss from secondary injury processes as well as in diminished glial scarring. Tract tracing demonstrated corticospinal tract fibres passing through the injury epicentre to the caudal cord, a phenomenon not present in untreated groups. Together with the evidence of enhanced local GAP-43 expression not seen in controls, these findings suggest a possible regeneration component. These results may suggest a new approach to SCI and, more broadly, may serve as a prototype for multi-disciplinary strategies against complex neurological problems.

Tysseling-Mattiace VM, Sahni V, Niece KL, Birch D, Czeisler C, Fehlings MG, Stupp SI, Kessler JA. Self-assembling nanofibres inhibit glial scar formation and promote axon elongation after spinal cord injury. J Neurosci. 2008;28: 3814–23.

Peptide amphiphile (PA) molecules that self-assemble in vivo into supramolecular nanofibres were used as a therapy in a mouse model of spinal cord injury (SCI). Because self-assembly of these molecules is triggered by the ionic strength of the in vivo environment, nanoscale structures can be created within the extracellular spaces of the spinal cord by simply injecting a liquid. The molecules are designed to form cylindrical nanofibres that display to the cells in the spinal cord the laminin epitope IKVAV at nearly van der Waals density. IKVAV PA nanofibres are known to inhibit glial differentiation of cultured neural stem cells and to promote neurite outgrowth from cultured neurons. In this work, in vivo treatment with the PA after SCI reduced astrogliosis, reduced cell death, and increased the number of oligodendroglia at the site of injury. Furthermore, the nanofibres promoted the regeneration of both the descending motor fibres and ascending sensory fibres through the lesion site. Treatment with the PA also resulted in significant behavioural improvement. These observations demonstrate that it is possible to inhibit glial scar

formation and to facilitate regeneration after SCI using bioactive three-dimensional nanostructures displaying high densities of neuroactive epitopes on their surfaces.

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Tissue Engineering for Peripheral Nerve Regeneration

13

Andrew Hart, Giorgio Terenghi, and Mikael Wiberg

13.1 Introduction

The neurobiological foundations of peripheral nerve injury and regeneration were established a century ago [23, 170, 171], although the importance of the Schwann cell [23, 66, 170, 171], and the molecules and structures responsible for trophism and tropism [124] has only been elucidated more recently [119, 204]. It is now over 60 years since clinical classifications of nerve injury, and the foundations of peripheral nerve reconstruction were established [44, 181–183, 197, 198]. Accurate approximation of nerve ends, the elimination of tension at the repair, and the use of magnification during microsurgical suture repair remain the keystones of nerve surgery. Since tension impairs regeneration, the management of segmental defects requires the use of nerve autograft [142], with associated donor morbidity and shortage of supply for large injuries such as to the brachial plexus [142, 143, 217]. There is no evidence that autografts function better when applied as vascularised flaps rather than simple grafts, except in very select circumstances (for example, heavily irradiated wound beds), since they neovascularise efficiently [73, 120, 142].

Nerve injury results in neuronal death [70], and although nerve repair leads to some regeneration, only 50–60% of neurons will cross a repair (at most) [218]. Clinical outcomes remain poor if results are critically assessed in comparison to other forms of reconstructive surgery, and normal function cannot be recovered after even minor injuries (for example, injury to the digital nerve in the hand) [119]. After injury to the major nerve trunks in the forearm, fewer than 60% of patients return to work [18], and outcomes are dramatically worse in more severe or proximal injuries such as those to the brachial plexus [32, 94, 102].

It has become clear that despite the technical developments in and exactitude of microsurgical repair techniques, surgical manipulation of the nerve stumps cannot adequately address many of the neurobiological hurdles to peripheral nerve regeneration, and the restoration of function. Nerve surgeons have therefore turned to neurobiologists to define the cellular events [66, 119]. With the exception of neuroprotection [70], tissue engineering has emerged as the discipline most likely to deliver solutions to the modulation of these peripheral events, and the neurobiological requirements have been established [51, 201].

This review will delineate the relevant features of the peripheral nervous system and its response to injury and repair that provide the framework for the subsequent review of tissue-engineered solutions.

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13.1.1 Neurobiology of Peripheral Nerve Injury

13.1.1.1 Peripheral Nerve Anatomy

Peripheral nerves are macroscopic structures that carry axons from the cell bodies located in the spinal cord (lower motor neurons) or dorsal root ganglia (first order sensory neurons) to the target organs (sensory receptor, or motor endplate) in the periphery. Autonomic axons also run with the nerves. The nerves have a strong outer epineural sheath of collagen fibres and flattened epithelium, and contain multiple intercommunicating axon bundles enclosed within a perineurium. These "fascicles" lie within a matrix of glycosaminoglycans, collagen, fibroblasts, cells of the non-specific immune system, and vasculature [44, 86, 122, 199]. The fascicles contain hundreds to thousands of axons, each with its supporting glia ("Schwann cells"), contained within a dedicated endoneurial tube of reticular connective tissue [203]. Each nerve receives vascular pedicles that enter at variable intervals along its length and feed longitudinal vessels within the perineurium that supply intricate plexi around fascicles and endoneurial tubes [122].

Schwann cells are peripherally migrated neural crest cells that provide trophic and metabolic support to the axon, along with electrical insulation for the myelinated fibres [203] to facilitate rapid saltatory conduction of action potentials. The proportion of sensory and motor axons varies according to the anatomical target of the nerve. Sensory neurons, in particular, show subdivisions of sensory function and of neurotrophic factor dependency and receptor profiles [121, 201].

An essential anatomical feature is that each neuron has but one axon, whose course may traverse much of the intercommunication between nerve plexus elements proximally, and between fascicles within its named nerve distally (the fascicle pattern seldom remains constant for more than a few millimetres proximally, and a few centimetres distally within the nerve [86, 199]). That course is the result of the axon lying within a unique endoneurial tube, with the result that after injury and repair, the regenerating nerve will be directed to its final destination on the basis of which endoneurial tube it re-enters at the repair site. The potential for misdirection is vast, both in terms of inappropriate anatomical territory ("topographical specificity"), and the type of target organ (sensory/motor: "type specificity").

13.1.1.2 Injury Events: Inflammatory, Anterograde, Retrograde (including death), Phenotypic Change

Nerve transection is accompanied by haemorrhage, retraction of the nerve stumps due to intrinsic elasticity, and mushrooming of contents from the cut ends. An inflammatory reaction occurs causing fibrin deposition, scarring, and the release of neurotoxic cytokines. The axon retracts proximally, to the next node of Ranvier in myelinated nerves [120, 201, 204], as a part of the process of somatofugal axonal atrophy [55] and chromatolysis within the perikaryon [55, 120, 191, 201, 203, 219]. Disruption of the axonal continuity to the target organ results in loss of neurotrophic support. This induces a phenotypic change within the injured neurons, triggering cell death and regenerative pathways that ultimately lead to the death of 30–70% of sensory neurons, and 0–80% of motor neurons depending upon the injury specifics [70]. The surviving neurons assume a regenerative phenotype and try to re-establish the innervation of target organs.

Distal to the injury events also commence to prepare the way for axonal regeneration. Axons lose nutritive support from their cell body, so detach from their myelin sheath and are phagocytosed by macrophages and activated Schwann cells [59, 120, 144, 172, 201], in the process of Wallerian degeneration [66, 103, 120, 201]. Denervated Schwann cells in the distal stump shrink away from the endoneurium to form solid columns, the "Bands of Büngner" [66, 144, 202], while those at the injury site migrate out towards other neural tissues and try and bridge any defects in nerve continuity [120]. Cells in the nerve trunk, most notably Schwann cells, initially proliferate and up-regulate nerve growth factor (NGF), leukaemia inhibitory factor (LIF), glial-derived neurotrophic factor (GDNF), growth-associated protein (GAP-43), and glial growth factor (GGF) receptor (p185erbB2/neu) mRNA [37, 62, 66, 67, 172, 184, 185, 195], and increase NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-4 (NT4), ciliary neurotrophic factor (CNTF) and LIF secretion [38, 39, 59, 120, 172, 184, 185, 201]. Damaged cells are an important source of CNTF [172, 185]. Schwann cells are essential for the guidance and support of regenerating axons [49, 51, 62, 65, 66, 172] and GGF [110] is the principal neuregulin controlling that interaction [51, 62, 66, 172, 174]. Under the influence of macrophage-derived interleukin-1 (IL-1) [59, 184, 201]

Schwann cells proliferate, and up-regulate the expression of c-erbB2&4 (parts of the neuregulin receptor complex) [114].

Target organs atrophy during the period of denervation, but the sensory organs fare somewhat better than muscle. In the skin, Merkel's discs disappear by 35 days in cat [19], Meissner's corpuscles persist for 6 months before degenerating [43], and although Pacinian corpuscles atrophy, they survive for at least 10 months in rat [229]. In clinical practice, it is generally held that if muscle cannot be reinnervated within ~18 months, it is unlikely to recover significant function, although exceptions are reported.

Thus in the initial period after injury, the surviving axotomised neurons spontaneously try to regenerate, while the denervated distal nerve stump becomes a highly receptive environment for the ingrowth of regenerating axons, and produces tropic and trophic cues to direct them towards it. If the process of surgical repair needs to merely approximate one to the other then the task would be slight, but unfortunately enormous complexities arise because of the impact of axonal misdirection, issues of specificity, the slow rate of regeneration and deleterious time-related changes, and the effect of segmental defects.

13.1.2 Neurobiology of Nerve Regeneration After Repair

If nerve continuity is lost and early repair not performed, then, in the distal stump, conditions for regeneration begin to deteriorate. If denervation is prolonged, then Schwann cells become unresponsive to GGF after 2–6 months [114, 144, 202], although they remain poorly able to myelinate axons [164]. After 10 weeks Schwann cells begin to atrophy, after 20 weeks to apoptose, and by 50 weeks there is severe Schwann cell atrophy [47, 66, 144, 179, 202]. The endoneurial fibrosis that results from denervation and Schwann cell atrophy begins within 28–35 days after transection [203], reducing their calibre [144, 179] to only 1% after 2 years in man [203], and impeding regeneration. Mean fascicular area falls by 39% after 3 months, 64% after 6 months, and 79% after 12 months, with comparable reductions in endoneurial tube diameter [144, 179]. The regenerative capacity of the neurons also deteriorates with time [50, 51, 114]. Complete loss of regenerative capacity through denatured muscle grafts occurs after more than 56 days [53], and axonal ingrowth into the distal stump is reduced after repair onto the native [114], or freshly divided distal stump [52]. If repair is delayed by 2 months or more, then the amount of axonal ingrowth into the distal stump is half that found after an immediate repair [114]. Prolonged axotomy therefore impairs the distal nerve stump's capacity to promote nerve regeneration after any subsequent repair due to endoneurial fibrosis, atrophy and loss of GGF responsiveness of the Schwann cell population [108]. This also correlates with the events after even the primary repair of the proximal injuries in long nerves, because the distal segment is not reinnervated for many months [51].

If the stumps of a transected nerve are brought into apposition by repair, then the Schwann cells, fibroblasts (mainly epineural derived), and capillaries migrate ahead of any axonal outgrowth from the proximal nerve stump to bridge the "interstump gap" [120], which initially fills with fibrinous clot. Schwann cells can only migrate several millimetres ahead of the neurites, and so growth comes preferentially from the proximal nerve stump if a gap persists. Axons begin to sprout [51] after a delay of around 3–14 days [12, 203]. Nerve fibres grow by sprouting ~50–100 neurites that advance through the repair site only to be pruned down to around 5 neurites per parent axon when the endoneurial tubes of the distal stump are reached [51] and may follow "pioneer axons". This is enhanced by the adoption of the regenerative phenotype [51, 120], partly in response to "injury factors", such as LIF [39, 152], and is facilitated by tropic and trophic influences [51, 124] that cause preferential neurite growth into the distal stump over the neighbouring tissues. These include contact guidance from neurite outgrowth promoting factors and cellular adhesion molecules [124, 211, 213], particularly those found upon Schwann cells, and concentration gradients of diffusible molecules including NGF, LIF, and GDNF [56, 189]. Schwann cells are an absolute requirement for nerve regeneration, providing neurotrophic and tropic guidance, and cellular adhesion molecules [61, 62, 65, 66, 119], and may determine the preferential motor reinnervation in the selection of endoneurial tubes by regenerating axons by the expression of moieties such as L2 carbohydrate [16, 65, 66, 82, 120]. Maki et al. have presented contrary evidence that promotes preferential sensory reinnervation as the selective process [131, 132].

Axons regenerate at around 0.25 mm/day across the repair site. Speculative growth is subject to competitive pruning that results in a single axon regenerating down a single endoneurial tube in the distal nerve stump at 1–8.5 mm/day. The rate of regeneration in the distal nerve is inversely proportional to the distance from the cell body [120, 214], but an overall figure of 1 mm/day is widely accepted in clinical practice [120]. A significant proportion of axons will never reach an endoneurial tube and instead leave the repair site, contributing to a neuroma in continuity. The remaining 50–60% [218] enter an endoneurial tube that will railroad them to the target organ. It is this selection that determines whether or not the regenerated axon will subserve any useful function since if topographical specificity is wrong (e.g. a sensory neuron embryologically mapped to innervate the thumb that regenerates to the ring finger) then central plasticity is required to restore function. If type specificity is wrong (e.g. a motor axon is directed to a sensory organ) then regeneration is entirely wasted. There is some evidence for a degree of preferential reinnervation of motor (PMR) tubes by motor axons [4, 16, 175], and this may be enhanced by leaving a millimetre gap between the nerve faces during repair.

Readers are referred to descriptions by Lundborg [120], Diao [44] and Fu and Gordon [51, 57, 218] for further details of injury and regeneration-associated events within the peripheral nerve.

13.1.3 Summary of the Requirements for Optimal Nerve Repair

Clinical experience has revealed that regeneration fails if nerve repairs come apart, and that interpositional nerve graft should be used whenever the nerve defects are sufficient that excess tension is required to maintain the nerve faces together. The precise length of the defect that this refers to is contested, and depends upon the type of nerve, anatomical site, timing of repair, and positioning of the limb. Most would agree that defects of greater than 1–2 cm require interpositional nerve graft in almost all the cases (in certain nerves excursion defects of less than 1 cm may require grafting) [44, 142, 143]. Neurobiology reveals that peripheral nerve has an innate capacity for regeneration, axons and Schwann cells can migrate short distances with preferential growth into the nerve tissue, and Schwann cells are an absolute requirement for nerve regeneration.

It is therefore apparent that for optimal regeneration, a nerve repair construct must maintain the nerve stumps within a few millimetres of one another, and optimise the tropic and trophic guidance cues that draw neurites to the distal nerve and reduce loss to neuroma formation, while blocking the ingrowth of non-neural tissues that would impede regeneration. In addition, Schwann cell migration and neurotrophic support should be facilitated, microscopic orientation of fascicles restored to maximise topographical specificity, and at the cellular level, any process supporting the type-specific reinnervation of the distal stump endoneurial tubes must be promoted. Macroscopic integrity will be required for around 1 month in an end-to-end nerve repair, and considerably longer in a gap repair (possibly up to 1 year for long gap repairs clinically). Microsurgical suture repairs at best provide macroscopic fascicular orientation, and longitudinal physical support to the repair, but contribute little else. A tissue-engineered construct could deliver greater benefits, but must be biocompatible, safe, and practical in terms of logistics and surgical handling.

13.2 Aim of the Discipline: The Tissue-Engineered Neurosynthesis

As a minimum requirement, a nerve repair (neurosynthesis) must maintain the cut nerve faces in close approximation. Microsurgical suture repair (either epineurial placement for epineurial repair, or perineurial placement for group fascicular repair) remains the clinical standard, but necessitates deleterious tissue handling and placement of foreign material within the nerve. Both induce scar formation, may result in endoneurial herniation, and prolong the compartmentation phenomenon [120]. Suturing does little to prevent axon escape, and cannot both block external scar ingress and leave a millimetre gap between the nerve faces to promote the type specificity of reinnervation. As a result various techniques have been experimentally tested to replace the suture. These include laser welding and a variety of glues [70, 80, 107, 108, 141], of which only Tisseel™ (human fibrin matrix, Baxter

International Inc.) [154], which reportedly has some growth factor content, has reached widespread acceptance. None have shown significant neurobiological benefit over suture repair, and fibrin glue does not have an adequate biomechanical profile to obviate the need for suture placement entirely [70, 158, 200].

If further elements are to be added to enhance the neurobiology of repair then a construct is required. The elements that could be added include protection from the ingress of external scar, localisation of tropic and trophic factors, directional growth cues, and the addition of exogenous pro–regenerative mediators such as NTFs or SCs. This is an extension of the concept of isolating the repair within a cuff of epineurium [138, 226], which has limited evidence and may be impractical in many clinical circumstances.

13.3 State of the Art: Tissue Engineering Technologies for Peripheral Nerve Repair

13.3.1 Entubulation /Wraparound Repair

A simple, biologically inert tube in which the nerve stumps are oriented and held approximated would serve the first two purposes, for which silicon tubing has a long history, both experimentally and in a long-term clinical trial [123, 125]. It has been found to maintain the repair, reduce compartmentation and locally concentrate the regenerative factors at the repair site [120], and to block scar ingress with minimal tissue reaction [40]. At 5-year follow-up after median or ulnar nerve repair (maximum 8 mm gap), silicone entubulation resulted in less cold intolerance, but was otherwise comparable to suture repair [123, 125]. The ability to leave a small gap (1–3 mm) between the nerve faces is postulated to enhance the type specificity of reinnervation [124], and studies have shown the technique to have certain advantages [34, 40, 41, 66, 115, 210], although other authors disagree [17, 66, 131, 155].

Silicone entubulation does not add further elements to the repair, and because the material is permanent, it is felt to require removal. Evidence has been presented that regeneration across longer defects is impaired by non-permeable constructs [99, 176], and so identification of a material suitable for use in a variety of repairs is preferable. Research has moved towards identifying a bioresorbable material that can further enhance regeneration. A range of biocompatible materials have been assessed experimentally for tubulisation repair, including collagen type 1, [178], and poly-glycolic acid [76, 216], but only PGA has robust clinical trial data published (and for terminal nerves in the hand only). The results for the injuries where direct epineurial repair would have been indicated are similar to the silicon entubulation study – comparable to the standard repair with subtle improvement in certain outcomes. Unlike silicone, no bioresorbable material has been clinically studied for large nerve repair, although encouraging early data on five obstetric brachial plexus cases repaired with a collagen tube is reported [6].

Furthermore, since nerves come in varying diameters and the dimensions of tubes relative to the nerve impact upon regenerative profile [21], tubes of multiple diameters would require to be held in stock. It is therefore of practical benefit if the repair material comes as a sheet which is formed into a tube around the nerve repair – the "wraparound repair". A range of biocompatible materials have been assessed experimentally for wraparound repair, including PHB [1, 72, 87], and bioglass [1, 72, 87], but only PHB has published clinical trial data. In that trial, 12 patients with median/ulnar nerve injury in the forearm not indicating nerve graft were followed for 18 months with a validated battery of tests, concluding that PHB wraparound repair was safe, and as efficacious as epineurial suture, with a weak trend towards enhanced improved outcome [1, 72, 87]. It is a common finding that results are similar to those of silicon entubulation repair. Motor and sensory outcomes as assessed by standard clinical tests are comparable to suture repair, with no clear evidence of significant benefit, but repairs are generally somewhat easier to perform. It seems likely that no single material will hold dramatic benefit over any other in terms of nerve regeneration, and that if outcome is to be enhanced, further active elements will need to be added. Discussion of these elements is best considered within the more challenging setting of the gap repair, which has become the experimental model of choice, because it is likely that elements which enhance regeneration in that setting will also have benefit in wraparound repair.

13.3.2 Gap Repair: Extending the Entubulation Concept to the Nerve Conduit Tube

13.3.2.1 Clinical Background: Nerve Graft Repair

When the transected ends of a nerve cannot be brought into direct apposition without excess tension (sufficient to cause local ischaemia and impair regeneration), then a nerve gap exists that must be bridged. The gap carries all the problems associated with a direct repair, plus the added impact of needing to maintain character for a longer time (until the nerve regeneration reaches the distal stump), and needing to promote nerve regeneration over distances that are beyond the physiological norm until the distal stump is reached. At present, gap repair is addressed clinically by the use of nerve autograft(s) harvested from a nerve subserving a less important function (typically the sural nerve) [142, 143].

The nerve autograft contains all the elements that are required to promote regeneration – barrier function from the epineurium, physical integrity to maintain a repair, appropriate mix of immune compatible support cells, directional guidance cues from the endoneurial tubes, and a vascular plexus that can be recruited within 3 days by graft inosculation. However, autograft is frequently not ideal in terms of size match for the recipient nerve, and handling characteristics during repair. Autografting incurs donor morbidity in terms of scarring, sensory loss, and potential complications. There may be insufficient graft available for major injuries [217], and there is increasing concern that sensory nerves may not optimally support motor regeneration [13, 157]. An alternative solution would be the use of a construct to act as a nerve conduit, and the search has been ongoing since the use of demineralised bone in 1882 [82]. Conduits have most recently been reviewed by de Ruiters et al. [26, 42], to which the reader is referred for a more detailed appraisal.

13.3.2.2 The Nerve Conduit: Initial Experimental and Clinical Constructs

In the preparation of a conduit, several elements are required, most probably all the elements underpinning the aforementioned utility of a nerve autograft. A brief investigation of the literature reveals that the more elements that are added, the better is the regenerative profile of the construct, yet none match the nerve graft in reproducible experimental studies. The most fundamental requirement for a construct is to maintain longitudinal strength in the repair to prevent loss of continuity. It must then minimise compartmentation, prevent the ingress of scar that would block nerve regeneration, and to maintain a passageway that favours nerve growth must maintain its cross-sectional profile. Further benefit would come from a matrix that supports cell migration and provides directional cues to longitudinal axonal growth (which may come secondary to Schwann cell orientation and migration), and from added tropic and trophic support. Vascularisation is necessary.

Various materials have been employed historically as simple tubes [82]. Autologous veingraft delivers longitudinal integrity, and localises regenerative factors, with no benefit from invagination [31, 96, 215]. It has been used to bridge gaps up to 3 cm with acceptable results for digital nerves [31, 215], and the regenerative profile is experimentally improved by the incorporation of a matrix such as collagen gel [34], Schwann cells [192, 230], denatured muscle [8, 73], or segments of nerve graft. It has not received widespread clinical acceptance due to concern about kinking, luminal obliteration, poor results in major nerves, the need for donor site scars, and potential size mismatch. It would be preferable to create a synthetic conduit, and several have been studied.

Silicon tubing will support regeneration to a gap length of around 2 cm in animal models, but is not clinically attractive for the reasons discussed in relation to entubulation repair, and lacks the permeability necessary to facilitate cell survival in the middle reaches of conduits in long gap repair (>2 cm). Other materials have been investigated and promoted as reviewed recently by Lundborg [120]. They include poly-3-hydroxybutyrate (PHB) [228], poly-glycolic acid (PGA) [76, 167]/poly-lactic acid (PLLA) and composites thereof [81, 109, 118], poly(organo) phosphazene [106], polytetrafluoroethylene (PTFE) [169, 190], bioglass [111, 190], polylactide-caprolactone [118, 176], poly(2-hydroxyethyl methacrylate-co-methyl methacrylate) hydrogel [9, 10, 93], collagen [3, 20, 85, 105], polyester [75], gelatin [26], chitin [232], and more recently, modified fibrin glue (Tisseel derived) [91]. Direct comparison of

experimental studies is unrewarding due to variance in animal model, nerve gap, surgical technique, outcome measures, and the length of follow-up. Further difficulties in comparison arise from the fact that the production methods will affect the regenerative profile (e.g. Pierucci et al. [168]). However, it is a general finding that the regenerative outcomes in rat models are roughly comparable with all the materials when used as empty tubes. None support good quality regeneration of gaps over >2–3 cm (rabbit/cat/primate models) without further adaptation, although regeneration has been reported across a 4 cm gap in an empty PHB conduit [228], and a 5 cm gap with an empty collagen-based nerve guide [105].

Tubes made from four materials have published data from small scale studies, have obtained FDA approval, and reached the clinical market: collagen type I (NeuraGen – Integra Neurosciences; Neuromatrix/Neuroflex – Collagen Matrix Incorporated), polyglycolic acid (PGA, Neurotube – Synovis), and Poly-(DL-Lactide-ε-Caprolactone) (Neurolac – Polyganics BV). All are selectively permeable, bioresorbable, and the Neuroflex and Neurotube conduits are corrugated to prevent kinking. They have recently been reviewed in detail by Meek and Coert [139], with the conclusion that robust clinical data (multiple centres, controlled prospective studies) is lacking for all but the Neurotube. Concern was expressed about complications, handling, and the efficacy of the Neurolac construct. The Neurotube has reassuring data on efficacy for distal nerve reconstruction (digital/terminal nerves only), and some outcomes are comparable to or better than direct repair or nerve grafting. These constructs are available only for gap lengths of a maximum of 3 cm, since animal studies suggest that regeneration will not occur through an empty conduit beyond this length.

Current clinically licenced nerve conduits and the initial experimental constructs were empty, or filled with an inert physiological solution such as saline. This has the benefit of providing no physical barrier to nerve growth, but does not facilitate the delivery of cultured cells (which would tend to concentrate under gravity, and may require physical interaction to optimise behaviour), or growth factors (a sustained release system would be preferable). Nor are topographical cues to directed growth provided within the lumen. This is all in contrast to the nerve graft which supports such favourable regeneration.

13.3.2.3 Refining the Nerve Conduit as a Biodynamic Construct: Matrix

Thus attention has turned from the wall of the conduit to the lumen, and the provision of a matrix to ensure appropriate dispersion and maintenance of implanted cells, enhance directional neurite growth, promote Schwann cell migration and phenotypic expression, and act as a sump for exogenous growth factors. The ideal matrix should provide these features while being biocompatible, either bioresorbable or neuroconductive, provide appropriate cell adhesion molecules, and physical directional guidance to longitudinal regeneration. The tissue-engineered solutions have largely fallen into three groupings – hydrogels, fibres, and semi-solid constructs with integral pores/channels.

The provision of a matrix to support and direct cellular migration in the same way that endoneurial tubes will do in native nerve is therefore considered important. There was a previous clinical interest in the use of denatured muscle (chemically or physically), after some initial encouraging animal model studies [28, 73, 145, 222], but robust evidence of clinically relevant efficacy has been lacking [73, 74, 177, 230] and it should be removed from clinical practice, certainly without being placed within a conduit structure. More promising are experimental studies that have investigated the materials and fabrication techniques to provide cell-friendly scaffolds to promote migration, or allow for the seeding of conduits with cells that promote regeneration.

Acellular nerve graft retains the basal lamina structure of endoneurial tubes, can be repopulated with Schwann cells [77], and supports nerve regeneration if used in isolation [98, 230]. However, the initiation of axonal growth is slower, to deleterious effect [105], and issues regarding the use of human tissues and the selection of appropriate-sized segments limit applicability. Since axons follow longitudinally oriented structures across a gap, and other axons build upon the lead axon, polyglactin fibres (8/0 suture) can guide nerve regeneration across a 7–15 mm gap in the rat [180], as can collagen fibres as shown by Nakamura et al. Fibres have been placed within conduit tubes as a means to improve the regenerative profile. Longitudinal PHB, or alginate fibres within the lumen of PHB conduits somewhat improve regeneration, (Terenghi et al., personal communication), as do collagen fibres within a PGA conduit [206]. Spider silk fibres show encouraging

capacity to enhance Schwann cell adhesion and migration [2], as do nano- or microscale constructs of polylactide [30, 156], or poly-caprolactone [30, 156]. The dimensions, surface characteristics, and coating of fibres affect cell behaviour [5, 15, 135, 220], and further elucidation will be necessary before design-based application is possible. However, the majority of research has focused upon the use of gels, or custom-fabricated constructs to provide matrix function.

Terenghi, Wiberg, and workers have demonstrated in the PHB conduit model of nerve gap repair that alginate hydrogel has some promising features in terms of function as a slow release vehicle for growth factors, and for the suspension of cultured cells. Regeneration is enhanced by the addition of fibronectin, Schwann cells, or growth factors [15, 69, 71, 146–149, 160]; however, the alginate fails to resorb at an appropriate rate, and may leave a residual barrier to nerve regrowth. More promising is the use of fibrin, following on from the demonstration of excellent regeneration across fibronectin mats [69, 221] (the cords dissolved too fast to be of use for long gap repair). Matrigel has been found to support regeneration well [100, 160, 212], but given its origin is unlikely to ever be clinically acceptable.

When prepared as a freeze-dried microporous sponge, alginate incorporated into the conduits supports regeneration over long distances (5 cm in the cat) [194]. Perhaps, most promising have been the studies by Nakamura's group, working upon the creation of an acellular conduit based on a PGA tube, and collagen matrix sponge [81, 84, 135, 205]. Successful regeneration across an 80 mm nerve gap has been demonstrated experimentally [135, 136, 205], and the construct has been used clinically with reported sensory regeneration across a 20 and a 65 cm nerve gap [84], and motor regeneration across a facial nerve gap [83]. If these findings are reproducible, then an acellular construct may prove to be a clinical rival to nerve autograft, but comparative, adequately controlled studies are required, and at present, the consensus is that a biologically inert, acellular construct is not optimal.

13.3.2.4 Refining the Nerve Conduit as a Biodynamic Construct: Therapeutic Delivery of Cultured Schwann Cells

The creation of microchannels in a PLGA foam was investigated by Vacanti et al., with the aim of producing a neo-nerve, ultimately demonstrating impressive results in a short gap sciatic nerve repair model in the rat, using multiple 60–550 μm channels with laminin surface coating and pre-culture with syngeneic Schwann cells [63]. Further technical refinement in manufacturing [104, 196] and tissue engineering [104, 196] has been described subsequently. Other workers have delivered the cells within a variety of matrices, all with beneficial effect.

Which cells to use? Autologous Schwann cells are the gold standard, as they drive the normal regenerative process, are immune competent, and secrete a favourable cocktail of neurotrophic and tropic cues. Autologous Schwann cells, both human and animal [112, 113], have been successfully cultured, can be labelled [207], and been shown to enhance regeneration across short gaps (~1 cm) in terms of improved quantitative morphological measures, electrophysiological measures, and functional scores [48, 64, 150]. They also increase the gap length that can be bridged by nerves when compared to acellular constructs [193] or allograft [77], and may contribute to neuroprotection [70]. They can be genetically modified to enhance the regenerative effect [58, 61, 62]; however, the clinical practicalities of use are not currently favourable. It takes several weeks to culture sufficient autologous cells to fill even a short, narrow diameter conduit unless a large nerve segment is harvested (in which case, donor morbidity approximates to that of nerve graft harvest), but early repair of nerve injuries reduces neuronal death [70], and is clinically indicated in the majority of cases. There are concerns regarding the use of animal-derived culture media and cancer cell line, or animal-derived feeder cells, and viral transfection techniques have always had some opposition. Strategies to overcome certain of these problems are under development [62, 89].

Allogeneic Schwann cells could be cultured and stored for rapid use should an injury present. They enhance regeneration through short gaps, although not to the same extent as syngeneic SC, but are rejected too quickly to assist long gap regeneration [147], unless immunosuppression were to be instituted. Immunosuppression is not detrimental to nerve regeneration [88, 153, 187], and as has been found clinically with the use of nerve allograft [127], may not be required long term since the native Schwann cells could repopulate the graft, and obviate the need for long-term nerve support by the allogenic population. Yet, ethical issues relating to transplantation and

potential transmission of infective agents would persist, and so a source of rapidly available autologous cells would be preferable.

13.3.2.5 Refining the Nerve Conduit as a Biodynamic Construct: Therapeutic Delivery of Stem Cells

Stem cell harvest offers this potential after the demonstration that multi-potent stem cells that can be induced to trans-differentiate can be derived from adult bone marrow. Undifferentiated mesenchymal stem cells accumulate at nerve injury sites [36, 231], and enhance regeneration [29, 166], but only to a limited extent. Their potential for therapeutic use in peripheral nerve injury was reviewed by Tohill and Terenghi [97, 209], and experimental studies have addressed the potential use of differentiated mesenchymal stems cells placed within a variety of nerve conduit models [33, 78, 90, 97, 186, 208, 209, 225, 231], and within acellular nerve allograft in a primate model [79]. Induced expression of morphological, molecular, and functional Schwann cell phenotypic markers has been demonstrated for bone marrow-derived stem cells of both rat [22, 129] and human origin [14]. Importantly, differentiated mesenchymal stem cells synthesise and secrete neurotrophins [130], and in vitro they up-regulate myelin genes and protein expression when co-cultured with neuronal cells (Mantovani, Kingham and Terenghi – personal communication). Similarly, other studies have shown the potential of differentiated mesenchymal stem cells to express various levels of growth factors [27, 35] and to myelinate axons [95].

They enhance nerve regeneration in vivo [27, 208], but are potentially hard to harvest in sufficient numbers given the clinical requirement for rapid access to large numbers of cells in major injuries. Adipose-derived stem cells would be available in large numbers by lipo-aspiration given the increased frequency within this tissue type [140], and the adiposity of most patients. Rat adipose-derived stem cells proliferate at a faster rate than bone marrow-derived mesenchymal stem cells and can be stably differentiated into glial cells expressing all the markers found in Schwann cells [101] (whose neurite promoting behaviour in a co-culture with neuronal cells they approximate) suggesting a differentiation into Schwann cell phenotype. Such cells when differentiated to express Schwann cell phenotypic markers enhance peripheral nerve regeneration [224]. Neural progenitor cells within the conduits may also be beneficial in vivo [151].

A future requirement for the large scale implementation of any cultured cell as a therapeutic technique would be the establishment of techniques that permit culture and expansion of cells without the need for animal, or human donor-derived products. In particular, it would be desirable to eliminate the need for feeder cell lines, and non-synthetic growth factors from culture systems; the use of 3-dimensional culture systems made hold promise in that regard. A further area for research is upon the effect of different number of passages, or cell culture environments upon the biological function of cells once implanted within the nerve conduits.

13.3.2.6 Refining the Nerve Conduit as a Biodynamic Construct: Therapeutic Delivery of Exogenous Growth Factors

An alternative route to making the conduits bioactive is the incorporation of elements that actively enhance cell function (either neuronal or non-neuronal cells) to promote regeneration. The initial therapeutic target in this regard should be axotomy-induced neuronal death, since this is the most fundamental barrier to optimising nerve regeneration. The main determinant of the magnitude of cell death is the loss of distal neurotrophic support; early nerve repair is partly neuroprotective [68, 126], presumably because of axonal reconnection to neurotrophic factors from the denervated Schwann cells in the distal stump. Entubulation repair with PHB confers a similar degree of neuroprotection to epineurial repair [115], and the addition of cultured Schwann cells to a gap repair improves the effect [70, 115].

Given their essential role in peripheral nerve injury and regeneration [57], the targeted delivery of exogenous growth factors (neurotrophic factors [227] or others) within a nerve conduit might be expected to reduce neuronal death, enhance regeneration, and support the Schwann cell population. This is the case in experimental studies [25, 51, 116, 119, 159, 161, 165, 173, 201] investigating the use of NGF [49, 51, 116, 165, 173, 223, 232], BDNF [159, 161, 163], NT-3 [60, 116], CNTF [117, 120], insulin-like growth factor 1 (IGF-1) [92, 165], fibroblast growth factor (FGF) [120, 165], and GDNF [7, 11, 46, 49, 51, 134] for peripheral nerve repair with a range of delivery systems, including

incorporation into range of conduits (generally by incorporation into slow-release hydrogels). Targeted local delivery is the preferable route due to the clinical complexities of delivery into the CNS, and the problems encountered with systemic treatment using neurotrophic factors in other conditions [137]. Terenghi and Wiberg plus co-workers have investigated a wide range of factors delivered by incorporation into comparable repair constructs, and readers are referred to individual studies for more detailed assessment. It is likely that growth factor manipulation will have a greater impact upon the initiation and rate of regeneration than upon neuroprotection, and the effect of incorporating factors into the conduit upon the exit of regrowing axons into the distal nerve stump remains to be adequately defined.

Despite the logical rationale to the incorporation of growth factors into nerve repair constructs, and experimental evidence that isolated factors confirm benefit to select sub-populations of neurons, or to select elements of nerve regeneration "growth factors have had no real effect on the clinical management of nerve injuries..."[204]. Although 10 years old, that statement still resonates: no clinical data is yet available, and to the authors' knowledge, no trials are underway. This is most probably because of licencing sensitivities, and since neuronal sub-populations exhibit specificity for individual neurotrophic factors, a cocktail of neurotrophic factors would be necessary for optimal effect. Yet, unexpected interactions between the factors have been demonstrated experimentally [54, 162, 163]. In addition, concerns exist about actions on other cells types [133, 188], and so the creation of an ideal cocktail of factors may not possible.

An alternative would be to use single agents that stimulate pleuripotent responses from cell groups. Potential agents here are LIF [24, 25, 45, 71] which as an injury factor stimulating the expression of regenerative phenotypic markers in a spectrum of neurons is of particular interest for late secondary nerve repair [24, 25, 71], and GGF as a Schwann cell mitogen that controls the normal neurite-Schwann cell interaction during regeneration and induces the secretion of a spectrum of neurotrophic factors by the activated Schwann cells [110, 128, 172]. Favourable effects upon regeneration have been found in a long gap model using a PHB/alginate conduit [69, 146], and a dramatic increase in nerve regeneration was found when a 1 cm gap in the rat sciatic nerve was repaired with a PHB/alginate conduit, both with and without cultured autologous Schwann cells [69].

13.4 Clinical Application

As described in the sections on entubulation/wraparound repair and nerve conduits, clinical translation of the more fundamental aspects of the tissue-engineered approach to peripheral nerve repair has begun. However, it is highly unlikely that these simple systems will dramatically alter clinical outcomes, as they do not radically alter the neurobiology of the repair site. In addition, when addressing the repair of a nerve defect, the constructs are up against a difficult adversary, since nerve autograft is, in many ways, such a beautifully engineered construct, albeit at the cost of donor morbidity.

The real impact of tissue engineering in nerve repair will come when autograft can be reserved for the more challenging scenarios, or when engineered solutions can radically alter the neuronal and Schwann cell behaviour to reduce axonal escape, increase type and topographic specificity, and increase the rate of regeneration across the repair and then out into the distal nerve stump. If constructs permit repair in situations not currently addressed clinically (e.g. pelvic nerve plexus reconstruction after prostate pelvic cancer surgery, and the sensory reinnervation of free tissue transfer in plastic surgery) then the horizons of reconstructive surgery can expand as previously occurred with the advent of microvascular tissue transfer. Furthermore, it must be remembered that the repair site is only one element of the neurobiology of nerve injury and repair – neuronal death, central plasticity, atrophy of target organs and the distal stump, and rehabilitation strategies must also be addressed if the patients are to be functionally restored after nerve injury.

13.5 Expert Opinion and 5-Year Perspective

Emerging technologies that will have further impact upon the design of nerve conduits for peripheral nerve repair will include the manipulation of nanotopography to modulate cell behaviour (both neuronal and

Schwann cell). Materials technology should provide enhanced, modulatable, biocompatible resorption for differing lengths of the conduit and enhanced matrix performance. The investigation of bioelectrics with regard to further enhancing cell migration and axon growth may impact upon conduit engineering or tissue culture sytems. Research will begin to define the advantages and disadvantages in terms of biology and the clinical reality of Schwann cells as opposed to Stem Cells (and the preferable source and extraction systems thereof). Culture systems devoid of cancer-derived feeder cells, and the potential sources of infectious agents should be investigated, with particular attention to the use of 3-dimensional culture systems, and the co-culture of multiple cell types, plus vascularising agents.

It will then be necessary to attend not only to the length of the defect across which regeneration can be induced, but also to the volume of regeneration that can be supported by constructs, in order to move to applicable systems for repair of large diameter nerves.

13.6 Limitations/Critical View

At present there is much to be encouraged. Experimental constructs are becoming more refined, as materials and fabrication systems are brought to bear upon the enhanced appreciation of the biological requirements of regenerating nerve and support cells. However, the translation from experimental system to clinically beneficial construct is difficult, and excessively slow. In all the work, it will be necessary to further attend to the issue of which experimental model will best reflect the clinical behaviour, and which clinical outcome measures and trial designs will deliver robust evidence, instead of the anecdotal norm that has beset the development of nerve research to date.

13.7 Conclusions/Summary

Peripheral nerve injury presents a hugely complex challenge to the clinicians who treat these common but functionally unrecoverable injuries, since the neurobiology of regeneration cannot be adequately addressed by the existing surgical techniques. It is apparent that a package of care is required to optimally rehabilitate the patient, and that tissue engineering has significant potential to improve the results of nerve reconstruction. If cell death is prevented, then the neurons of the peripheral nervous system are phenotypically driven to regenerate, and the supporting cells form no barrier. So the challenge to the neurobiologists in the field is to employ tissue engineering to provide the cells with an environment more conducive to regeneration into the distal nerve stump than microsurgical techniques have done thus far.

Considerable progress has been made, and encouraging experimental results are available in terms of defining the elements required for nerve repair, and supporting regeneration across long defects in animal models, even with acellular constructs. Clinical translation has begun using inert constructs, and bioactive elements should now increasingly be translated within the framework of well-designed, adequately powered trials using validated outcome measures.

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Tissue Engineering of Blood Vessels: How to Make a Graft

14

Sajoscha Sorrentino and Hermann Haller

14.1 Introduction

Cardiovascular diseases remain the leading cause of death in western countries and often require vascular reconstruction. So far, autologous arteries or veins are the most commonly used substitutes for coronary and peripheral bypass procedures. Albeit recent advances in secondary prevention and interventional cardiology [5, 101], coronary artery bypass remains an important therapeutic option, and the incidence of peripheral arterial disease and end-stage renal failure requiring vascular access is increasing [2, 28]. However, autologous veins are not available in up to 40% of patients as a result of trauma, underlying diseases, such as varicose veins or previous surgery [73]. The problem is further amplified by the chronicity of these diseases, resulting in a group of patients who will inevitably require reoperation.

14.2 Aim of the Discipline

A solution to these problems may be found by the application of tissue engineering to the creation of blood vessels. The general approach of vascular tissue engineering is to seed cells on scaffolds, followed by in vitro culture or in vivo implantation (Fig. 14.1). Ideally, the scaffolds will be gradually resorbed, leaving only the new tissue generated by the cells. Thus, the successful tissue regeneration relies on the seeding cells, the scaffolds, and the construction technologies [56, 89].

Tissue engineering has been defined in 1988 as "the application of the principles and methods of engineering, materials sciences and the life sciences to the fundamental understanding of the structure-function relationships in normal and pathological mammalian tissues at a fundamental level and the development of biological substitutes to restore, maintain or improve the tissue functions" [93].

The development of durable and functional blood vessels, however, remains an immense challenge thus far.

The challenges faced by the approach of tissue engineering for replacing blood vessels are substantial. They include sufficient strength and elasticity to withstand the hemodynamic cyclic strain, matched compliance with the adjacent native vessel, and a lining of the lumen with nonthrombogenic properties. While engineering approaches for other tissue substitutes can rely on in vivo remodeling to approach functionality with time, tissue-engineered blood vessels (TEBVs) should function immediately upon implantation [76].

Ideally, functional TEBVs should be nonthrombogenic, nonimmunogenic, and compatible at high blood flow rates and have similar viscoelasticity to native vessels [36, 68, 96]. Moreover, the grafts should be living tissues that could eventually integrate into the body and become indistinguishable from the native vessels (Table 14.1).

Thus, to accurately mimic a blood vessel, all of its elements should be taken into consideration. The techniques used in the tissue engineering of blood vessels can be broadly divided into the formation of the three layers of a normal vessel, the supportive scaffold used, in vitro evaluation, and finally, implantation and in vivo study.

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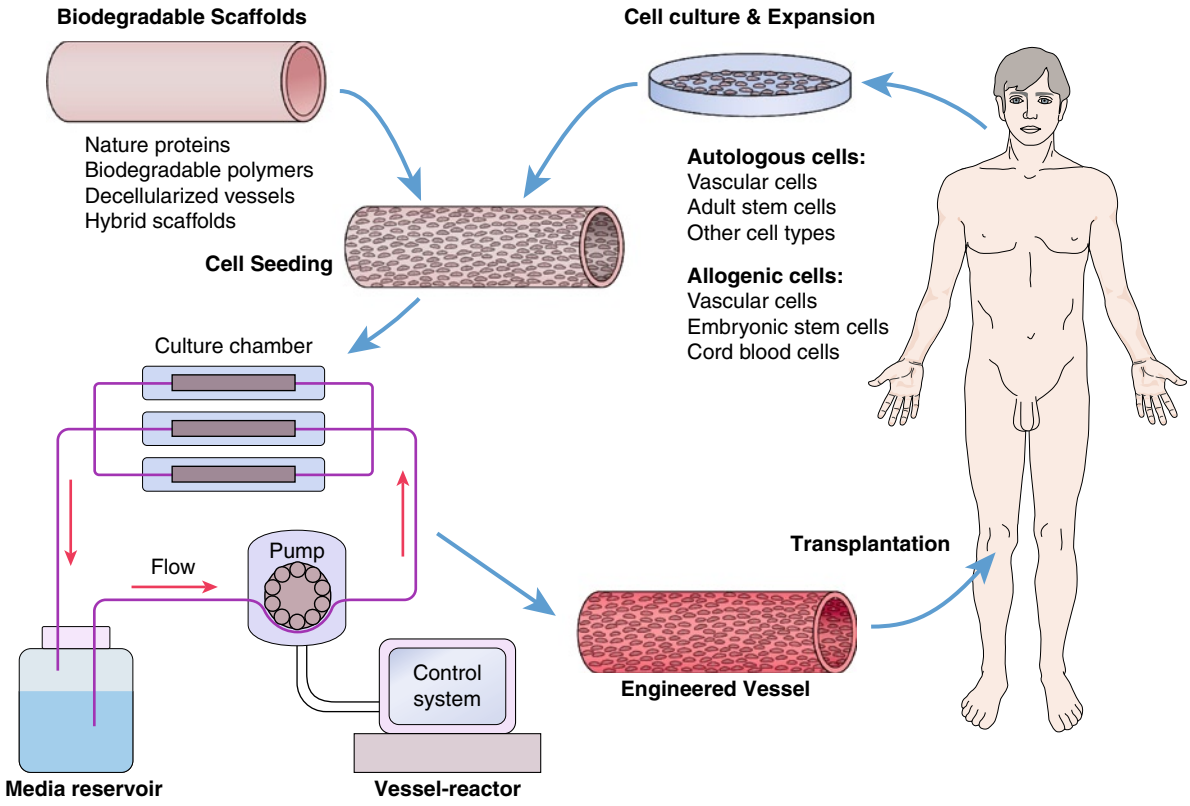

Fig. 14.1 Principle of vascular tissue engineering

Table 14.1 Ideal properties of a TEBV [75]

Biological	Mechanical	Commercial	Physical
Vasoreactive: dilate/constrict to neural and chemical stimuli	Strength to resist burst pressures	Can be tailored to individual's requirements, e.g., length and diameter	Leak-proof: avoids hemorrhage through its walls
Nonthrombogenic	Avoids kinking even over joints	Inexpensive to manufacture	Porosity for healing and angiogenesis
Biostable: does not weaken in vivo to result in aneurysms and/or rupture	Hold sutures under circumferential and longitudinal tension	Short time period from request to implantation	
Biocompatible: not inflammatory, toxic, carcinogenic, or immunogenic	Retains axial and radial compliance and pulsatility		
Infection resistant			

Native blood vessels provide a nonthrombogenic conduit for blood flow. The blood vessel wall is a trilammellar structure in which each layer plays an important role in the normal function of the vessel. The individual sections are called – from the luminal side outward – the (tunica) intima, the (tunica) media, and the (tunica) adventitia (Fig. 14.2).

In healthy blood vessels, the intima is an endothelial cell (EC) monolayer consisting of a confluent layer of flat, elongated cells, aligned in the direction of blood flow [85]. Over the last two decades, it has become evident that the endothelium is not merely an inert, single-cell lining covering the internal surface of blood vessels but in fact plays a crucial role

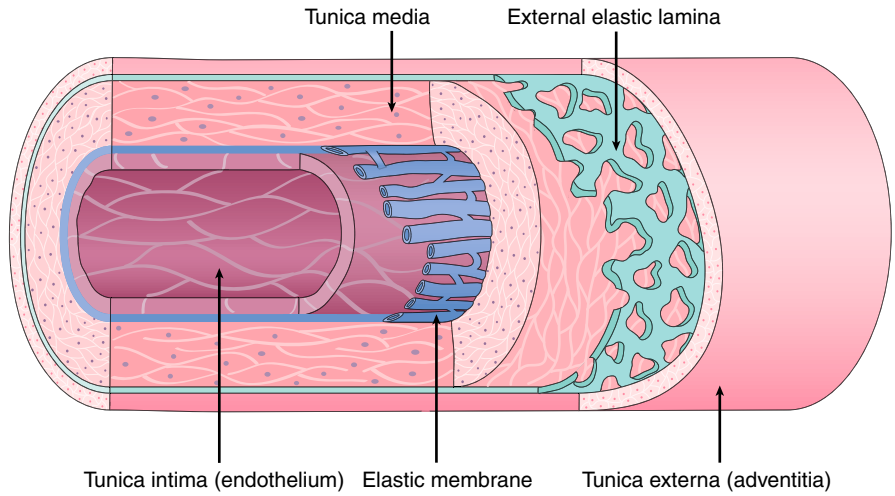

Fig. 14.2 Structure of a blood vessel (artery) showing the three tunics

in regulating vascular tone and structure. Importantly, a healthy endothelium inhibits platelet and leukocyte adhesion to the vascular surface and maintains a balance of profibrinolytic and prothrombotic activity, e.g., by secreting specific molecules [11, 54, 59, 80]. Namely, several studies have shown that nitric oxide (NO) is a crucial mediator in the regulation of platelet aggregation, EC proliferation, and EC migration, which can affect thrombus formation in blood vessels [29, 64, 102]. Moreover, NO inhibits proliferation and migration of smooth muscle cells (SMCs) responsible for intimal thickening [30, 82]. Located below this layer is the basement membrane, made of Type IV collagen, fibronectin, and laminin.

The multilayered media, accounting for the greatest volume of the vessel wall, confers mechanical strength to the vessel and is responsible for most of its mechanical properties. The media is formed from SMCs, elastin, Type I, III, and V collagen and proteoglycans. The SMCs have a nearly circumferential orientation in most vessels [43], controlling vessel caliber by contracting or relaxing [24]. Medial elastin keeps the vessel elastic enough to expand when necessary, while medial collagen prevents excessive dilation [19, 31, 85], thus allowing arteries to maintain sufficient blood pressure with variations in hemodynamic stress of the cardiac systole and diastole [22]. In addition to the mechanical responsiveness, elastin is also a potent autocrine regulator of vascular SMC activity, and this regulation appears to be important in the prevention of fibrocellular pathology. Elastin signals via a nonintegrin heterotrimeric G-protein-coupled pathway, induces actin stress fiber organization, inhibits SMC proliferation, and regulates migration [78]. In fact, elastin knockout studies and clinical observations have revealed an essential regulatory function. It was demonstrated that in the absence of extracellular elastin, SMC proliferation is responsible for stenoses of arteries [58].

The adventitia comprises fibroblasts, nerves, Type I collagen, and elastin fibers [43]. In bigger arteries, a vascular network, the vasa vasorum, is also present in the tunica adventitia. The adventitia also contributes to the mechanical properties of the blood vessel, mainly by anchoring to the surrounding connective tissue [85]. It appears that the adventitia is more than a simple elastic membrane, which surrounds the media. However, the extent of its contribution to vascular physiology, as well the mechanisms involved, remains to be clearly established and characterized.

14.2.1 Cell Sources for Vascular Tissue Engineering

The ideal cell source should be nonimmunogenic, functional, and easy to achieve and expand in culture. Mature vascular cells and embryonic and adult stem cells, as well as alternative cell types that could possibly replace the ECs and SMCs, have been tested in vessel engineering.

Nonimmunogenic autologous ECs and SMCs isolated from patients themselves are the first choice for vessel engineering. Cells isolated from autologous

vessels have been well used for engineering new vessels by many groups [52, 69, 98]. However, it is known that the majority of the cells in adult blood vessels are terminally differentiated. Even the cells isolated from umbilical veins have limited proliferation potential.

Adult stem cells on the other hand can be obtained from patients themselves, which can avoid the immunorejection and ethical problems. In addition, adult stem cells are normally limited to certain lineages, which do not have tumorgenic capacity. Endothelial progenitors cells (EPCs) are one type of the adult stem cells that have the capacity to proliferate, migrate, and differentiate into mature ECs [3, 4, 8, 53, 87].

Furthermore, omitting the surgical vein harvesting for EC isolation and noninvasive harvesting of progenitor ECs from peripheral blood represent undoubted benefits for the patients.

EPCs have been well utilized in the endothelialization of synthetic vessel grafts as well as in vessel engineering [33, 48, 83].

Regarding SMCs, studies have shown that bone marrow-derived mesenchymal stem cells (BMSCs) could be differentiated into SMC phenotypic cells in the presence of certain factors [34, 45, 47].

14.2.2 Scaffolds for Vascular Tissue Engineering

Scaffold is another key factor for tissue engineering. The 3D structure of scaffold provides a template for supporting cell growth, migration, differentiation, and secretion of extracellular matrix (ECM) proteins, as well as for directing new tissue formation in the tissue regeneration process. Ideally, the scaffolds will be slowly resorbed in culture or after implantation, leaving only the tissue generated by the cells. In order to engineer a biocompatible vessel with growth potential and to avoid material-related side effects, the ideal scaffold for vessel engineering should be biodegradable. Varieties of materials have been utilized for vessel engineering, including the nature proteins, synthetic biodegradable polymers, and decellularized vessels (Table 14.2).

14.2.3 Vessel Reactors for Vascular Tissue Engineering

Due to the dynamic environment of the cardiovascular system, the engineered vessel should be fully functional at the time of transplantation, with a nonthrombogenic luminal surface, good mechanical strength, and physiological vasoreactivity. In addition, the mechanical and hemodynamic properties of vessel grafts are also crucial for their long-term survival [32]. To achieve such a functional graft in culture, a construct technology that could mimic the physiological vessel environment is required. Vessel reactors have been developed and successfully utilized for vessel engineering by many groups [15, 25, 40, 49, 52, 69]. Basically, the vessel reactor mimics the physiological stimuli that a native vessel received in the body, including the cyclic strain and shear stress [10, 63, 65, 86, 92, 100]. Cyclic strain could significantly improve the mechanical property of engineered vessel, while shear stress could change cell alignment and improve EC adhesion [13, 57, 67].

As the poor mechanical strength of collagen-based artificial vessels has been partially attributed to the longitudinal orientation of the SMCs before implantation, several strategies have been used to stimulate reorientation of the SMCs in a circumferential direction. L'Heureux et al. [50] hypothesized that

Table 14.2 Biological and synthetic scaffolds for vascular tissue engineering [71]

	Advantages	Disadvantages
Biological scaffolds	Naturally occurring Nontoxic Favorable for cell binding Generally biocompatible	May degrade rapidly May have weak mechanical properties Inconsistency between batches Chance of disease transmission
Synthetic scaffolds	Precise control over material properties Easily available and cheap Easy to process Little or no batch-to-batch variations	Toxic residual monomers or catalysts and degradation byproducts may illicit inflammation Poor cellular interaction

SMC orientation is directly influenced by tensions that oppose cell contraction. In this context, circumferential forces induced during the maturation of the artificial vessel are favorable, whereas longitudinal forces are not.

An innovative technique was introduced by Kanda et al. [46] to induce longitudinal orientation of the ECs, which enhances their stability under flow shear stress, and circumferential orientation of the SMCs, which is expected to increase the mechanical strength of the artificial vessel and improve its vasomotor reactivity. The investigators hypothesized that by applying pulsatile flow on a collagen-based artificial vessel, the ECs would orient parallel to the direction of flow, and the SMCs would align parallel to the direction of stretch (i.e., circumferentially). They utilized a nondegradable polyurethane graft with a collagen gel embedding canine SMCs. Autologous ECs were then seeded onto the media equivalent to create the framework of an intima, and the graft was subjected to pulsatile flow of culture medium. After 10 days of stress loading, the ECs aligned parallel to the longitudinal axis of the graft, and the SMCs were oriented in a circumferential direction.

14.3 State of the Art and History of Tissue-Engineered Blood Vessels

In 1986, Weinberg and Bell [98] reported the first TEBV created from collagen gels combined with bovine ECs, fibroblasts, and SMCs. Collagen and bovine aortic SMCs were casted together in culture medium to create an annular mold. The inner surface of the graft was seeded with bovine ECs and the outer with bovine adventitial fibroblasts. A Dacron mesh was embedded into the wall to enhance the mechanical strength of the model. Models made without the mesh dilated and ruptured at very low intraluminal pressures (<10 mmHg). A model with one mesh had a burst strength of 40–70 mmHg, whereas three layers of collagen lattice alternating with two meshes provided a burst strength of 120–180 mmHg. The absence of elastin, the longitudinal (instead of circular) orientation of the SMCs, and the low densities of the SMCs and collagen accounted for the poor mechanical properties of the model. However, well-differentiated SMCs and functional endothelium, which could produce von Willebrand's factor and prostacyclin, made this blood vessel model attractive and sparked further research into the tissue engineering of blood vessels.

Many different scaffolds and cell types have been used in these approaches. For the sake of clarity, we will group different approaches depending on the scaffolds used (see examples below in Tables 14.3–14.6).

14.3.1 Natural Scaffolds

The use of acellular natural scaffolds to enhance graft incorporation was first proposed by Rosenberg et al. [79] as early as 1966 by using tanned bovine carotid artery. Lantz et al. [55] developed a biological vascular graft material made from small intestine submucosa and tested it in a dog model.

Similarly, Huynh et al. [44] constructed a scaffold from a collagen biomaterial derived from the submucosa of the small intestine and type I bovine collagen. This scaffold was decellularized using Triton X-100, and the luminal surface was treated with heparin. This acellular graft was then implanted into rabbit aortas and explanted at a number of time points up to 90 days.

Bader et al. [7] used acellularized porcine aorta. These xenografts were then repopulated with human myofibroblasts and ECs from saphenous vein biopsies. Clarke et al. [20], using hypotonic water and ribonucleases, acellularized bovine ureters, which were then grafted into dog aorta with 100% patency and no aneurysms at 10 months.

14.3.2 Permanent Synthetic Scaffolds

Weinberg and Bell [98] used this methodology to develop a biological blood vessel substitute. In their landmark paper, bovine SMCs were used to contract a collagen gel around a tubular mandril. An external Dacron mesh sleeve reinforced the resulting cell-rich matrix. The outer surface was then seeded with bovine fibroblasts until further contraction resulted in a tubular structure that could be slipped off the central mandril, allowing ECs to be seeded into the lumen. This early Dacron-reinforced graft bore a fundamental resemblance to a muscular artery in that it produced prostacyclin. However, the graft lacked elastin, and the

Table 14.3 Examples for TEBVs with natural scaffolds

Author	Year	Cells	Scaffold	Implantation	Patency
Lantz et al. [55]	1993	None	Decellularized porcine/ canine small intestinal submucosa		75% at 5 years
Huynh et al. [44]	1999	None	Decellularized porcine small intestinal submucosa	Rabbit carotid artery	100% at 90 days
Matsuura et al. [61]	2000	None	Decellularized human femoral vein	Human arteriovenous fistula (AVF) for dialysis access	49% at 1 year
Bader et al. [7]	2000	Human myofibroblasts and endothelial cells (ECs) from saphenous vein biopsies	Decellularized porcine aorta	None	n/a
Clarke et al. [20]	2001	None	Decellularized bovine ureter	Canine abdominal aorta	100% at 13 months
Kaushal et al. [48]	2001	Ovine EPC	Decellularized porcine iliac arteries	Ovine carotid artery	100% at 130 days
Conklin et al. [21]	2002	None	Decellularized porcine carotid artery	Canine carotid artery	100% at 67 days
Borschel et al. [12]	2005	Rat endocardial ECs	Decellularized rat iliac artery	Rat femoral artery	89% at 4 weeks
Cho et al. [17]	2005	Canine bone marrow cells	Decellularized canine carotid arteries	Canine carotid artery	100% at 8 weeks
Martin et al. [60]	2005	None	Decellularized canine external jugular vein	Canine carotid artery	100% at 8 weeks
Darby et al. [23]	2006	None	Decellularized bovine ureter	Human dialysis access	95% at 1 year
Amiel et al. [1]	2006	Human saphenous vein ECs	Decellularized porcine carotid artery	None	n/a
Narita et al. [66]	2008	Canine peripheral vein ECs	Decellularized canine ureters	Canine carotid artery	100% at 24 weeks
Narita et al. [66]	2008	None	Decellularized canine ureters	Canine carotid artery	20% at 1 week

Table 14.4 Examples for TEBVs with permanent synthetic scaffolds

Author	Year	Cells	Scaffold	Implantation	Patency
Weinberg and Bell [98]	1986	Bovine aortic Smooth muscle cells (SMC), EC, and fibroblast	Collagen	None	n/a
Ratcliffe et al. [76]	2000	Smooth muscle cells	Nondegradable polyurethane	Canine carotid artery	100% at 4 weeks
Sparks et al. [88]	2002	Rabbit peritoneum	PTFE	Rabbit carotid artery	80% at 21 days
Rashid et al. [74]	2008	Human umbilical vein smooth muscle and ECs	Polycarbonate-ureaurethan	None	n/a

Table 14.5 Examples for TEBVs with biodegradable synthetic scaffolds

Author	Year	Cells	Scaffold	Implantation	Patency
Shum-Tim et al. [84]	1999	Ovine carotid SMC, EC, and fibroblasts	PGA-PHA	Ovine infrarenal aorta	100% at 5 months
Niklason et al. [69]	1999	Bovine aortic SMC and EC	PGA	Porcine saphenous artery	100% at 4 weeks
Watanabe at al. [97]	2001	Canine femoral vein SMC and fibroblasts	PGA-CL/LA	Canine inferior vena cava	100% at 13 months
Hoerstrup et al. [42]	2001	Ovine carotid fibroblasts and EC	PGA-P4HB	None	n/a
Hoerstrup et al. [41]	2002	Human umbilical cord cells	PGA-P4HB	None	n/a
Torikai et al. [94]	2008	None	Knitted polyglycolic acid compounded with collagen	Porcine thoracic descending aorta	100% at 4 weeks

Table 14.6 Examples for TEBVs with nonscaffold-based approaches

Author	Year	Cells	Scaffold	Implantation	Patency
L'Hereux et al. [50]	1993	Human umbilical vein SMC and EC, human skin fibroblasts	Type I and III collagen	None	n/a
Hirai et al. [37, 38]	1994	Canine jugular SMC and EC	Type I collagen	Canine posterior vena cava	65% at 6 months
L'Heureux et al. [52]	1998	Human umbilical vein SMC and EC, human skin fibroblasts	None (PTFE mandrel)	Canine femoral artery	50% at 7 days
Tsukagoshi et al. [95]	1999	Rabbit inflammatory reaction cells	None (silicone mandrell)	Rabbit femoral artery	80% at 8 weeks
He et al. [35]	2003	Canine EPC	Type I collagen	Canine carotid artery	92% at 3 months
Berglund et al. [9]	2003	Human coronary ECs, neonatal human dermal fibroblasts	Type I collagen	None	n/a

incorporated cells tended to adopt an unsatisfactory longitudinal orientation because the contraction of the matrix was largely radial.

Ratcliffe [76] used poly(ether)urethane scaffolds seeded with SMCs. These were allowed to proliferate under conditions of fluid flow termed "preconditioning" for 4 or more weeks, generating ECM proteins such as collagens, elastin, and proteoglycans. The cells orientated themselves perpendicular to the direction of flow as in native vessels. ECs were then seeded onto the luminal surface and cultured under fluid flow until confluent. The resulting grafts were implanted into the carotid arteries of dogs with 100% patency at 4 weeks.

14.3.3 Biodegradable Synthetic Scaffolds

Niklason et al. [69] used polyglycolic acid (PGA) scaffolds, chemically modified by sodium hydroxide to increase hydrophilicity and so enhance protein adsorption. Bovine aortic SMCs in suspension were pipetted on to this biodegradable scaffold. Grafts were then cultured for 8 weeks under conditions of pulsatile pressure before seeding with ECs. The SMCs migrated towards the lumen enveloping PGA fragments.

The resulting conduits were morphologically similar to native arteries and contracted in a measurable fashion when exposed to serotonin or endothelin 1.

Furthermore, they withstood burst pressures of over 2,000 mmHg. These conduits were eventually implanted into porcine right saphenous arteries. The pulsed grafts had a 100% patency rate at 4 weeks, whereas grafts that did not undergo cyclical radial strain developed thromboses within 3 weeks.

Shum-Tim et al. [84] used a copolymer of PGA and polyhydroxyalkanoate (PHA) as a scaffold onto which was seeded a mixture of smooth muscle and ECs and fibroblasts (cultured as explants from lamb carotid arteries). When implanted into lamb abdominal aortas, all remained patent at 150 days; acellular PGA-PHA copolymer controlled all thrombosed.

14.3.4 Nonscaffold-Based Tissue-Engineered Blood Vessels

Hirai et al. [37] have developed a tubular hybrid medial tissue by pouring a cold mixed solution of canine jugular SMCs and type I collagen into a tubular mold. After thermal gelatinization followed by 10 days of tissue culture, the tube was finally seeded with canine jugular ECs.

L'Heureux et al. [52] produced an innovative graft requiring no scaffold and using exclusively cultured human cells. An acellular layer, made by dehydrating a fibroblast sheet, was wrapped around an ePTFE-based circular mandril. Two further cellular sheets, comprising an inner SMC and outer fibroblast layer, were then added in a "Swiss roll" maneuver. The ePTFE mandril was then removed, and the graft lumen surface was seeded with ECs. The grafts were implanted in dogs, resulting in a patency rate of only 50% at 1 week.

14.4 Clinical Application: An Example

Hemodialysis access is probably the most challenging model of a TEBV with respect to the mechanical environment. In addition to the obvious challenges associated with repeated puncture, the high flow rates encountered generate considerable hemodynamic loads.

The incidence of end-stage renal failure treated by dialysis continues to increase in both Europe and the USA. Hemodialysis is the most common modality for dialysis. The gold standard for vascular access is the creation of a primary arteriovenous fistula (AVF) using native vein. In Europe and more recently in America, the benefits of this approach have been fully recognized, and this approach is formalized by the K/DOQI guidelines in the USA [70]. However, it is recognized that there will always be a percentage of patients for whom use of a native vein is not possible, as mentioned above. Hence, there is an extensive history in the use of synthetic arteriovenous grafts (AVG) for this purpose. The most widely used bridge graft material for hemodialysis is polytetrafluoroethylene (PTFE) [16, 18]. The main complications for PTFE grafts used for hemodialysis access have been thrombosis, reported to contribute to 70% of the failures [27, 39] and infection, which on average occurs in 10% of implanted PTFE grafts [18, 39, 62, 90].

Vascular tissue engineering aims to obviate these obstacles. Until recently, the "tissue-engineering" approach to vascular access for hemodialysis was confined to the use of xenograft materials (e.g., Artegraft®, made from bovine carotid arteries; ProCol®, made from bovine mesenteric vein; SynerGraft® SGVG 100, made from depopulated bovine ureters) or cryopreserved human blood vessels (e.g., CryoVein®, made from human superficial femoral vein). However, the use of biological grafts has yielded mixed results, often resulting in failures related to aneurysm formation, calcification, infection, and poor patency rates [6, 14, 26, 77, 81, 99].

Recently, L'Heureux et al. [51] reported promising results from a small clinical trial using tissue-engineered blood vessels in an adult arterial model. Ten patients with end-stage renal disease whose arteriovenous shunts were failing were included in the study. Blood vessels were engineered as described by L'Heureux et al. before. The grafts consisted of three components: an adventitia, a decellularized internal membrane, and an endothelium. The internal membrane was assembled by wrapping an 8-week-old fibroblast sheet around a temporary Teflon®-coated stainless-steel-support tube (three revolutions). After a maturation period of at least 10 weeks, the individual plies fused together to form a homogenous cylindrical tissue. This tissue was then dehydrated to form an acellular substrate for EC seeding. The internal membrane was also included to provide a barrier against cell migration towards the lumen. The adventitia was formed in a similar fashion by wrapping a living sheet around the internal membrane. After a second maturation phase, the steel tube was removed,

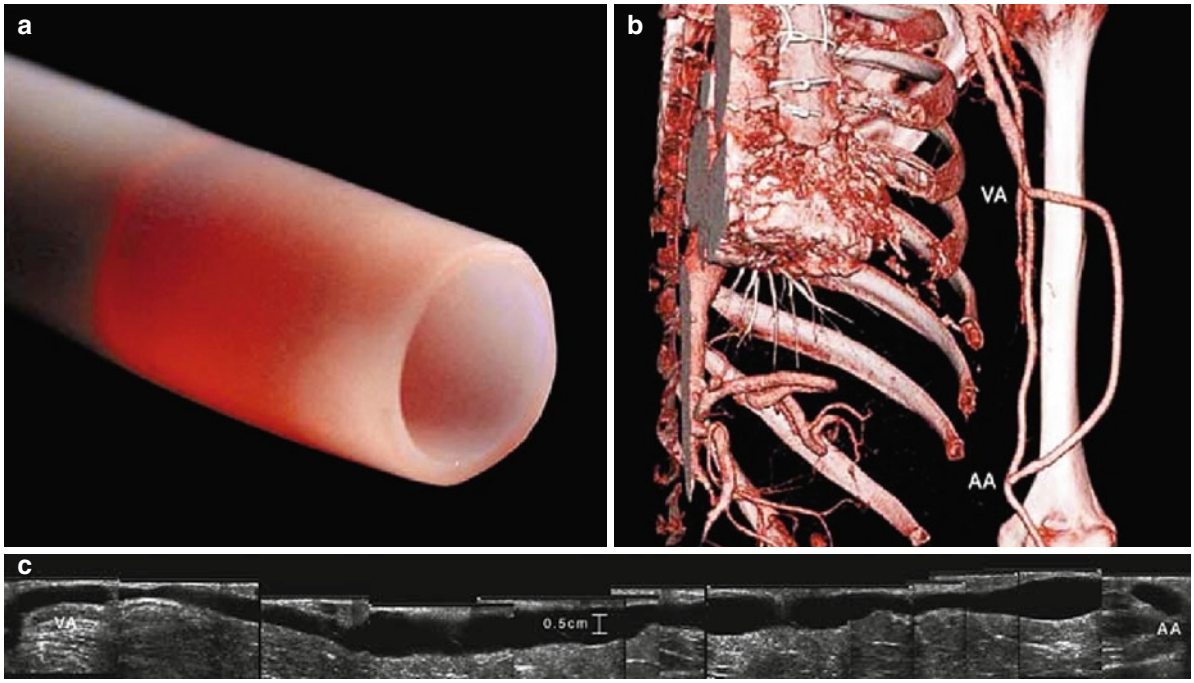

Fig. 14.3 The tissue-engineered blood vessel (TEBV) preoperatively (**a**), at 3 months after implantation (**b**, computed tomographic angiography), and at 12 months after implantation (**c**, Doppler ultrasonography). *VA* venous anastomosis; *AA* arterial anastomosis [51]

and ECs were seeded in the lumen of the graft. The vessel was then subjected to pulsatile flow that increased from 3 to 150 mL/min over a 3-day- preconditioning period [49].

After creation of an AVF with these grafts, a subsequent observation period of 3 months was added to assure in vivo stability of the tissue-engineered blood vessel. After that, grafts were punctured for hemodialysis access and have been used up to 13 months (Fig. 14.3).

14.5 Expert Opinion

An ideal TEVG could be constructed of a biodegradable and biocompatible material sufficiently strong and elastic to resist the high and dynamic pressure of blood flow in vivo. Selection of a reliable scaffold is critical for engineering vascular tissues under mechanically dynamic in vivo environments such as in blood vessels. Appropriate scaffolds for these applications should have elastic mechanical properties and a proper tensile strength. In addition, the scaffolds should allow for appropriate interactions with seeded cells to induce vascular tissue formation.

14.6 Five-Year Perspective

The discovery of circulating bone marrow-derived EPCs [3] may potentially ease some of the concerns associated with sourcing of autologous cells. EPCs are CD34+ cells derived from adult bone marrow that are committed to a vascular lineage. They can be isolated and cultured from the mononuclear fraction of circulating leucocytes [3]. Unlike pure stem cells, by definition, they have little or no potential for self-renewal [72], but they retain the ability to differentiate into ECs and in some situations vascular SMCs [48]. EPCs are easily harvested by venesection and thus can be obtained from a "nondepleting, self-renewing resource" (peripheral blood) for tissue engineering. In 2001, Kaushal et al. [48] used EPCs to seed a decellularized porcine scaffold, with promising results. When implanted as a xenogenic interposition graft in a sheep common carotid artery model, the engineered vessels

had a 100% patency rate (seven of seven grafts) either at 15 or 130 days after implantation, whereas non-seeded conduits thrombosed. On explantation, the grafts showed confluent EC layers at 15 days with a media repopulated with SMCs at 130 days. The grafts also displayed promising vasoactive properties, including contraction in response to noradrenaline and serotonin, high NO production, and NO-dependent relaxation. These were comparable to the properties of native sheep carotid artery and better than those of saphenous vein. Such a finding is encouraging as the last two properties are implicated in the better long-term patency rates achieved with native internal mammary artery than with vein grafts in cardiac bypass.

14.7 Limitations/Critical View

Meeting all criteria for the ideal tissue-engineered artery simultaneously remains a challenge. Despite recent advances in tissue engineering and new mechanical and biological approaches to replicate the structure and function of a small-diameter blood vessel, no tissue-engineered graft has yet been adopted in routine clinical practice. Indeed, this goal is some years away. Advances in cell sourcing, particularly using stem cell precursors, and a better understanding of extracellular matrices and their interactions will be crucial to the success of this type of endeavor. Advances in bioreactor design and pulsatile flow circuits are necessary to enhance graft maturation.

The "bench-to-bedside" applicability of the in vitro approaches is limited because of its long cultivation times. The limited conduit length generated in current bioreactors and the need for additional surgical interventions for sourcing autologous cells (vein harvest, liposuction, laparoscopy, and bone marrow aspiration) [91] are other issues that must be considered.

14.8 Conclusion/Summary

In summary, although great advances have been made towards an artificial artery, many open questions and obstacles remain. Answering these questions and overcoming these obstacles will require an interdisciplinary effort requiring critical contributions from biologists, engineers, and clinicians, with strong collaborations among these three fields being crucial to success. Hopefully, the scale of the clinical need will ensure that such efforts will continue to grow and drive research toward the goal of producing a functional vascular graft.

Suggested Readings with Abstracts

Lavik E, Langer R. Tissue engineering: current state and perspectives. Appl Microbiol Biotechnol. 2004;65(1):1–8.

Tissue engineering is an interdisciplinary field that involves cell biology, materials science, reactor engineering, and clinical research with the goal of creating new tissues and organs. Significant advances in tissue engineering have been made through improving singular aspects within the overall approach, e.g., materials design, reactor design, or cell source. Increasingly, however, advances are being made by combining several areas to create environments that promote the development of new tissues whose properties more closely match their native counterparts. This approach does not seek to reproduce all the complexities involved in development but rather seeks to promote an environment that permits the native capacity of cells to integrate, differentiate, and develop new tissues. Progenitors and stem cells will play a critical role in understanding and developing new engineered tissues as part of this approach.

Weinberg CB, Bell E. A blood vessel model constructed from collagen and cultured vascular cells. Science. 1986;231(4736):397–400.

A model of a blood vessel was constructed in vitro. Its multilayered structure resembled that of an artery, and it withstood physiological pressures. Electron microscopy showed that the endothelial cells lining the lumen and the smooth muscle cells in the wall were healthy and well differentiated. The lining of endothelial cells functioned physically, as a permeability barrier, and biosynthetically, producing von Willebrand's factor and prostacyclin. The strength of the model depended on its multiple layers of collagen integrated with a Dacron mesh.

Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, et al. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med. 2001;7(9):1035–40.

Arterial conduits are increasingly preferred for surgical bypass because of inherent functional properties conferred by arterial endothelial cells, especially NO production in response to physiologic stimuli. Here, we tested whether endothelial progenitor cells (EPCs) can replace arterial endothelial cells and promote patency in tissue-engineered small-diameter blood vessels (4 mm). We isolated EPCs from peripheral blood of sheep, expanded them ex vivo, and then seeded them on decellularized porcine iliac vessels. EPC-seeded grafts remained patent for 130 days as a carotid interposition graft in sheep, whereas nonseeded grafts occluded within 15 days. The EPC-explanted grafts exhibited contractile activity and NO-mediated vascular relaxation that were similar to native carotid arteries. These results indicate that EPCs can function similarly to arterial endothelial cells and thereby confer longer vascular-graft survival. Due to their unique properties, EPCs might have other general applications for tissue-engineered structures and in treating vascular diseases.

L'Heureux N, Dusserre N, Konig G, Victor B, Keire P, Wight TN, et al. Human tissue-engineered blood vessels for adult arterial revascularization. Nat Med. 2006;12(3):361–5.

There is a crucial need for alternatives to native vein or artery for vascular surgery. The clinical efficacy of synthetic, allogeneic, or xenogeneic vessels has been limited by thrombosis, rejection, chronic inflammation, and poor mechanical properties. Using adult human fibroblasts extracted from skin biopsies harvested from individuals with advanced cardiovascular disease, we constructed tissue-engineered blood vessels (TEBVs) that serve as arterial bypass grafts in long-term animal models. These TEBVs have mechanical properties similar to human blood vessels, without relying upon synthetic or exogenous scaffolding. The TEBVs are antithrombogenic and mechanically stable for 8 months in vivo. Histological analysis showed complete tissue integration and formation of vasa vasorum. The endothelium was confluent and positive for von Willebrand factor. A smooth muscle-specific alpha-actin-positive cell population developed within the TEBV, suggesting regeneration of a vascular media. Electron microscopy showed an endothelial basement membrane, elastogenesis, and a complex collagen network. These results indicate that a completely biological and clinically relevant TEBV can be assembled exclusively from an individual's own cells.

L'Heureux N, Germain L, Labbe R, Auger FA. In vitro construction of a human blood vessel from cultured vascular cells: a morphologic study. J Vasc Surg. 1993;17(3):499–509.

Purpose: The purpose of this study was to create a tubular vascular model exclusively made of human cells and collagen. *Methods*: The blood vessel equivalent was constructed with the three following human cell types: vascular smooth muscle cells, endothelial cells, and fibroblasts. A tissue-like structure was obtained from the contraction of a tubular collagen gel (human origin) by vascular smooth muscle cells, which created a media-like structure. An adventitia-like tissue was added around the media-like structure by embedding fibroblasts into a collagen gel. An endothelium was established within the tubular structure after intraluminal cell seeding. *Results*: Cell orientation and gel contraction were followed up over time. Vascular smooth muscle cells developed a complex tridimensional network and were oriented in a circular fashion around the tube's axis. In contrast, fibroblasts were randomly oriented. A viable, homogeneous, and well-characterized endothelium was observed. These endothelial cells showed a slightly elongated structure and were oriented parallel to this vascular equivalent axis. *Conclusion*: An in vitro tridimensional vascular model that exhibits some phenotypic characteristics of in vivo vascular cells could be useful in the study of events that lead to atherosclerotic plaque formations.

Lantz GC, Badylak SF, Hiles MC, Coffey AC, Geddes LA, Kokini K, et al. Small intestinal submucosa as a vascular graft: a review. J Invest Surg. 1993;6(3):297–310.

Continuing investigations of vascular graft materials suggest that unacceptable graft complications continue and that the ideal graft material has not yet been found. We have developed and tested a biologic vascular graft material, small intestine submucosa (SIS), in normal dogs. This material, when used as an autograft, allograft, or xenograft, has demonstrated biocompatibility and high patency rates in aorta, carotid and femoral arteries, and superior vena cava locations. The grafts are completely endothelialized at 28 days post-implantation. At 90 days, the grafts are histologically similar to normal arteries and veins and contain a smooth muscle media and a dense fibrous connective tissue adventitia. Follow-up periods of up to 5 years found no evidence of infection, intimal hyperplasia, or aneurysmal dilation. One infection-challenge study suggested that SIS may be infection resistant, possibly

because of early capillary penetration of the SIS (2–4 days after implantation) and delivery of body defenses to the local site. We conclude that SIS is a suitable blood interface material and is worthy of continued investigation. It may serve as a structural framework for the application of tissue-engineering technologies in the development of the elusive ideal vascular graft material.

Cho SW, Lim SH, Kim IK, Hong YS, Kim SS, Yoo KJ, et al. Small-diameter blood vessels engineered with bone marrow-derived cells. Ann Surg. 2005;241(3):506–15.

Objective: The objective of this study is to investigate if bone marrow-derived cells (BMCs) regenerate vascular tissues and improve patency in tissue-engineered small-diameter (internal diameter = 3 mm) vascular grafts. *Summary Background Data*: BMCs have demonstrated the ability to differentiate into endothelial-like cells and vascular smooth muscle-like cells and may offer an alternative cell source for vascular tissue engineering. Thus, we tissue-engineered small-diameter vascular grafts with BMCs and decellularized arteries. *Methods*: Canine BMCs were differentiated in vitro into smooth muscle alpha-actin/smooth muscle myosin heavy-chain-positive cells and von Willebrand factor/CD31-positive cells and seeded onto decellularized canine carotid arteries (internal diameter = 3 mm). The seeded grafts were implanted in cell-donor dogs. The vascular tissue regeneration and graft patency were investigated with immunohistochemistry and angiography, respectively. *Results*: The vascular grafts seeded with BMCs remained patent for up to 8 weeks in the canine carotid artery interposition model, whereas nonseeded grafts occluded within 2 weeks. Within 8 weeks after implantation, the vascular grafts showed regeneration of the three elements of artery (endothelium, media, and adventitia). BMCs labeled with a fluorescent dye prior to implantation were detected in the retrieved vascular grafts, indicating that the BMCs participated in the vascular tissue regeneration. *Conclusions*: Here, we show that BMCs have the potential to regenerate vascular tissues and improve patency in tissue-engineered small-diameter vascular grafts. This is the first report of a small-diameter neovessel engineered with BMCs as a cell source.

Narita Y, Kagami H, Matsunuma H, Murase Y, Ueda M, Ueda Y. Decellularized ureter for tissue-engineered small-caliber vascular graft. J Artif Organs. 2008;11(2):91–9.

Previous attempts to create small-caliber vascular prostheses have been limited. The aim of this study was to generate tissue-engineered small-diameter vascular grafts using decellularized ureters (DUs). Canine ureters were decellularized using one of four different chemical agents (Triton-X 100 (Tx), deoxycholate (DCA), trypsin, or sodium dodecyl sulfate (SDS)), and the histology, residual DNA contents, and immunogenicity of the resulting DUs were compared. The mechanical properties of the DUs were evaluated in terms of water permeability, burst strength, tensile strength, and compliance. Cultured canine endothelial cells (ECs) and myofibroblasts were seeded onto DUs and evaluated histologically. Canine carotid arteries were replaced with the EC-seeded DUs ($n = 4$). As controls, nonseeded DUs ($n = 5$) and PTFE prostheses ($n = 4$) were also used to replace carotid arteries. The degree of decellularization and the maintenance of the matrix were best in the Tx-treated DUs. Tx-treated and DCA-treated DUs had lower remnant DNA contents and immunogenicity than the others. The burst strength of the DUs was more than 500 mmHg, and the maximum tensile strength of the DUs was not different to that of native ureters. DU compliance was similar to that of native carotid artery. The cell seeding test resulted in monolayered ECs and multilayered alpha-smooth muscle actin-positive cells on the DUs. The animal implantation model showed that the EC-seeded DUs were patent for at least 6 months after the operation, whereas the nonseeded DUs and PTFE grafts become occluded within a week. These results suggest that tissue-engineered DUs may be a potential alternative conduit for bypass surgery.

Shum-Tim D, Stock U, Hrkach J, Shinoka T, Lien J, Moses MA, et al. Tissue engineering of autologous aorta using a new biodegradable polymer. Ann Thorac Surg. 1999;68(6):2298–304; discussion 2305.

Background: Ovine pulmonary valve leaflets and pulmonary arteries have been tissue-engineered (TE) from autologous cells and biodegradable PGA-polyglactin copolymers. Use of this cell-polymer construct in the systemic circulation resulted in aneurysm formation. This study evaluates a TE vascular graft in the systemic circulation, which is based on a new copolymer of PGA and polyhydroxyalkanoate (PHA). *Methods*: Ovine carotid arteries were harvested, expanded in vitro, and seeded onto 7-mm diameter PHA-PGA tubular scaffolds. The autologous cell-polymer vascular constructs were used to replace 3–4 cm abdominal

aortic segments in lambs (group TE, $n=7$). In a control group ($n=4$), aortic segments were replaced with acellular polymer tubes. Vascular patency was evaluated with echography. All control animals were sacrificed when the grafts became occluded. Animals in TE group were sacrificed at 10 days ($n=1$), 3 ($n=3$), and 5 months ($n=3$). Explanted TE conduits were evaluated for collagen content, deoxyribonucleic acid (DNA) content, structural and ultrastructural examination, mechanical strength, and matrix metalloproteinase (MMP) activity. *Results*: The four control conduits became occluded at 1, 2, 55, and 101 days. All TE grafts remained patent, and no aneurysms developed by the time of sacrifice. There was one mild stenosis at the anastomotic site after 5 months postoperatively. The percent collagen and DNA contents approached the native aorta over time (% collagen = 25.7% ± 3.4 (3 months) vs. 99.6% ± 11.7 (5 months), $p<0.05$; and % DNA = 30.8% ± 6.0 (3 months) vs. 150.5% ± 16.9 (5 months), $p<0.05$). Histology demonstrated elastic fibers in the medial layer and endothelial specific von Willebrand factor on the luminal surface. The mechanical strain-stress curve of the TE aorta approached that of the native vessel. A 66 kDa MMP-2 was found in the TE and native aorta but not in control group. *Conclusions*: Autologous aortic grafts with biological characteristics resembling the native aorta can be created using TE approach. This may allow the development of "live" vascular grafts.

Torikai K, Ichikawa H, Hirakawa K, Matsumiya G, Kuratani T, Iwai S, et al. A self-renewing, tissue-engineered vascular graft for arterial reconstruction. J Thorac Cardiovasc Surg. 2008;136(1):37–45, 45.e31.

Objective: Various tissue-engineered vascular grafts have been studied to overcome the clinical disadvantages of conventional prostheses. Previous tissue-engineered vascular grafts have generally required preoperative cellular manipulation or use of bioreactors to improve performance, and their mechanical properties have been insufficient. We focused on the concept of in situ cellularization and developed a tissue-engineered vascular graft for arterial reconstruction that would facilitate renewal of autologous tissue without any pretreatment. *Methods*: The graft comprised an interior of knitted PGA compounded with collagen to supply a scaffold for tissue growth and an exterior of woven poly-L-lactic acid for reinforcement. All components were biocompatible and biodegradable, with excellent cellular affinity. The grafts, measuring 10 mm in internal diameter and 30 mm in length, were implanted into porcine aortas, and their utility was evaluated to 12 months after grafting. *Results*: All explants were patent throughout the observation period, with no sign of thrombus formation or aneurysmal change. Presence in the neomedia of endothelialization with proper integrity and parallel accumulation of functioning smooth muscle cells, which responded to vasoreactive agents, was confirmed in an early phase after implantation. Sufficient collagen synthesis and lack of elastin were quantitatively demonstrated. Dynamic assessment and long-term results of the in vivo study indicated adequate durability of the implants. *Conclusion*: The graft showed morphologic evidence of good in situ cellularization, satisfactory durability to withstand arterial pressure for 12 postoperative months, and the potential to acquire physiologic vasomotor responsiveness. These results suggest that our tissue-engineered vascular graft shows promise as an arterial conduit prosthesis.

L'Heureux N, McAllister TN, de la Fuente LM. Tissue-engineered blood vessel for adult arterial revascularization. N Engl J Med. 2007;357(14):1451–3.

No abstract available.

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Biohybrid Strategies for Vascular Grafts

15

Robert van Lith and Guillermo A. Ameer

15.1 Introduction

Despite the enormous advances realized in cardiology and cardiovascular surgery, approximately 500,000 deaths due to coronary heart disease occur annually in the US [166]. The high mortality is primarily due to atherosclerotic disease, a gradual buildup of plaque in the vascular system. This build up, which may ultimately lead to occlusion of these blood vessels or sudden detachment of plaques, leads to embolisms that can cause blockages elsewhere in the body. As a result of atherosclerotic disease, the number of surgical procedures for coronary bypass and percutaneous coronary interventions has risen to approximately one million annually (~469,000 and 620,000 procedures, respectively, in 2005) [166]. Furthermore, another 100,000 bypass procedures are being performed annually for the treatment of peripheral arterial disease [148]. Finally, approximately 340,000 end-stage renal disease patients are eligible for a synthetic arteriovenous (AV) graft for long-term vascular access [54]. Hence, the total number of patients for which vascular synthetic grafts may be used approximates 1.5 million. Despite all the advances in surgical techniques and improved pharmaceutical regimens, the need for improved vascular repair technologies remains. Synthetic grafts are usually not suitable for smaller diameters, whereas vein grafts, the current standard-of-care, are often severely affected by the condition of systemic atherosclerosis and/or suffer from restenosis or have been harvested for prior procedures.

15.1.1 Current Status of Vascular Grafts

For approximately 50 years, the use of synthetic vascular grafts has been common practice in vascular surgery [216]. Ever since Voorhees used a Nylon graft for aortic reconstruction for the first time in 1952 [216], the research on synthetic grafts has continued to be a top priority. As of now, usually polyethyleneterepthalate (PET) is used for larger vessels (ID>10 mm), whereas for medium-sized arteries (ID 6–10 mm) poly(tetrafluoroethylene) (PTFE) is the preferred choice [220]. PET, also known as Dacron, is a nondegradable polyester that can be used in a woven form or the more porous knitted form. In the latter case, usually preclotting is necessary or the use of a sealant like fibrin, collagen, or albumin is required. On the one hand, PET prostheses have mainly met success in thoraco-abdominal surgeries and abdominal aortic aneurysm surgeries. PTFE, on the other hand, is a chemically inert, hydrophobic polymer that does not degrade over time. The microporous form, expanded poly(tetrafluoroethylene) (ePTFE) with luminal leakage-resistant internodal distances of 30 μm, is typically used in vascular prostheses. ePTFE grafts have largely been the choice of graft for femoropopliteal bypasses. In larger-diameter vessels, both PET and ePTFE grafts are used, although ePTFE remains the more satisfactory choice in terms of mechanical strength and thromboresistance. In coronary and below-the-knee peripheral applications, however, both PET and ePTFE grafts experience rapid occlusion due to acute thrombus formation or long-term failure due to neointimal hyperplasia, which led to the

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virtual abandonment of PET and ePTFE for those purposes, unless suitable native conduits are unavailable. Recently, polyurethane (PU) grafts have arrived to the hospital setting. Poor compliance matching between native arteries and PET/ePTFE grafts is thought to be the main determinant of anastomotic hyperplasia in small-diameter vessels [81], which led to the interest in the superior elastomeric properties of PUs among existing polymers. Unfortunately, PUs often undergo changes in their compliance characteristics upon implantation because of poor biostability. So far, one PU graft has been FDA-approved for hemodialysis (Vectra graft, Thoratec, Corp.), although recently a compliant PU, called MyoLink, with improved biostability was developed by Seifalian and co [204] and is currently undergoing trials.

In general, autogenous grafts from the internal mammary artery or saphenous vein are still the gold standard for bypass procedures. However, in 20–30% of patients, vein grafts are not available in the adequate size or the vessels are diseased [38]. Furthermore, vein grafts also often experience occlusion at a later stage due to neointimal hyperplasia, with up to 40% failing within 10 years [124]. Improved patency rates have been observed with arterial grafts, but thrombosis and neo-intimal hyperplasia (NIH) remain an issue [83]. Good results with permanent synthetic grafts have been obtained in large- and medium-diameter arteries. Nevertheless, the use of these grafts remains fraught with complications that threaten long-term patency when used in small-diameter blood vessels [90]. The excellent track record of ePTFE and PET prostheses in larger blood vessels, together with the current suboptimal solution of autogenous grafts, continue to fuel research aimed at abating small-diameter synthetic graft complications.

15.1.2 Failure Mechanism

Graft failure in small-diameter vascular conduits (defined as vessels with a diameter <6 mm) usually arises as a result of thrombosis or NIH. The former is associated with early graft failure (within several months), whereas the latter causes long-term failure occurring within a few years after implantation. The cause for these failure modes is multifactorial and not yet completely understood. In the case of synthetic materials without an endothelial cell (EC) monolayer lining the lumen, the material's properties typically lead to platelet adhesion and activation of the coagulation mechanism. This surface-induced thrombosis is initiated by rapid amphiphilic protein adsorption from the surrounding plasma within seconds upon implantation. Platelets can subsequently adhere to the synthetic graft through interaction of their GPIIb/IIIa receptors with the adsorbed plasma proteins [155, 226]. A similar process occurs in vascular injury, when denudation of endothelium exposes underlying substrate proteins to which platelets may adhere, especially collagen and fibronectin. A complex cascade of events follows, leading to recruitment of more platelets and the formation of a thrombus (Fig. 15.1). Another important process leading to thrombosis is the inflammatory reaction to implants or vascular injury. The rolling of leukocytes usually happens on ECs as part of natural mechanisms. However, they can also adhere to damaged ECs or platelets, after which they may release factors that inhibit anticoagulant molecules and/or activate platelets. Furthermore, leukocytes have the ability to interact with platelets leading to mutual activation. Similarly, complement activation may interact with platelets as well. In fact, it is now recognized that the events leading to thrombosis are caused by the interwoven systems of complement activation, coagulation, and inflammation. For a comprehensive review see [48].

Over time, the initiation of the coagulation cascade may promote smooth muscle cell (SMC) overproliferation and migration, as well as superfluous extracellular matrix (ECM) deposition, leading to the advancement of NIH. Platelets secrete a multitude of factors that regulate the coagulation pathways, and some of these factors may also play an important role in the development of NIH. Platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) are the best-known examples. These proteins are released from platelets upon activation and are known to have a potent mitogenic effect [66, 194, 195]. They are widely accepted as two of the main signaling culprits for neointimal hyperplasia. Vascular endothelial growth factor (VEGF) has also been conspicuously present at the site of anastomotic NIH [169]. Although originally thought to be an important candidate for vascular graft therapy due to its angiogenic potential, experiences have been disappointing and several reports have outlined its deleterious effects on NIH [16, 221].

Other factors contributing to the onset of SMC overproliferation include the initial inflammatory response to foreign materials or vascular injury, leading to chemo-attraction of macrophages and lymphocytes

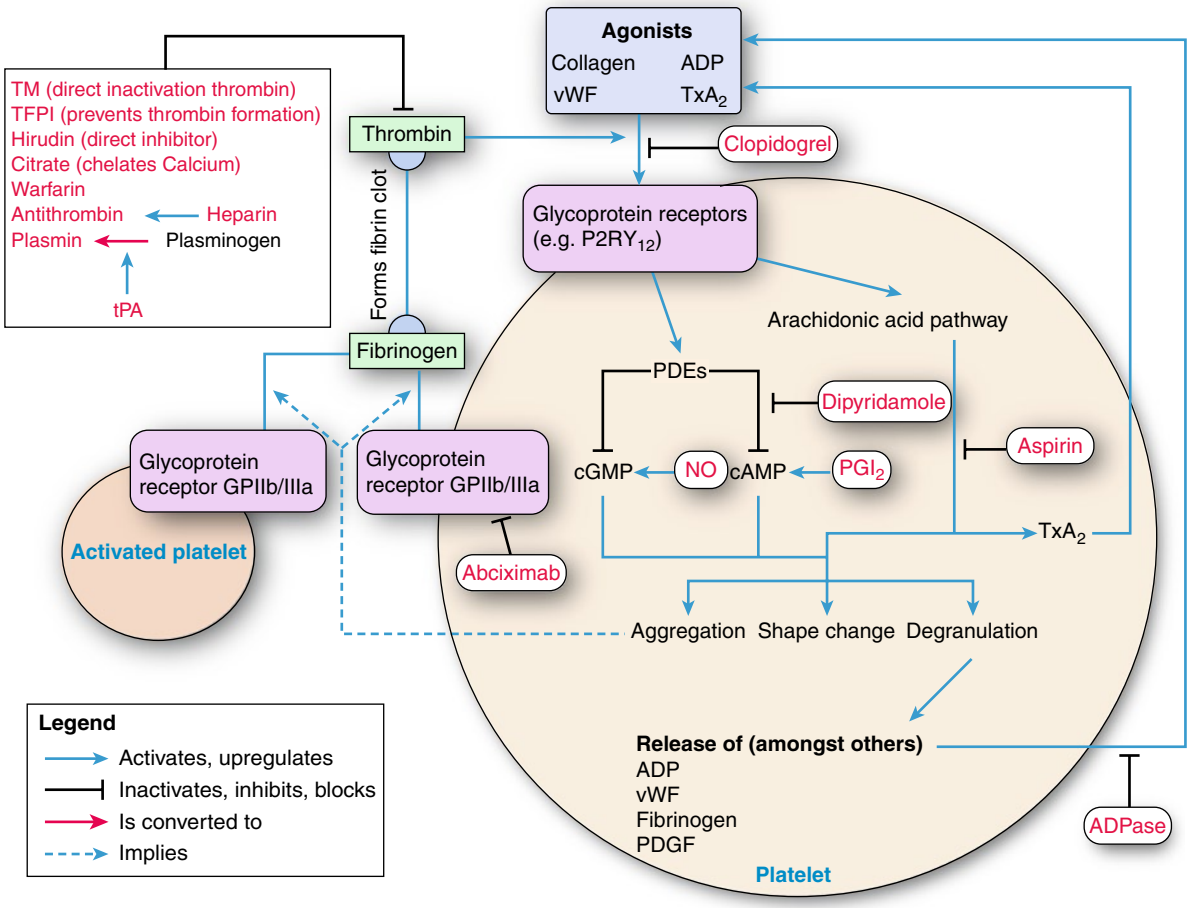


Fig. 15.1 Overview of thrombus-formation and major anti-thrombotic agents explored in vascular graft modifications to prevent thrombosis. Anti-thrombosis agents are printed in *red*. Activated platelet depicted smaller for schematic purposes. *TM* thrombomodulin; *TFPI* tissue factor pathway inhibitor; *tPA* tissue plasminogen activator; *TxA2* thromboxane; *ADP* adenosine diphosphate; *vWF* von Willebrand factor; *PDGF* platelet derived growth factor; *PGI2* prostacyclin; *NO* nitric oxide; *PDEs* phosphodiesterases; *cGMP* cyclic guanosine monophosphate; *cAMP* cyclic adenosine monophosphate

that can secrete cytokines and growth factors (among others, again, PDGF). Furthermore, the compliance mismatch between native artery and synthetic graft, together with a diameter mismatch between artery and graft, results in an aberrant hemodynamic environment with abnormal shear stress profiles and turbulent flow, which also contribute to rapid development of NIH, especially at the distal anastomosis [55]. Traditionally, the overproliferation of the SMCs in the medial layer was thought to be the primary cellular source of NIH. However, recently researchers have started to give more attention to the role of the adventitial layer. The group of Alfred Cheung showed that in a porcine AV ePTFE graft model, it was mainly overproliferation of the adventitial layer that seemed to result in the formation of the neo-intima at the site of anastomosis [113]. The shift in paradigm on NIH and the enhancement of our understanding of the pathogenesis behind it will likely result in the advent of novel preventive strategies. In fact, anti-proliferative therapies directed at SMCs from within the graft may not affect the actual culprits, the graft-extrinsic cells, possibly explaining the conspicuous failure of anti-proliferative strategies to inhibit NIH in human trials. One can already observe an increase in research projects investigating the use of perivascular therapies in the treatment of autogenous graft NIH [36, 75, 82, 133, 141, 152, 243]. The direct contact of any pharmacological agent with the adventitia may, indeed, be more effective than the traditional luminal delivery of anti-NIH drugs.

15.2 Aim of the Discipline

In the field of vascular engineering, an exponential growth in the development of novel materials for synthetic vascular grafts has taken place within the past decades, focusing mainly on optimization of these materials for tissue engineering-based therapies. Consequently, new biomaterials programs world-wide have led to a rapid expansion of the available types of materials for implantation in the body. A large range of material properties and characteristics such as tensile strength, elasticity, and porosity have been reported, but despite the vast number of materials described and tested at a preclinical level, the diversity of materials actually approved for and used in a clinical setting is limited (see next paragraph). The enormous variety of biomaterials that have been reported for vascular repair in combination with the virtually stagnant translation to clinical practice raises the question whether further development of novel materials for vascular repair is still warranted. Reevaluation of the rationale for vascular repair strategies may be necessary to make valid judgments on what type of research to embark upon. One not only needs to consider theoretical feasibility, but also practical and cost-based issues, as well as how current healthcare regulations may or may not support novel technologies.

The field of vascular grafts aims to create an optimal solution to assist, sustain, or replace the function of damaged blood vessels, and when looking at the requirements for vascular grafts, we can make a distinction between those for functional performance and for clinical/commercial performance.

Functional performance	Clinical/commercial performance
Biocompatible (anti-thrombogenic, nontoxic)	Ease of manufacturing
Compliant (similar to native artery)	Off-the-shelf availability in various sizes and diameters
Suturable	
Strong (burst and tensile)	Economical
Biostable	
Infection resistant	

The development of new biohybrid strategies that fulfill all these requirements is interdisciplinary, involving collaboration between materials scientists, chemical engineers, and clinicians to come up with solutions for all current clinical issues. The design or selection of a specific combination of material and biological components depends on the relative stringency of the various requirements, and primary physical requirements include compliance, strength, suturability, and long-term dimensional stability. In addition, predicting the consequences of interactions between host and material is an important and unique consideration when using synthetic materials, and two particularly important issues are bio-compatibility and bio-stability. Currently, optimizing the interactions that occur at the surface of implanted biomaterials represents the most significant challenge to further advancement of graft technology. An increasing understanding of complex biological materials in the development of novel biomaterials custom-designed at the molecular level is therefore necessary.

Although the functional performance requirements may be met in due time with scientific progress, the other set of requirements poses different types of problems. For most state-of-the-art strategies investigated, particularly the tissue-engineered blood vessel (TEBV) approach, especially off-the-shelf availability and ease of manufacturing remain significant hurdles. Although an important niche does exist for strategies that are not instantly available for patients, e.g., renal failure patients, the majority of vascular repair procedures prescribe off-the-shelf products (for more on this, see Sect. 15.5).

In this chapter, we focus on current state-of-the-art strategies to improve vascular repair outcomes, with a particular emphasis on so-called "biohybrid" approaches to design vascular grafts. The term biohybrid (or biosynthetic) in this respect indicates utilizing knowledge on processes naturally occurring in the human vasculature to design synthetic grafts with partially biologic components to mimic blood vessels' natural properties or with agents to regulate biologic processes for improved performance. This includes "living" blood vessels that can adapt to the immediate environment and/or self-repair, but also synthetic grafts for which the biologic components act passively. The biologic components can involve cytokines, growth factors, and proteins, but also drugs that are known to achieve beneficial effects in terms of reduced thrombogenicity and/or NIH. The way of incorporation of these components may vary, including a more traditional method of coating a graft, the employment of drug-delivery vehicles in the graft's

wall and immobilization by covalent attachment to the graft's polymer backbone. Although the focus is on vascular grafts, these strategies may apply to other areas as well. The first part will discuss approaches for tailoring the host response to the material, with particular emphasis on the latest developments in biohybrid modifications to ePTFE and PET grafts. The second section will depart from this and discuss the current status of TEBV technology, since recent developments in that area may open up new possibilities for widespread use of such an approach.

15.3 State of the Art: Biohybrid Strategies for Synthetic Grafts

Although ePTFE and PET may permit cell adhesion and growth to a limited extent, their surface characteristics are not conducive to selective EC adhesion, migration, and proliferation, leaving a prolonged thrombogenic surface exposed after implantation. Attempts to decrease the thrombogenicity of synthetic grafts may include mimicry of native vascular anti-thrombogenic phenotype by immobilizing biomolecules on scaffold surfaces, the immobilization or release of anti-thrombogenic factors/drugs or by achieving truly stable confluent EC monolayer formation [26]. In addition to targeting thrombogenicity, which also underlies NIH development, strategies to specifically target NIH are actively pursued. NIH, characterized by overproliferation of SMCs and superfluous ECM deposition, can be specifically targeted by halting or slowing down cell proliferation, for instance by immobilization or release of anti-proliferative drugs. Such biohybrid scaffolds may be capable of enhanced anti-thrombogenicity, either by resisting platelet/cell adhesion in general or by the existence of a functional endothelium, thereby possibly facilitating long-term patency.

15.3.1 Modification with Other Materials to Decrease Thrombogenicity

Ever since it became clear that the use of ePTFE and PET for small-diameter blood vessel replacement does not result in satisfactory patency rates, a great deal of attention has been given to the improvement of these materials. One approach involves shielding the blood components from the thrombogenic surface by means of coatings. The first attempts for coatings mainly focused on impregnating ePTFE or PET with ECM proteins such as collagen and fibronectin, sometimes followed by EC seeding before implantation [18, 22, 96, 121, 144, 156]. Besides their use for preclotting of the grafts to prevent exudation of blood components, these proteins are known to support cell adhesion and therefore are also meant to stimulate EC monolayer formation (see also Sect. 15.3.3.1), as well as accelerate vascular repair mechanisms. Although some success was met in terms of increased EC adhesion, both fibronectin and collagen are also known to cause thrombosis and increase the risk of NIH [40]. New coating options are therefore being actively developed.

The initiation of thrombus formation takes place by adsorption of thrombogenic proteins, for example, fibrinogen. Hence, a logical approach to address the problem involved the engineering of so-called bioadhesion-resistant surfaces. These surfaces resist protein adsorption and subsequent platelet deposition/thrombus formation. Protein adsorption is favored by hydrophobic interactions between the material's surface and adsorbed proteins, as well as electrostatic interactions. This process is accompanied usually by a local increase in entropy following the displacement of water molecules and counter ions from the first nanolayers of the surface. Passivation of the polymer's surface by reducing the entropic effects that drive protein adsorption and platelet adhesion is therefore an interesting concept. The surface of ePTFE bears a negative charge, which may slow down or prevent the adhesion of platelets and bacteria. However, its hydrophobic nature makes if susceptible for protein adhesion and subsequent thrombus formation. Currently, there are two main avenues in the prevention of bioadhesion leading to thrombosis, being the passivation of surfaces by preadsorption of synthetic or natural molecules.

15.3.1.1 Coating of Grafts with Synthetic Materials

Carbon

One of the oldest and widely-investigated methods involves supplying ePTFE and PET grafts with a carbon-based coating. Carbon is known to be highly biocompatible and has been used as a thrombosis

deterrent since several decades ago, for it has been shown to prevent platelet adhesion and fibrin deposition [15, 76]. Of all coating methods explored, indeed the application of this concept is one of the few that has been FDA-approved for commercialization by Bard PV (Carboflo grafts). However, a recent multicenter, clinical study did not show significant differences between carbon-impregnated and control ePTFE grafts when used in femoral-anterior tibial bypass surgeries [84]. It remains therefore doubtful to what extent carbon actually assists in graft survival.

Polyethylene Glycol

Probably the best-known and most widely-explored molecule for surface modification of implant materials is polyethylene glycol (PEG). It was observed in the 1970s that PEG passively adsorbs onto glass surfaces, which leads to decreased adsorption levels for both platelets and thrombin [223]. Subsequent studies demonstrated lower protein and cellular adhesion onto PEG than any other known polymer [134]. This phenomenon is thought to be caused by the presence of a strongly hydrophilic structural repeat unit, $[CH_2–CH_2–O]n$, which gives rise to a water-solvated structure that can form a "liquid-like" surface with highly mobile molecular chains without a systematic molecular order [142]. Unlike other polymers that present similar water-solvated structures, PEG is unique in having only small hydrophobic methylene groups. Moreover, no surface charges are present. These properties are thought to account for the lack of protein or cellular binding.

Surface modification of synthetic grafts with PEG has been attempted using several methods, including bulk modification [125, 187, 218], covalent grafting [54], and physical adsorption [86, 240]. In most cases, investigators have been able to demonstrate increased resistance to protein binding in vitro. Preliminary animal studies are still scarce, but Karrer et al., for example, coated ePTFE with polypropylene sulfide–PEG and tested these grafts in a porcine model of an extracorporeal femorofemoral AV shunt [86]. Grafts were assessed for cellular and microthrombi deposition within 9 min. The polypropylene sulfide–PEG-coated ePTFE showed decreased thrombogenicity [86]. In vivo results have been inconsistent though, and clinical studies using PEG-modified synthetic grafts have yet to be performed. The biggest challenge with respect to PEG-based protein-resistance appears to be firm bonding to polymer surfaces, with PEG-ylated surfaces often rapidly losing their integrity in vivo and thus anti-adsorption qualities. For PEG-grafted grafts, another issue is the chance of oxidation of the terminal hydroxyl-group of PEG in vivo, which leads to an aldehyde-group that may react with proteins [59], annulling the beneficial property of PEG. Instead of using PEG as the sole material modification, it has often been used as a spacer molecule to attach molecules such as peptide-fragments or anti-thrombotic drugs to synthetic grafts [25, 200].

Phosphatidylcholine

Phosphatidylcholine is another popular candidate for coating of polymer grafts. Phosphatidylcholine is the predominant glycerolphospholipid found in the animal cell membranes and has been shown to limit protein, platelet, and cell adhesion in vitro [91, 122, 212]. It has been proposed that this phenomenon is due to the zwitterionic nature of the phosphorylcholine head group that, while carrying both positive and negative charges, is electrically neutral. Strong binding of water occurs, creating a hydration layer that resists protein adsorption in a similar fashion as PEG [69]. Efforts have been aimed at the formation of stable "membrane-mimetic" films through protein anchors [80] and in situ polymerization of synthetically modified polymerizable phospholipids [129]. The protein and cell-resistant properties of the exposed phosphatidylcholine layer are usually retained. Chaikof et al. recently applied a polymerized membrane-mimetic film onto the luminal surface of a 4-mm-diameter ePTFE vascular prosthesis and demonstrated dramatically reduced platelet adhesion in a baboon femoral AV shunt model [77]. Besides using phospholipids for modification of ePTFE grafts, a variety of PU-based polymers have also been synthesized that incorporate the phosphorylcholine head group within the polymer backbone [73], and phospholipids have also been applied to stents [110]. However, animal studies of phosphorylcholine-coated grafts [24] and stents [99, 228] have had inconsistent results, whereas the first clinical experience [45] did not indicate a unique benefit of the coating.

Poly(Diol Citrate) (PDC)

Polyesters elastomers composed of poly(diol citrates) (PDCs) are a novel family of materials for biomedical applications that were developed in Dr. Ameer's laboratory. In particular, poly(1,8 octanediol citrate) (POC) can easily be coated onto ePTFE grafts without significantly affecting graft mechanical properties such as compliance [236]. At the same time, it offers improved biocompatibility when compared with uncoated ePTFE. The hydrophilicity is increased by the coating, which improves cell-adhesion properties and POC itself has mild anticoagulant properties [139]. POC has been tested both in vitro, showing retarded blood clotting and reduced platelet adhesion, and in an in vivo pig model, showing low inflammatory reactions, less fibrin coagulum, and lower platelet counts (see Fig. 15.2) [236]. These results all indicate favorable modification of ePTFE to reduce thrombogenicity and potentially decrease NIH. Furthermore, it was shown that both porcine and human endothelial-like cells isolated from peripheral blood attached and proliferated well on the POC-coating, indicating support for EC retention after seeding. Although research on POC is ongoing, results so far suggest that POC improves the thromboresistance of ePTFE grafts and may serve as a mediator for mechanical interlocking between ECs and the underlying ePTFE material without altering the mechanical properties of ePTFE grafts.

15.3.1.2 Coating of Grafts with Natural Materials

Albumin

Albumin, one of the most abundant proteins in blood, is known to resist platelet deposition [71] and has therefore been exploited for surface passivation of synthetic grafts by means of creating conditions that favor albumin adhesion instead of other, more thrombosis-sensitive proteins such as fibrinogen or fibronectin [149]. Albumin, although hydrophobic as opposed to the hydrophilic PEG, is considered to resist cell, protein, and platelet adsorption because of the lack of peptide sequences for interaction with those blood constituents. The direct covalent attachment of functional albumin to the surface of ePTFE and PET surfaces has been attempted [71], as well as increasing the selective affinity for endogenous albumin [35]. An albumin-coated PET graft by Bard Co. was commercially available for large-diameter vessels and showed good biocompatibility [95, 128], but has been discontinued since, possibly related to the lack of improved patency numbers in clinical studies [4, 98]. Notable issues with albumin passivation of surfaces include the possibility of eventual displacement by other proteins in the case of adsorption and potential denaturation of the protein, hampering long-term effectiveness.

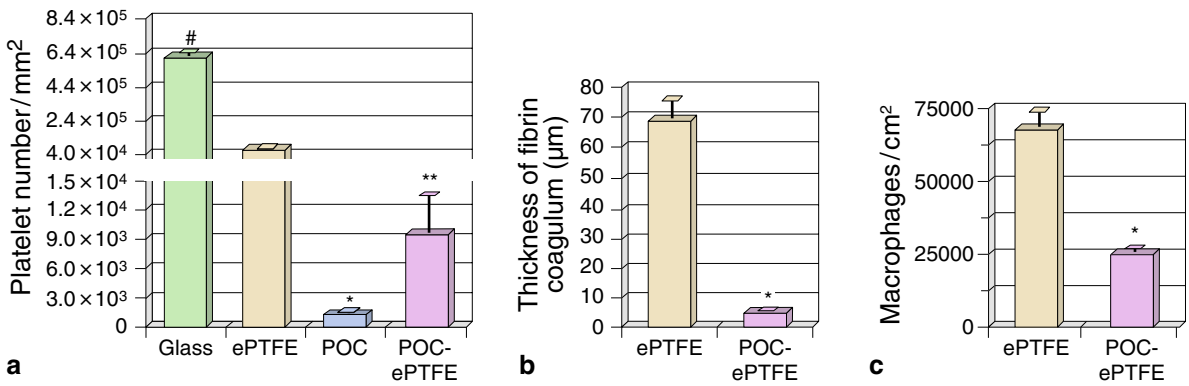


Fig. 15.2 Poly(1,8-octanediol-co-citrate) (POC) coating of expanded poly(tetrafluoroethylene) (ePTFE) grafts (POC-ePTFE) results in a lower number of adhered platelets in vitro compared with glass and ePTFE (**a**). In vivo, after 1 week of implantation in a porcine iliac artery model, less coagulum formation (**b**) and macrophage density (**c**) was observed, amenable to graft survival. Asterisks, double asterisks, and hash indicate $p<0.05$ vs. ePTFE. Data is mean ± SD, $n=6$. Reprint from [236]

Elastin

Although collagen and fibronectin may have lost their charm as coating candidates because of their risk of thrombosis by platelet activation, increased knowledge of the biology of blood vessels has spawned interest in another ECM protein, elastin. Elastin, the protein responsible for the recoil property of blood vessels, is present in the medial layer of vessels in the form of laminae. It is well-known as the "missing link" in TEBV technology [151], for it has proven to be a difficult task to trigger and control elastin production by cells in vitro. Besides providing elasticity, elastin has also been observed to elicit minimal platelet adhesion and activation [14]. Moreover, it was shown that elastin inhibits monocyte adhesion and leukocyte transmigration [203], as well as inhibits mitogenic activities of SMCs [117]. All these factors diminish thrombogenicity and inhibit subsequent NIH development, contrary to the highly thrombogenic collagen. Since then, a number of groups have embarked upon the synthesis of elastin-like polypeptides (ELP) in combination with synthetic grafts. ELP is an example of an artificial ECM component that may be engineered to be biosynthetically produced with tailored biological properties to mimic natural elastin [185]. Woodhouse et al. passively adsorbed a recombinant ELP of the human elastin gene on PET-, ePTFE-, and PU-based grafts and observed decreased platelet adsorption and activation in vitro [232]. In a similar fashion, Jordan et al. developed a recombinant elastin-mimetic amphiphilic protein polymer with the peptide-sequence valine-proline-glycine-valine-glycine (VPGVG) as main functional constituent. Impregnation of ePTFE grafts with this polymer was tested in an in vivo baboon AV model and minimal thrombogenicity was observed over a 60-min timeframe, as well as negligible fibronectin deposition compared with the control ePTFE grafts [78]. These results, albeit still preliminary, support further investigation into the use of elastin-mimicking coatings for vascular graft modification.

15.3.2 Protein and Drug Immobilization and Release

Apart from coating grafts with so-called thromboresistant materials, immobilization or release of proteins, peptides, and/or small-molecule drugs that interfere with the thrombolytic pathways and/or events leading to NIH has been given considerable attention. Especially for thrombosis, virtually every step in the cascade leading to its development has been targeted by the incorporation of a selective inhibitor or activator (Fig. 15.1). The inhibition of the coagulation pathway and platelet aggregation/activation is usually perceived as the task of emulating functionality of the natural EC monolayer that is known to secrete a number of pro- and anti-thrombosis factors. Promising results have indeed been achieved using this biomimetic approach both with bioactive agents that intervene with either thrombin or platelet aggregation and activation. NIH inhibitory agents that are most often explored operate by nonselectively preventing cell growth progression, such as paclitaxel and sirolimus, while other agents prevent proliferative stimulators, for example, PDGF or fibroblast growth factor (FGF), to exert their mitogenic effects.

15.3.2.1 Agents That Inhibit Thrombin

The agent that has been used clinically during cardiovascular surgeries as the anticoagulant of choice, heparin, has also historically been the most popular drug to use in combination with vascular grafts (for a review, see [147]). Heparin binds to antithrombin III, which becomes active and subsequently inactivates thrombin, thereby inhibiting the polymerization of fibrin. Heparin has been immobilized using electrostatic immobilization [193], covalent grafting [25], and loading in the bulk of a polymeric coating [123], e.g., a hydrogel or fibrin gel [39]. Initial results have not been solely positive, which is mainly attributed to the usual transient nature of heparin immobilization. Still, several heparin-coated grafts have been FDA-approved recently, including a PET-based graft (Intergard Heparin, InterVascular Co.) and an ePTFE-based one (Propaten, W.L. Gore). A nonrandomized clinical trial on the use of Propaten grafts for peripheral procedures suggested better results compared with regular ePTFE grafts, although it should be noted that adequate controls were not included in the study [19]. A 2-year study on Propaten grafts for below-the-knee bypasses, nevertheless, showed primary patency rates of 85% [34]. Another randomized clinical study on heparin-bonded ePTFE and Dacron grafts for femoropopliteal usage failed to show any significant benefit after 5 years [33], whereas a 5-year study on the

intergard heparin graft for femoropopliteal bypass surgery showed patency rates similar to vein grafts [177]. Although preclinical trials show promising results, there is no consensus whether those results translate to equally successful clinical outcomes. Nevertheless, new technologies involving heparin bonding have been developed, showing potential [107, 115], so it is unlikely that the efforts to fabricate heparin-incorporating grafts will subside soon. An important drawback to heparin is the need for cofactor antithrombin III and its inability to inactivate clot-bound thrombin. Thus if any thrombus-formation occurs in the graft, heparin is incapable of dissolving that thrombus. Therefore, scientists have actively searched for alternatives to heparin.

Extensions of the EC-emulating approach to inhibit thrombin for prevention of fibrin formation include the incorporation of thrombomodulin (TM) [112, 119, 192, 231, 233] and tissue factor pathway inhibitor (TFPI) [159, 170, 196, 197]. TM binds directly to thrombin to inactivate it, while TFPI inhibits factors in the upstream coagulation cascade, most notably the factor VII-tissue factor complex, ultimately inhibiting the conversion of thrombin from prothrombin. TM was shown by the group of Dr. Butler to have not only antithrombotic effects, but also anti-proliferative effects after deploying a TM-coated ePTFE stent in a porcine carotid artery [231]. After 4 weeks, NIH was reduced when compared with bare ePTFE grafts, although this was only significant (27% less intimal thickness) at the proximal end [231]. TFPI passively adsorbed to albumin-coated PET grafts was shown to inhibit thrombosis and reduce intimal thickness from 0.57 to 0.26 mm after 3 months of implantation in mongrel dogs. An alternative direct thrombin inhibitor that is not constitutively expressed by ECs is hirudin. Wyers et al. [234] covalently attached hirudin to BSA-coated Dacron grafts and after 2 h exposure to nonheparinized blood in a canine model, less thrombus- and pseudointima-formation was observed.

15.3.2.2 Agents That Inhibit Platelet Activation

Although the inhibition of thrombin and subsequent fibrin clot formation remains a highly important and fertile field for research, recent studies have demonstrated that antiplatelet therapy is associated with a lower incidence of cardiac events and a lower risk of hemorrhage when compared with anti-coagulatory interventions. Therefore, agents that can antagonize platelet adhesion, activation, and/or aggregation are highly appealing alternatives.

ECs in their healthy, uninjured state secrete prostacyclins (PGI2), which raise cyclic AMP (cAMP) levels in platelets. However, low cAMP levels are responsible for platelet activation. Therefore, the use of prostacyclins or analogs thereof is anticipated to inhibit platelet aggregates to form. For this purpose, Chandy et al. have successfully combined PGI2 with both Dacron and ePTFE grafts [23]. Another group combined iloprost, a PGI2 analog, with an alginate hydrogel, and they showed a significant reduction in the in vitro formation of thrombus [214]. Of the non-EC drugs for platelet inhibition, the oldest and a most widely investigated one is aspirin or acetylsalicylic acid. The drug interferes with the production of thromboxane A2 (TxA2), which activates neighboring platelets to aggregate. Literature on incorporation of aspirin-like substances in vascular grafts is still limited, though with varying results on efficacy reported. Nevertheless, acute platelet deposition was reported to decrease in a canine ex vivo model [74, 164, 171]. For dipyridamole as an antiplatelet agent, so far only one publication has reported reduced platelet adhesion [2]. Although their PU graft with surface-conjugated dipyridamole experienced improved patency in vivo in a goat model, this effect may also have been caused by the well-known antiproliferative effect of the drug (see below). Finally, there exist multiple candidates for antiplatelet therapy that block several receptors on the platelets surface. The receptor for TxA2 can be blocked by clopidogrel, but so far, no research has been done on the application of this compound in vascular grafts. Another receptor, the GPIIb/IIIa glycoprotein, is essential for platelet aggregation, which form bridges between them by fibrinogen binding to these receptors. Abciximab is a selective blocker of the latter, which effectively prevents the formation of these bridging connections. A clinical study from 2006 on abciximab-coated stents showing a decrease in stenotic area from 37.9 to 18.9% after 60 days on placement indicates the potential of this agent for vascular grafts as well [89].

15.3.2.3 Agents That Inhibit Neo-Intimal Hyperplasia

Besides the anti-platelet and anticoagulatory agents mentioned above, inhibitors of NIH have received

considerable attention. Agents preventing the over-proliferation of SMCs (or, as recently discovered, fibroblasts) may have an advantage over biological reagents as often used for the prevention of thrombosis. Anti-NIH drugs are usually small molecules and therefore are less likely to elicit an immunologic response. The delivery of such agents may take place by luminal coating or immobilization of the drugs, injecting the drug at high-at-risk locations like the anastomoses or perivascular placement of a drug depot. In the case of stents, drugs may be coated onto the struts. On the basis of the recent discovery of the role of adventitial cells in NIH-development, a perivascular approach may be advantageous, for the drug can be in proximity to the cells critical for NIH formation compared with application on the luminal side. Therefore, for vascular grafts or the treatment of vein grafts, many groups are looking at ways to deliver anti-NIH drugs to the external side of the graft [37, 173, 184], especially around anastomosis-sites, where NIH is more pronounced.

Although no anti-proliferative drug-coated synthetic vascular grafts have been FDA-approved for clinical use, the successful track record in coronary interventions has led to numerous attempts to achieve similar reductions in the degree of stenosis for ePTFE and PET grafts. For example, both a paclitaxel-coated ePTFE graft in a porcine AV model [109] and a canine AV ePTFE graft where the paclitaxel was applied perivascularly at the time of surgery using a thermosensitive hydrogel as carrier [130] showed reductions in stenosis development. Most recently, the first clinical trial was conducted on vascular grafts, in which 109 patients received ePTFE grafts for femoropopliteal bypass conduits [132]. One group received control grafts, whereas in the other group, a paclitaxel-eluting PLGA polymer film was wrapped around the distal anastomosis. After 2 years, the average patent diameter of the anastomosis in the control group was 2.1 mm less than in the paclitaxel-treated group, without any adverse effects associated with the wraps [132].

A sirolimus-coated ePTFE graft was implanted in a porcine iliac artery by Cagiannos et al. [21], and they found a significant reduction in NIH from 28.5 to 16.2%. Rotmans found that in ePTFE AV grafts in a porcine model, placement of a sirolimus-eluting stent at the anastomosis-location was beneficial, although their use of a stent in combination with an ePTFE graft is undesirable [168]. Dipyridamole, already mentioned for its anti-thrombotic function, has been reported to have an anti-proliferative effect as well and this combination of advantageous properties would make it an excellent candidate for use in vascular grafts. It acts on the receptor for PDGF, thereby preventing the mitogenic effect of the latter on SMCs. It may therefore be more specific than paclitaxel and sirolimus, which, as anti-cancer/immunosuppression drugs, block cell proliferation in general, possibly also preventing reparative events such as endothelialization by ECs. However, reports on the success of dipyridamole vary [100, 188] and the low potency of this drug compared with paclitaxel and sirolimus may prevent it from reaching significant NIH prevention at clinically safe dosages.

15.3.2.4 Nitric Oxide

Vascular graft therapies revolving around the local delivery of nitric oxide is currently among prime topics in cardiovascular therapy strategies. EC-derived nitric oxide (NO), secreted at a surface flux of an estimated 10^{-7} mol/cm^2/day [209], is synthesized from L-arginine by nitric oxide synthase (NOS) in cells and has been shown in the past decades to be of paramount importance in the human vasculature, for it is highly vasoprotective by inhibiting platelet adhesion and activation, leukocyte chemotaxis, SMC proliferation and migration, and promoting EC proliferation and survival [217]. This has led to an exponential increase in research on mimicry of endothelial NO-release as a therapeutic way to alleviate thrombosis and neointimal hyperplasia (NIH) after vessel injury/in vascular grafts by remediating NO deficiency as a result of a lacking functional EC monolayer. NO, as a highly active free radical, is captured immediately in the bloodstream by oxygen and hemoglobin and therefore, highly specific localized delivery is required as well as controlled, sustained release. Recent work on NO donors has already shown the potential of this strategy to reduce platelet adhesion to graft walls under both dynamic and static conditions and to inhibit SMC overproliferation, indicating that NO delivery may introduce a way to prevent thrombosis and NIH, at least during the period following the intervention.

Recently, many different approaches have been investigated to deliver NO to vein grafts for a prolonged

period of time and thereby to reduce early thrombosis and NIH, but a remaining critical issue is the short-lived effect of local NO delivery. Although it has repeatedly been reported that NO release kinetics are controllable depending on the molecular architecture of the NO donor, the short durability of NO release still imposes a major limitation to achieve satisfactory therapeutic efficacy for on-demand applications. Therefore, chemical compounds capable of releasing NO are continuously investigated for long-term NO release. Several compounds that are studied regularly in the context of vascular therapies are N-diazeniumdiolates and S-nitrosothiols. Diazeniumdiolates have the major advantage that NO-release is spontaneous in a physiologic solution and their release kinetics is highly predictable and easily tuned by changing the nucleophile to which the diazeniumdiolate moiety is attached. In a seminal paper by Larry Keefer et al., diazeniumdiolated ePTFE-based grafts were prepared and exposed to the baboon circulatory system for 1 h using an AV shunt system [189]. Platelet adhesion was significantly reduced compared with untreated grafts. Although their method altered the grafts mechanical properties, rendering it unsuitable for further in vivo testing, they did convincingly show the plausibility of using NO-donors for vascular grafts. Since then, multiple groups have elaborated on their groundwork on diazeniumdiolate-based strategies, incorporating the compounds using microparticles [157] and polymer films [42, 140, 160, 200]. Interesting results were recently obtained, when it was shown in a rat artery injury model by Dr. Kibbe's group that the perivascular application of a diazeniumdiolated drug simply in its powder-form was more effective than when a gel was used as the delivery vehicle. Moreover, they showed that a relatively short-acting NO-donor was more successful at inhibiting NIH [82, 152]. These results support currently upheld theory that overcoming the early inflammatory response and thrombosis may be sufficient to prevent NIH and subsequent late-graft failure.

S-nitrosothiols function in human cells and vasculature as a reservoir and transporter of NO. These include S-nitroso-albumin, S-nitroso-cysteine, and S-nitrosoglutathione. Nitrosothiols are interesting candidates for NO delivery because they naturally occur in vivo and they can release their NO load via exposure to light or decomposition catalyzed by copper ions and/or vitamin C [190]. Frost et al. where the first to demonstrate the feasibility of this concept by embedding a silica-particle anchored nitrosothiol in a PU film, which released NO triggered by light, but not by physiologic solutions [43].

15.3.2.5 Combinatory Approaches

Positive effects of both anti-coagulant and platelet inhibitors have clearly been shown when combined with synthetic grafts. However, in animal models and clinical trials, the extent to which each particular therapy limits both thrombosis and/or NIH has been variable. The one-dimensional approaches usually tested may indeed not be potent enough to reach a clinically relevant threshold since only partial thromboresistance or protection from NIH is provided. Moreover, the depot nature of virtually all preclinically tested concepts offers little flexibility to tune regulation of the thrombosis/NIH responses. A potential solution to these issues is a combination approach, incorporating multiple concepts to reach a synergistic effect. Indeed, several groups have published results on a variety of combinatory graft therapies, often co-incorporating multiple thrombosis-inhibiting drugs [51, 58, 205] with antiproliferative drugs [70, 233]. Although the multitargeting concept in grafts is relatively new, two in vivo trials have already garnered very encouraging results. Heise et al. used 4 mm ePTFE grafts, applied a PLLA/PEG coating with iloprost (anti-platelet) and hirudin (anti-thrombin) to the surface and implanted them in the femoropopliteal position in 18 pigs [58]. After 6 weeks, the flow rates of drug-coated grafts was not reduced, while untreated grafts showed a reduction in flow rates of approx. 70%. NIH levels were also reduced, although the reduction was only significant in part of the anastomosis. It should be pointed out that the authors failed to include a control group with PLLA and PEG included in the graft. Given the known non-fouling effect of PEG, it remains unclear to what extent the improvement in patency of the grafts was due to the combination of drugs or due to the included PEG. In another in vivo study, Ishii et al. combined sirolimus and heparin in a PU graft, including hyaluronic acid and collagen as graft fillers that served as the drug reservoirs. At 6 months after implantation in the rabbit abdominal aorta, sirolimus-heparin grafts showed an additive beneficial effect on neointimal formation when compared with grafts with heparin alone [70], supporting a combination approach.

15.3.3 Stimulation of Endothelial Cell Monolayer Formation

It has been unequivocally accepted that the successful and permanent formation of a functional EC monolayer is the holy grail of vascular repair. ECs physiologically form the luminal lining of all blood vessels and serve as the bioregulator of vascular functions. It provides a selectively permeable barrier between circulating blood and the vascular wall. Functional ECs secrete antithrombogenic factors that prevent platelet adhesion, activation, and aggregation. Furthermore, leukocyte adsorption is inhibited, as well as overproliferation and migration of the underlying SMCs. The emulation of EC functionality, as discussed in the previous paragraph, by the incorporation of all these phenotypical features in vascular repair technologies would be beneficial to graft survival, but also presents a daunting task to investigators worldwide, especially when surface modifications of polymers need to exhibit all properties at once. It should therefore come as no surprise that the number of publications on the actual (re)-endothelialization of synthetic grafts, stents, and autologous grafts is staggering. However, virtually all these results are severely confounded by the fact that the majority of animal models are poorly chosen.

Zilla et al. [244] pointed out that vascular graft lengths that are typically studied are too short, that is less than 10 cm, thereby facilitating complete transanastomotic endothelialization that cannot realistically be obtained in clinically used graft lengths, which are often much longer than 10 cm. Moreover, short graft lengths usually make it challenging to distinguish with any degree of certainty whether midgraft endothelialization takes place by transanastomotic ingrowth and migration or by perivascular infiltration of surrounding ECs. Furthermore, it has become clear that humans do not possess the same endothelialization potential as animals. Finally, animal models often consist of juvenile animals, which are known to reendothelialize grafts more rapidly than senescent ones. Since graft applications typically involve older patients, the issue of age is also an important parameter. It seems therefore that the current knowledge base on the reason for the lack of clinical translation of endothelialized grafts should stimulate improvement of in vivo models.

These issues not withstanding, at present, encouraging confluent EC monolayer development on synthetic grafts is still a highly active field. For a long time, it was thought that ECs must be in proximity to other cells such as SMCs to exert their functonality. It is now known that that the modulating effect of endothelium can occur without proximity to SMCs. Hence, endothelialization of the standard-of-care synthetic grafts and scaffolds remains a viable concept [145]. For the purpose of this chapter, we will therefore point out the most recent and exciting concepts in the latter area. Two broad methodologies can be distinguished: (1) Incorporation of techniques/molecules to improve endothelialization following implantation (so-called EC fishing); (2) Improvement of EC retention after implantation of EC-seeded grafts. The first approach is especially interesting, for it could lead to a truly off-the-shelf biohybrid synthetic graft, while optimization of the second is of grave importance for TEBV concepts.

15.3.3.1 In Situ Endothelialization

Spontaneous endothelialization of synthetic vascular grafts may occur through several mechanisms: (1) transanastomotic ingrowth/migration of adjacent ECs; (2) perivascular transmural ingrowth of surrounding cells; (3) homing and adhesion of circulating ECs from the bloodstream. In animals, rapid endothelialization typically occurs and it is thought to take place primarily through transmural migration. In humans, however, re-endothelialization is conspicuously absent and EC layers on both ePTFE and Dacron-type grafts fail to develop properly [52, 180]. Although this problem may reflect simple differences in species, it may very well be that the chosen animal models, as pointed out before, are poor reflections of the clinical situation in humans. Clinically, transanastomotic ingrowth is extremely limited, while transmural migration is limited to SMCs, which contribute to the development of NIH. Although reendothelialization of synthetic grafts has continued to be very limited, it is, nevertheless, thought that the human vasculature does harbor the potential for successful EC monolayer formation by circulating cells, presumably through circulating endothelial progenitor cells (EPCs). EPCs were identified 10 years ago as nature's "emergency kit" for injured blood vessels [10]. They are partially differentiated stem cells derived from bone marrow cells, with the potential to differentiate into ECs for re-endothelialization of damaged EC monolayers. It is this cell source that has attracted considerable interest

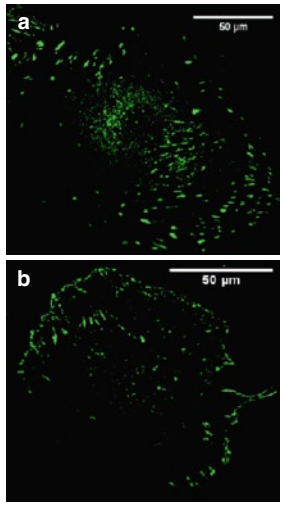
from the scientific community. However, flow conditions are likely unfavorable for EPC adhesion in vivo after implantation, whereas the number of circulating EPCs is low. Together with the usual inherent thrombogenicity of synthetic grafts and the lack of physiologic cues for EPC recruitment and adherence, limited graft endothelialization should not be surprising. The use of biological cues for homing and increased adhesion of EPCs to the graft is therefore actively investigated. Currently, multiple strategies can be identified to increase adhesion of ECs and/or EPCs. The earliest attempts to enhance the adhesion of ECs to synthetic surfaces revolved around the application of ECM components to render biomaterial surfaces cell-adhesive and augment their interaction with cells.

Extracellular Matrix Components

ECM components such as fibronectin, fibrin, collagen, and laminin are known for their supportive function and favorable structure for cell adhesion. Although many reports are available that give exceptional numbers for endothelialization when using coatings/fillings of collagen [121, 144, 156] and fibrin gels [49], fibronectin [121, 215] and/or laminin and combinations thereof [213], the exact mechanisms and outcomes in vivo of applying such complex proteins are not trivial. Beside issues of pathogen transfer and immunogenic risks, ECM proteins present cells with a plethora of cell binding and growth factor-binding domains, complicating the control over cell adhesion selectivity. Moreover, cell adhesion domains in large ECM proteins may often be sterically unavailable to approaching cells because of protein unfolding upon adsorption to a material. To circumvent these problems and enhance control over cell adhesion, nowadays often only particular ECM protein domains are used that function as basic subunits for cell adhesion and proliferation by interaction with cell surface-specific integrins. Such short peptide segments can be easily synthesized, are more stable than large ECM proteins, and have lower immunogenic potential.

The minimal peptide segment derived from fibronectin required for cell adhesion, for example, is arginine-glycine-aspartic acid (RGD), and numerous studies have shown that the incorporation of the peptide sequence greatly enhances cell adhesion to substrates [7, 60]. However, RGD is a nonspecific cell adhesion ligand and platelets and several cell types besides circulating ECs and EPCs can interact with the peptide. Hence, adhesion peptides that are more specific to ECs are of interest. A peptide derived from laminin, YIGSR, which does not support platelet adhesion, has been used extensively to promote EC adhesion, e.g., by Taite et al. [200] as a cell adhesive component of PU grafts. In their experiments, it was shown that YIGSR inhibited platelet adhesion while increasing EC adhesion and proliferation. An example of specific peptides that have recently garnered attention is CRRETAWAC, with high affinity to the $\alpha 5\beta 1$ integrin, mainly prominent in ECs. Larsen et al. [108] have shown that CRRETAWAC is superior to RGD or fibronectin, since it promotes platelet adhesion to a far lesser extent and adhering ECs secreted higher amounts of anti-platelet prostacyclin [108]. REDV and P15 are two other peptides with increased EC specificity currently under investigation [67, 111].

Anamelechi et al. [8] acknowledged the rather weak affinity of protein-integrin bonds as they occur between ECs and ECM proteins or peptide segments thereof. They postulated that this low affinity does not lead to sufficiently matured cell layers on substrates after seeding for short periods and leads to significant cell loss upon implantation and exposure to flow. They therefore sought to increase the binding affinity for ECs by employing streptavidin-biotin ligand-receptor technology, which is known for its high affinity. In brief, they biotinylated a standard fibronectin (Fn) coating on ePTFE grafts to provide the grafts with a biotin receptor exposing surface (b-Fn). Human umbilical vein endothelial cells (HUVECs) were incubated on Fn-surfaces, b-Fn surfaces, and b-Fn surfaces with streptavidin-conjugated RGD (RGD-SA) coated on the b-Fn surface. They showed that biotinylation of Fn does not interfere with the formation of focal adhesion contacts between ECs and Fn, while additional available RGD-domains due to the RGD-SA-b-Fn complexes resulted in an increase in average number of focal adhesion contacts per EC, as well as an increase in focal adhesion contact size (Fig. 15.3). The latter is a sign of maturation of the focal adhesion. Although no in vivo testing has been done as of yet, this extra "loading" of RGD-domains for graft coatings does offer potential for improving EC retention for one-stage seeding procedures, while possible substitution of EC-specific peptide segments for RGD may provide a sufficiently strong and specific EC capture tool. In short, it has been successfully



c

surface treatment	percent of cells with focal adhesions	av no.of focal adhesions per cell	av size of focal adhesions (μm^2)
FN (A)	100	308.3 ± 30.7	1.73 ± 0.19
bFN (B)	100	309.0 ± 18.0	1.57 ± 0.10
bFN + WT-SA	62.4 ± 23.1[b]	203.2 ± 12.2	1.22 ± 0.15
bFN + RGD-SA	100	430.0 ± 19.9[c]	1.84 ± 0.17

a Values are reported as average SEM for n = 3. Column statistics was performed using ANOVA and Tukey-Kramer post hoc tests.
b Significantly different compared to all other treatment group ($p < 0.01$).
c Significantly different compared to all other treatment group ($p < 0.05$).

Fig. 15.3 Focal adhesion formation of a human umbilical vein endothelial cell (HUVEC) bound to fibronectin (FN) (**a**); A HUVEC bound to RGD-streptavidin-biotinylated FN (RGD-SA-bFN) (**b**). Quantitative analysis of focal adhesion for HUVEC seeded onto glass cover slips for 1 h with various surface treatments (**c**). Adapted from [8]

demonstrated that short peptide sequences instead of the bulk ECM proteins can selectively enhance EC layer formation. Together with new technologies for micropatterning of such peptides, a high level of spatial control over EC layer organization may soon be achieved.

Growth Factors

Besides the use of ECM components and peptide-fragments thereof to influence cell adhesion, the immobilization of growth factors could modulate subsequent cell proliferation, differentiation and activity, which may lead to enhanced monolayer formation. A variety of growth factors have been used to attract ECs, for example, by chemotaxis. Greisler et al. have reported the beneficial effect of acidic fibroblast growth factor (aFGF) and heparin in fibrin glue in which the ePTFE grafts were impregnated. aFGF is a potent mitogen and heparin enhances aFGF activity [20, 50]. A significant increase in luminal EC proliferation and functional monolayer formation was reported. Another well-studied angiogenic growth factor, bFGF, has recently been incorporated into PEG hydrogels for vascular graft applications [32]. The resultant materials could guide cell alignment and migration of cells, indicating its usefulness in controlling tissue formation in synthetic grafts. Wissink et al. [230], on the other hand, showed that binding of bFGF to immobilized heparin on grafts stimulated the proliferation of HUVEC. Although the use of bFGF is promising, bFGF adsorbed to PET grafts tested in a canine model showed an additional mitogenic effect on fibroblasts, which is a potential risk for NIH development [201].

Zisch et al. [245] have coupled a cell membrane growth factor, ephrin-B2, into fibrin matrices and shown that fibrin-bound ephrin-B2 stimulated EC angiogenic responses. This work has demonstrated that matrix-bound ephrins can evoke prolonged and local signaling events in adjacent cells and tissues. In more recent work, ephrin-A1 was incorporated into PEG hydrogels and was found to stimulate EC adhesion and spreading [138]. Moreover, ECs cultured on that surface spontaneously reorganized themselves into extensive vasculature-like networks, indicating possible additional effects that are beneficial to graft survival.

VEGF incorporation in vascular grafts to promote endothelialization [29, 158, 222] has been a popular approach for many years because of its angiogenic effects. Yet a recent report by Walpoth et al. [222] indicates increased NIH development over time compared with bare ePTFE grafts. In a pig carotid artery model, the difference in endothelial coverage of the lumen was insignificant between bare ePTFE, fibrin-coated ePTFE, and VEGF-containing fibrin-coated ePTFE after 1 month. However, the progression of NIH was more severe in VEGF-containing grafts, indicating that the effect of VEGF signaling may not be purely inhibitory on SMC proliferation. Indeed, similar detrimental effects on NIH have been reported when VEGF was used to enhance ePTFE endothelialization in a rabbit abdominal artery interposition models [158], although

in that report EC coverage was significantly enhanced under the influence of VEGF. It is possible that although the chemokine effect of VEGF on EC mobilization is well established, VEGF is not as specific as previously thought and in vivo SMCs or progenitors thereof may also be recruited in addition to ECs.

Specific EPC Capture

There is no clear consensus on what subpopulation of the mononuclear circulating cells may be classified as EPCs; nevertheless, most investigators consider EPCs to be the subpopulation of cells that express both of the surface proteins CD34 and CD133 (although it should be noted that there is increasing evidence that at least one other subpopulation exists with different cell markers that may differentiate into ECs). One of the earliest attempts to capture circulating EPCs selectively to rapidly endothelialize synthetic grafts or stents was therefore based on the immobilization of CD34 antibodies. A stent coated with CD34 antibodies has been developed by Genous and is currently in phase II of clinical trials, showing very promising results in first clinical evaluations. The clinical feasibility and safety was shown in a prospective study, wherein 129 patients received a CD34-antibody-coated stent, while receiving a single dose of statin right after the procedure. Statins are thought to promote circulating EPC levels and homing to injury sites. One year postimplantation, the percentage of patients with major adverse cardiac events (MACE) was only 9.2%, while no late thrombosis has been observed. It should be noted, however, that later follow-up data are not yet available [28].

In spite of these positive results on anti-CD34 stents, results with similarly coated synthetic grafts have been disappointing. Rotmans et al. [167] reported that AV implantation of ePTFE grafts with anti-CD34 in pigs leads to EC coverage of 95% vs. 5% in uncoated grafts after 3 days and 88% vs. 32% after 4 weeks. In that respect, the increased EC adhesion and retention seems to be on par with the stents. However, at the venous anastomosis, the NIH level was tripled in coated grafts. This may indicate that EPCs may develop into cells other than ECs, or that pro-mitogenic and/or pro-angiogenic factors are released from adherent cells that can stimulate not only the ECs, but may also activate other cells.

As pointed out, it is not clear what actually constitutes the cell population in peripheral blood that can develop into ECs. Recently, for example, it was shown convincingly in vitro that a subpopulation of circulating mononuclear cells that does not express CD34, but instead is highly positive for CD14, can develop into functional ECs [97]. Since these cells are found in much higher abundance than CD34+ cells (10–20% vs. 0.01–0.1%), it seems far more appropriate to target that fraction in favor of CD34+ cells, for example by providing anti-CD14 coatings similar to the anti-CD34 coatings. However, CD14+ cells are also known to differentiate into monocytes and macrophages and more characterization studies, both in vitro and in vivo, are needed to prevent catastrophic differentiation into inflammatory cells upon adhesion to synthetic grafts. Another group that has also recognized the potential issue with CD34 recruitment recently used immobilization of an antibody against vascular endothelial growth factor receptor 2 (VEGFR2) to capture a more appropriate cell population. Mounting evidence suggests that VEGFR2, expressed on the cell surface of progenitor cells, may be a far better indicator of exclusive endothelial differentiation potential than CD34 [153, 165], while less than 1% of CD34+ cells express VEGFR2 and vice versa [153, 165]. Thus, targeting VEGFR2 might result in capturing a more appropriate cell line than with CD34. Markway et al. [127] immobilized protein G on glass coverslips and anti-VEGFR2 antibody was bound with proper VEGFR2-binding group orientation. Their experiments showed exciting results, where VEGFR2-positive cells were selectively captured from a mixed cell population, with VEGFR2-negative cells not interfering with capture potential, even when the VEGFR2+ cells only made up less than 1% of cells. This result indicates the high specificity of this method and potential for a more appropriate EC monolayer creation in vivo. Although their method shows very promising results, only ~0.5% of the VEGFR2-positive EC population was captured, even though flow rates were at subphysiological levels. However, if the capture efficiency can be improved, VEGFR2 targeting may be a superior alternative to CD34.

15.3.3.2 Endothelial Retention After Ex Vivo Endothelialization

The EC seeding concept of ePTFE and Dacron grafts has been around for approximately 30 years. The use of autologous cells may only be feasible if one of the

cell adhesion strategies as just discussed offers sufficiently potent, instantaneous, reliable EC adhesion for a single-stage seeding approach to be performed. In that case, ECs may be harvested at the time of surgery, seeded onto the graft and the graft implanted concomitantly. However, currently this is still largely an utopian thought, and multiple-stage (i.e., isolation of cells during an additional surgical procedure and expansion in vitro before seeding onto grafts) seeding strategies seem a much more reliable option, even though this means that only nonemergency situations may be treated. At present, two issues limit the success of multiple-stage EC seeding. First, the cell source to be used continues to be problematic. Harvesting the patients own cells during an extra procedure is clearly undesirable, while the isolation and expansion of such EC populations is not a trivial task in its own right, with risks of contamination and dedifferentiation of cells. The alternative of using allogenic or xenogenic ECs is generally precluded for immunogenic reasons. Second, even when ECs (whether autologous or not) are seeded and preconditioned under dynamic flow to form a stable confluent layer of cells in vitro, surgical handling upon implantation together with a sudden (re-)exposure to flow in vivo upon implantation generally results in loss of a large percentage of the EC layer, rendering the graft thrombogenic. Although most techniques and strategies as discussed in the previous paragraph are applicable to preimplant cell seeding as well, there are two main additional avenues being pursued to increase success rates: (a) the use of alternative autologous cell sources and (b) the genetic modification of harvested autologous cells.

Alternative Cell Sources

A complicating matter in EC seeding ex vivo is the need for autologous differentiated ECs, which are obtained by harvesting a blood vessel from the patient. Besides the obvious dilemma of this extra surgical procedure, the use of these cells is usually heavily hampered by an inability to isolate and culture adequate cell numbers to seed conduits with a confluent cell layer. The use of alternative cell sources is therefore advocated strongly to avoid these issues. Besides targeting circulating EPCs for endothelialization as discussed before, other cells sources are also considered. Mesothelial cells, for example, are easily obtained in large quantities from omental fat tissue through laparoscopic procedures. Indeed, phenotypical expression profiles of high tPA [207] and TM expression [210] similar to vascular ECs indicated the potential of this cell type. Unfortunately, in vivo experiments in dogs did not yield consistent results, with Pasic et al. [150] reporting graft survival after 52 weeks of implantation of no less than 95% for omental cell-seeded PET grafts compared to 13% for bare grafts due to decreased intimal thickening. In contrast, Verhagen et al. did not find any anti-thrombogenic properties or anti-NIH effects in mesothelial cell-seeded ePTFE grafts [211].

The use of microvascular cells derived from subcutaneous fat through liposuction are easily obtained in large quantities and can be used without any culture period, which may indicate the possible birth of a viable single-stage procedure. Even though results of early animal studies were highly encouraging [57, 229], the clinical studies undertaken so far failed to show comparatively positive results. A small-scale study in which nine patients received either a microvascular cell-seeded ePTFE grafts for bare ePTFE grafts for hemodialysis access was terminated after the discovery of severe intimal thickening in the cell-seeded grafts, more pronounced than in bare control grafts [178]. Although these results are disappointing, it has been suggested that contamination of the seeded cell population with non-ECs may have been the underlying issue in this conspicuous and rather catastrophic failure [9]. More reliable procedures for procuring pure microvascular cell populations may be needed for this concept.

Currently, the most popular cell source for in vitro endothelialization of vascular grafts is arguably the earlier-discussed EPC fraction. Although self-endothelialization of grafts in situ is the ideal solution for emergency procedures, the fact that EPCs are easily obtained by venopuncture instead of the more intrusive procedures for isolation of mature ECs, mesothelial or microvascular cells, makes EPCs also a highly interesting candidate for in vitro endothelialization. Indeed, many groups consider the use of EPCs as cell source the ideal solution and are actively pursuing the creation of EPC-covered synthetic grafts. In a seminal paper by Kaushal et al. [87], EPCs derived from ovine peripheral blood were seeded on decellularized porcine arteries, preconditioned under flow for 2 days and implanted in sheep for up to 130 days. All EPC-seeded

grafts remained patent at 130 days, with no signs of thrombus-formation or NIH development and affirmed vasoreactivity to nitric oxide. He et al. [56] have studied the performance of EPC-seeded collagen coated PU grafts as carotid artery replacements in a canine model and reported thrombus-free luminal surfaces and complete patency after 3 months of implantation. Furthermore, there was no sign of NIH development observed [56]. In addition, the same researchers have proven the feasibility of translation of this strategy to human EPCs by seeding a PU graft with human peripheral-blood derived EPCs and static in vitro cultivation for 4 days, followed by 12 h of supraphysiological flow stimulation of 30 dyn/cm^2. The resulting EPC monolayer exhibited nonthrombogenic behavior, with significant endothelial nitric oxide synthase (eNOS) expression and NO and tPA production [182].

Further progress in the use of EPCs was made by Allen et al. [6], who used a clinically more relevant ePTFE-based graft for endothelialization with circulatory EPCs. It was shown that porcine EPCs cultured on poly(1,8-octanediol-co-citrate) (POC)-coated ePTFE grafts under physiological shear stress conditions exhibit EC-like behavior, with NO secretion comparable to porcine aortic endothelial cells (PAECs) (2.5 ± 0.15 and 8.8 ± 0.9 μM/10^3 cells vs. 3.3 ± 1.0 and 4.9 ± 0.08 μM/10^3 cells, for baseline levels and after VEGF-stimulation, respectively) and clotting kinetics, as well as platelet adhesion, similar or superior (see Fig. 15.4). Recently, they extended their research to human EPCs and, using a similar approach, it was shown that human EPCs possessed EC-like behavior, with eNOS, prostacyclin, and tPA levels comparable to HAECs and HUVECs, respectively. Moreover, clotting kinetics and the inhibition of platelet adhesion were similar to HAECs and HUVECs, while cell retention at physiological flow levels was successfully demonstrated [5]. Although the need for ex vivo expansion, approximately taking 2 weeks, remains a drawback for widespread applicability, these results do indicate the tremendous potential for EPC-endothelialized synthetic grafts.

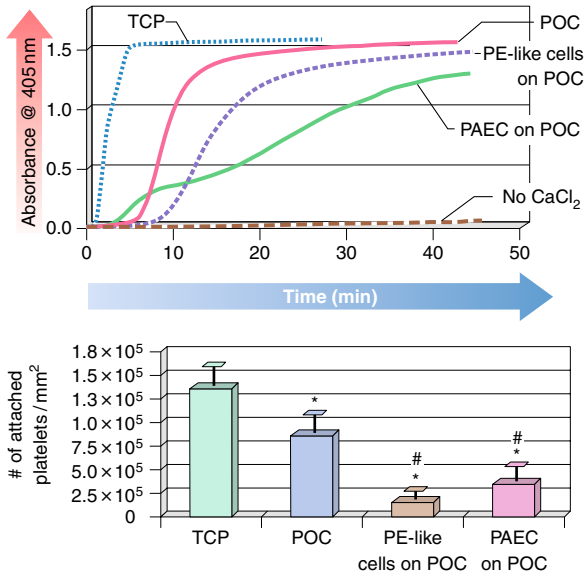


Fig. 15.4 Functionality assessment of porcine endothelial progenitor cells (EPC) isolated from peripheral blood and cultured on poly(1,8-octanediol-co-citrate)-coated ePTFE grafts. (**a**) Clotting kinetics assessment using a plasma recalcification assay. An increase in absorbance indicates clot formation. (**b**) Levels of attached platelets after 1 h incubation of a platelet suspension on the various substrates. Porcine endothelial-like (PE-like) cells on POC were compared with porcine aortic endothelial cells (PAEC) on POC, POC alone and tissue culture plastic (TCP) alone. Data shown are means ±SD (n=3). For the clotting kinetics, SD bars are omitted for visual clarity. Reprint from [6]

Genetically Engineered Cells

Long cell culture times are required to form EC monolayers on synthetic grafts. As a result, genetic enhancement of cellular properties has been investigated as a method to improve cell adhesion to the graft and antithrombotic factor expression. For example, Zhu et al. [241] transfected circulating EPCs with the gene for A20 expression, a factor known to prevent atherogenesis, and after in vitro cultivation cell-seeded grafts were implanted in the carotid arteries of rats. After 6 months of implantation, the proliferation index of neointimal cells was shown to be much smaller when compared with implanted control grafts. Furthermore, fewer SMCs were present, as well as apoptotic cells. An alternative way to increase functionality of EPCs is by overexpression of eNOS, achieved by Kong et al. [93], who observed enhanced EC vasoprotection as a result, as well as inhibition of NIH.

An interesting approach to increase endothelialization of grafts by means of genetic engineering is under investigation by Niklason et al. [161]. They acknowledged the issue of the limited proliferation potential of isolated cells in vitro and sought to solve this by overexpression of the human telomerase reverse transcriptase subunit (hTERT), the catalytic subunit of the telomerase

enzyme. Reduced expression of telomerase is associated with the aging process (i.e., diminished cell division potential) in humans [118] and therefore induced expression of this molecule could extend the lifespan of cells. Niklason et al. [161] observed indeed an extension in the lifespan of SMCs, without any adverse effects. Even though the effect was shown in SMCs, extending this concept to ECs to enhance endothelialization is just a step away. Recently, Schachner et al. [173] were able to deliver a VEGF-2 plasmid in a vascular stent. They found that the treatment group had an improved EC coverage, improved luminal cross-sectional area and vessel function as measured by nitric oxide production in hypercholesterolemic rabbits [173]. These approaches show much promise, but single gene therapy may not yield straightforward results. For example, although VEGF does improve endothelialization consistently, its effects on NIH are conflicting in the literature [16, 158]. It is therefore likelier that pleiotropic gene therapy is indispensable to ascertain the desired efficacy.

Other

In an attempt to improve cell adhesion strength and cell distribution on polymer scaffolds, Sinclair and Salem [187] converted native sialic residues on the surface of cells into nonnative aldehydes, which can then be reacted with biotin hydrazide to obtain biotinylated cells. The polymer they used consisted of a biotinylated PEG-PLA copolymer, to which avidin can be attached with high level of control over spatial distribution using a PDMS stamp technology [187]. The spatial distribution of avidin results in selective binding of biotinylated ECs onto the polymer substrate. The high-affinity biotin-avidin cell adhesion may have high potential for preimplant cell seeding, although it has been reported that cell adhesion is only enhanced during the first hour [17], offering little or no progress in two-stage seeding. However, for single-stage EC seeding, this technology may offer sufficiently improved cell retention of ex vivo seeded cells upon exposure to blood flow.

15.3.4 Gene Therapy for Vascular Grafts

Maintaining full bioactivity of local delivery systems for proteins, cytokines, and growth factors is a well-known problem, which may lead to unnecessary high doses to improve treatment outcome [162]. This makes such systems expensive and involves increased safety issues of potential dose dumping into the bloodstream, which could cause severe adverse effects. Gene therapy supposedly negates these issues by local protein expression, instead of relying on compounds maintaining their bioactivity. Additionally, gene therapy offers the potential benefit of sustained and controlled (over)expression or downregulation of target proteins. Finally, gene therapy may provide a more specific and better controlled solution than the delivery of vascular cells, which can result in a variety of local, unintended functional effects. Bypass grafts, whether synthetic or native, are ideally suited for gene therapy, as genetic approaches can be performed ex vivo prior to implantation. Furthermore, complexation of DNA or RNA with polymers protects against degradation and facilitates cellular internalization [31].

Not only preimplantation genetic engineering of scaffold-seeded cells is possible, as addressed in Sect. 15.3.3.2, but also the localized delivery of genetic constructs such as naked DNA, small interfering RNA (siRNA), antisense oligonucleotides (ODNs), and plasmid vectors by incorporation in scaffolds. The scaffold material in that case functions as the delivery vehicle and can be utilized to delay the release of the genetic material in situ to prolong effects. Gene vector encapsulating biomaterial scaffolds can also be applied perivascularly, for example, as a wrap or in gel-form, to deliver gene therapies locally to external graft surfaces postimplantation. This technique can be applied for synthetic, vein and TEBV grafts alike. Vein graft and TEBVs, alternatively, may also be supplied with genes to various vessel layers prior to transplantation.

15.3.4.1 Current Status of Genetic Engineering for Vascular Grafts

Although the prospect of genetic modulation using local delivery through scaffolds seems appealing, studies on gene delivery using standard permanent vascular synthetic grafts are scarce. Indeed, most researchers focus on genetic engineering to improve patency rates of vein grafts. In general, such studies use local infusion of plasmids, naked DNA, or viral vectors in vein grafts, usually using catheter-based delivery or by direct

injection into grafts preimplantation. Some of those approaches have even reached the phase of clinical trials (for an overview, see [163]). For example, a large-scale phase IV trial using preimplantation injection of E2F decoy ODN in saphenous vein grafts was performed. The E2F DNA was thought to block the activity of E2F, which is known to stimulate NIH, and thus prevent or reduce NIH. However, the phase IV trial did not show any improvement in patency rates [3], severely tempering expectations for future genetic interventions to improve bypass performance. Other clinical trials, mostly targeting VEGF or FGF expression using viral vectors, report inconsistent results, which mainly indicates an incomplete understanding of in vivo processes and parameters to be controlled [163]. Nevertheless, positive in vitro and preclinical results are widespread, indicating that further investigation is warranted.

Kibbe et al. [88], in an attempt to modulate the levels of pleiotropic NO, fabricated an adenoviral construct for inducible nitric oxide synthase (iNOS) and transfected porcine jugular veins, which were subsequently anastomosed to the carotid arteries of pigs. After 21 days, they observed increased NO secretion accompanied by a reduction in NIH of approximately 30%. Another study by Pfeiffer et al. [154], specifically targeting NIH in ePTFE grafts, injected a liposome-complexed adenoviral iNOS-encoding plasmid into the carotid artery wall of dogs at the anastomoses of implanted grafts. After 6 months, a reduction in NIH was reported at both the distal and proximal site, ranging from 40 to 80%. Another study specifically targeting the field of synthetic grafts, using a perivascular approach, where in Lahtinen et al. [105] tested the effect of $VEGF_{165}$ and FGF-2 encoding plasmids when applied on the external surface of ePTFE and polyester grafts in rats, rabbits, and dogs. Positive effects of $VEGF_{165}$ on endothelialization levels were observed in all species for ePTFE grafts with 60 μm internodal distances, while for 30 μm internodal distance or hybrid grafts, results were inferior. The latter might reflect the limiting effect of the smaller pores on transmural endothelialization, indicating the necessity of sufficiently large pores if the goal is cell infiltration. In their study, polyester grafts tended to endothelialize faster under influence of $VEGF_{165}$ [105]. Interestingly, a combination of $VEGF_{165}$ and FGF-2 was deleterious, explained as an inhibitory feedback loop being triggered. Nevertheless, the effects of $VEGF_{165}$ as well as earlier-mentioned iNOS are encouraging and indicate the usefulness of genetic modulation in permanent synthetic grafts.

Similar results of VEGF gene delivery in ePTFE grafts were obtained by Tao et al. [202], albeit in vitro by impregnating ePTFE grafts with the VEGF-encoding plasmid in a fibronectin solution. Control grafts were seeded with fibronectin only, fibronectin with a green fluorescent protein encoding plasmid or fibronectin with control vector pCDI. A burst release of DNA from the ePTFE graft took place within the first 8 h, where after continuous release for 3 days was observed. HUVECs seeded on ePTFE grafts encoding VEGF showed increased proliferation rates within 24 h after seeding and after 5 days, surface coverage of ePTFE with HUVECs was almost identical to that of a control surface of tissue culture plastic, which is known to be highly supportive of cell adhesion and proliferation. Moreover, although VEGF expression levels remained constant in cells on control surfaces, VEGF production in HUVECs on VEGF-plasmid containing ePTFE increased over time, until more than 50 times as high as control cells on day 5 [202]. Apart from VEGF targeting, other target proteins that may augment EC functionality include TM, TFPI, eNOS, prostacyclin, and c-type natriuretic receptor when overexpressed, whereas interference with thrombin may prevent thrombosis. The prevention or inhibition of NIH in vascular repair, likewise, may be achieved by overexpression of factors that initiate apoptosis, such as fas ligand, or halt cell division, such as p53 [46]. Alternatively, interference with molecules essential for normal (smooth muscle) cell cycle progression is possible. The E2F factor discussed earlier is an example of this group of molecules. For an overview of major potential target molecules in vascular grafts, see Table 15.1.

15.3.4.2 Prospects

An exciting concept to improve transfection efficiency may be the incorporation of fusogenic peptides or pH-responsive polymers that mimic endosomal escape elements of viruses. For example, Dr. Irvine [65] has shown the successful cytosolic delivery of proteins by encapsulation in pH-responsive core-shell nanoparticles. The particles were endocytosed by natural mechanisms, where after the decrease in pH triggered osmosis-based disruption of the particle shells and release of the protein in the cytosol.

Table 15.1 Major pathophysiologic processes relevant to vascular graft occlusion and potential target molecules for gene therapy

Target	Overexpression candidates	Inhibition candidates
Endothelial cells	VEGF, eNOS, iNOS, A20, HGF	
Thrombosis	TM, TFPI, PGI2, ADPases, tPA	Thrombin, COX, PAF
Smooth muscle cells	p53, fas ligand, survivin, iNOS	E2F, PDGF, TGF-1, midkine, cell cycle genes
Inflammation	VCAM, ICAM, SOD, HO-1, IL-10	MCP-1, LPL
Extracellular matrix	TIMPs	TGF-β

VEGF vascular endothelial growth factor; *eNOS* endothelial nitric oxide synthase; *iNOS* inducible nitric oxide synthase; *PGI2* prostacyclin; *HGF* hepatocyte growth factor; *TM* thrombomodulin; *TFPI* tissue factor pathway inhibitor; *PAF* platelet activating factor; *HO-1* heme oxygenase 1; *tPA* tissue plasminogen activator; *COX* cyclooxygenase; *TGF-1* transforming growth factor 1; *VCAM* vascular cell adhesion molecule; *PDGF* platelet derived growth factor; *ICAM* intracellular cell adhesion molecule; *SOD* superoxide dismutase; *MCP-1* monocyte chemoattractant protein 1; *LPL* lipoprotein lipase; *TIMP* tissue inhibitor of metalloproteinases; *TGF-β* transforming growth factor *β*; *IL-10* interleukin 10

By encapsulating vectors for genetic therapy, this technology may easily be adapted for increased transfection efficiency. Cell-specific gene delivery, likewise, may be induced by equipping polyplex-, lipoplex-, or microparticle-based systems with specific cell surface receptor ligands. Thus, it is reasonable to predict that future solutions may consist of nonviral vectors that can transfect a very specific cell population, utilize the cells' inherent molecular and chemical mechanistics for highly efficient intracellularization of vectors and effectively modulate the cells' expression profiles.

15.4 State of the Art: Tissue Engineering of Small-Diameter Blood Vessels

Limitations for the use of synthetic grafts (with or without an EC layer) lie, as discussed, in the fact that essentially nonliving grafts are employed, which mainly address mechanical and physical deficiencies and not the functionality of tissue. Therefore, the level of remodeling and vasoreactivity that can be obtained is limited. Therefore, the hope remains that the use of synthetic grafts will be a bridging technology until TEBVs become a reality. Although the widespread acceptance of TEBV technology currently faces significant obstacles such as off-the-shelf availability and the harvest and cultivation of autologous cells, significant progress has been made in the past decade and the potential for clinical use therefore cannot be ignored.

15.4.1 The Central Paradigm

Ever since the seminal work done by Weinberg and Bell published in 1986 [225], leading to the advent of vascular tissue engineering, the field has developed into a well-established and highly investigated research area. Tissue engineering of blood vessels involves the use and manipulation of cells to generate new functional tissue capable of biological cue-dependent remodeling and growth. At the center of the TEBV paradigm lies ex vivo regeneration of functional vascular tissue, usually through the use of biomaterial scaffolds seeded with cells, although this is not necessarily a component of TEBVs. The scaffold imparts structural support to seeded cells and sometimes provides stimulatory chemical and/or mechanical signals to cells to assist the development of functional tissue (Fig. 15.5). The scaffold itself may degrade over time after/during maturation of tissue, although this is not a mandatory requirement per se. The adaptation of tissue to altering environmental mechanical and chemical cues over time may be hampered if some type of nonliving structure remains behind as it also poses a risk for chronic inflammatory responses. Therefore, the majority of research groups pursuing TEBV use scaffolding materials that are expected to degrade or dissolve entirely over time.

The degree of cell manipulation necessary to generate functional tissue by proliferation, migration, and ECM deposition depends on the cell type used, but generally involves dynamic stimulation with mechanical signals provided in an ex vivo bioreactor environment. Often, mechanical stimulation of cells is supplemented by biochemical signals provided by relevant cytokines,

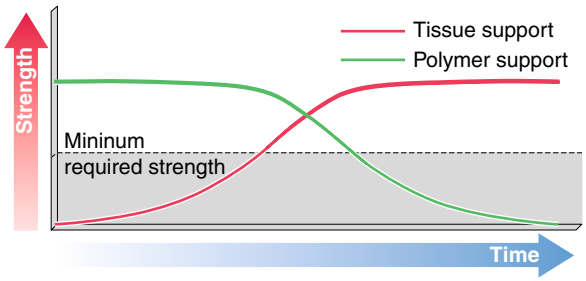


Fig. 15.5 Initially, the polymer scaffold provides the bulk of the mechanical support (*left*), but maturation of tissue, either in vitro or in vivo, is expected to gradually take over part of the tissue engineered blood vessel (TEBV) mechanical properties (*center*) and eventually provide a mechanical structure similar to native tissue after complete degradation of the scaffold (*right*)

growth factors, or other molecules. After maturation of a TEBV construct, implantation is expected to lead to incorporation of the tissue within the host vasculature. Although this paradigm is not necessarily rigid, as discussed later on, several groups have departed from this basic concept by omitting biomaterial frameworks or by regenerating the TEBV tissue in vivo rather than in vitro [30, 104].

15.4.2 Design Criteria

In order for a TEBV construct to become a worthy alternative to standard synthetic or native grafts for small-diameter vessel reconstruction, the technology has to lead to considerably improved patency rates and demonstrable long-term safety. The grafts should also be easy to handle. To reach that goal, there are design criteria that have to be followed, which should lead to a TEBV that fundamentally incorporates functional requirements and lead to successful clinical use. The strength (tensile and radial) requirement is the most important requirement that has to be met, for a TEBV needs to withstand physiological stress upon implantation. It is also a requirement for which investigators over the years have struggled to reach sufficient values even after extended culture times in vitro, especially for burst (radial) strength. Although a burst strength of several times the value of physiological blood pressure may suffice for vessels to function without the chance of sudden rupture, generally the generation of a desirable burst/rupture strength of the same order of magnitude as vein or arterial grafts is strived for, which is approximately 1,680–2,270 mmHg [104, 106] for human saphenous veins and 2,031–4,225 mmHg [101] for human arteries. Furthermore, when using biodegradable scaffolds, the continuously changing mechanical properties of the scaffold during implantation are a vital design aspect. For example, if degradation of the scaffold takes place too rapidly or unevenly, this process may lead to weak tissue fractions and the risk of aneurysm formation and sudden catastrophic rupture.

A second structural requirement for surgeons to consider TEBVs is its suturability, meaning ease of creating the anastomosis without chances of sudden rupture or tearing of the vessel under both longitudinal and circumferential stresses. Although this aspect is generally considered only to be on a surgeon's "wish list," one should keep in mind that difficulties in terms of suturability are likely to lead to inferior anastomoses with an increased risk of structural and dynamic environments favorable to the development of NIH. Moreover, if surgeons do not feel comfortable suturing a TEBV, the chances of acceptance, no matter how well the graft performs, are slim.

In addition to suturability and strength requirements, the TEBV compliance and stability thereof caused by scaffold degradation or vessel remodeling are considered essential determinants of graft patency. It is widely accepted that one of the main reasons for graft failure (whether synthetic or natural) is the local difference in compliance between native vessel and replacement conduit. Localized differences in shear stresses caused by compliance mismatch can be detrimental to an already unstable and/or injured EC monolayer. Local low shear stress areas can cause stasis with increased interactions between platelets and the vessel wall. Furthermore, excessive stretching of SMCs due to inferior mechanical properties may lead to their overproliferation. These factors ultimately lead to high rates of either thrombosis or NIH (or both), mainly around the site of anastomosis, since that area is prone to mismatched compliance. Indeed, the failure of ePTFE and Dacron grafts may largely be caused by their relatively low compliances, which is on the order of 1.0–2.0%/mmHg^{-2} [137, 199]. In contrast, vein and arterial grafts typically are reported to range from 2.0 to 8.0%/mmHg^{-2} [137, 199, 219]. TEBV must achieve compliances of the same order of magnitude as native vessels. One way to achieve compliance

matching may be to use elastomeric biomaterial scaffolds that have compliance values comparable with the target blood vessel. A popular approach has been the use of highly compliant inner porous tubes with a stiffer external sleeve to emulate the increasing influence of collagen at higher pressures as seen in native blood vessels [183, 224].

Even when initial compliance requirements are met by the scaffold/tissue combination upon implantation, a critical issue is the maintenance of adequate compliance over time. Many TEBV materials suffer from a change over time of compliance, leading to increasing mismatch and decreasing mechanical properties possibly leading to increased risks of NIH development or aneurysm formation (see Sect. 15.2). Native vessels have an inherent large capacity to adapt their structure to changing mechanical environments to match compliance requirements, but since polymers do not possess that capacity, it is vital that during the initial period after implantation graft compliance support proper tissue development. The ability to meet this condition is directly related to the timeframe of degradation/elimination in the body. For example, if degradation is too fast, the tissue fractions may not have matured enough and possible aneurysm formation may occur. Furthermore, rapid degradation of materials may lead to local buildup of acidic moieties (in the case of a polyester scaffold), negatively affecting the vessel's development. Degradation that is too slow, on the other hand, poses a continued risk of infection and inflammation, negatively influencing graft patency. Gradual stress transfer during degradation is therefore considered vital when designing scaffolds for TEBVs.

Besides optimization of degradation kinetics for appropriate structural support and gradual stress transfer, the expected degradation products must be known and monitored. Degradation of biomaterials often leads to localized buildup of basic or acidic moieties, residual catalysts, and micron or submicron particles, which may degenerate the TEBV construct. Also, breakdown products from biomaterials have the potential to lead to overproduction of ECM by SMCs, leading to occlusion [62]. Therefore, degradation products should not elicit severe inflammatory or immunogenic reactions, should not involve any carcinogenicity or toxicity, or cause any adverse effects on the tissue construct.

Pore size and porosity are another set of essential design criteria for TEBV scaffolds to facilitate tissue infiltration. The term porosity can be defined in several ways, but for TEBVs, the essential aspects are overall porosity and the extent to which pores are truly interconnected, accommodating cell ingrowth, cell–cell interaction and angiogenesis.

Finally, the cell source to be used for cell seeding in vitro is as much an issue as it is for endothelialization of permanent grafts. To avoid the additional surgery to isolate SMCs and ECs that may not have sufficient proliferative potential and are at risk for dedifferentiation in vitro, new sources are actively investigated, including previously mentioned mesothelial and microvascular cells. The focus currently seems to be on using autologous pools of stem or progenitor cells. Types of specific interest for TEBV include a multipotent subpopulation of bone marrow stem cells identified by Yoon et al. [237] and the EPCs mentioned in Chap. 4. Besides the EPCs, thought to selectively differentiate into ECs, it has been reported that there also exists a distinct SMC progenitor population in peripheral blood [186]. These findings suggest that the potential exists for the isolation of both progenitor subpopulations from one blood donation followed by differentiation and expansion in vitro.

15.4.3 Current Status

15.4.3.1 In Vivo Tissue Regeneration Methods

In vivo tissue engineering of blood vessels usually involves the implantation of a scaffold that already fulfills many of the above-mentioned requirements for an ideal vascular substitute, after which gradual remodeling of the construct takes place, either with or without eventual degradation of the initial structure. The appeal of this approach lies in the fact that no tissue culture period is needed, accommodating potential off-the-shelf TEBVs. An approach widely investigated has been the use of decellularized biological scaffolds, such as small intestinal submucosa (SIS) or decellularized blood vessels to generate elastin- and collagen-based scaffolds. Although the underlying theory of using the natural ECM architecture to provide structural support and biological cues for tissue development is valid, in the past it has been proven to be challenging to achieve sufficient strength, whereas immediate thrombogenicity is also a major concern, especially using collagen-based scaffolds. Moreover, in in-vivo tissue engineering,

immunogenicity risks remain present. A hyaluronan-based scaffold has shown excellent results after transplantation into a porcine carotid artery. Five months postimplantation, the biomaterial was almost completely degraded, while profuse tissue maturation and remodeling had taken place, including the advent of elastin layers identical to native arteries [239]. Since elastin has been identified as the "missing link" of TEBV technology so far, the results obtained in this study are particularly encouraging. However, the constructs in this study all suffered from severe stenosis, with three out of ten vessels occluding before the end of the study. Therefore, more research is necessary to clarify whether decoupling of elastin biosynthesis and SMC overproliferation is possible.

An unorthodox approach for in vivo tissue engineering was reported by Campbell et al., in which silastic polymer tubes were inserted into the peritoneal cavity of rabbits to generate an autologous graft by taking advantage of the host response to foreign materials. Within 2 weeks, a capsule had formed around the tubes and after removal of the tube and inversion of the capsule, trilayered constructs were available with a vascularized adventitial layer, contractile "media" including elastin and collagen and a lumen with confluent mesothelium. An intriguing notion was that the luminal mesothelium exhibited EC-like behavior and displayed immunohistochemical features of ECs, indicating that the choice of cell source for vascular grafts may be less important than traditionally assumed. Implantation of these grafts as arterial replacement in the same rabbits showed patency of 67% of the grafts after 4 months, without any antiplatelet therapy. Although the approach is fascinating and results encouraging, it seems hardly applicable to clinical practice. Besides the maturation period necessary, implantation of a silastic tube in the patients' peritoneal cavity brings risks along in terms of potential abdominal adhesion and access site infection.

15.4.3.2 In Vitro Tissue Regeneration Methods

Natural scaffolds that have been investigated over the years include collagen [11]-, elastin [191]-, and fibrin [198]-based matrices, as well as decellularized arteries [27]. Crosslinked collagen scaffolds have been attractive, because they are easy to obtain and can support cell adhesion. However, a concern with collagen remains the inherent thrombogenicity and suboptimal mechanical properties remain an issue [47]. Fibrin-based scaffolds generally have similar issues associated with them, although fibrin, in contrast to collagen, allows for elastin-production by seeded SMCs [120, 198], an important factor in potential TEBV survival.

In one of the most-cited original papers on scaffold-based TEBVs, Niklason et al. [143] utilized tubular polyglycolic acid (PGA) scaffolds to engineer an artery in vitro. The scaffolds, which were placed over a silicon tube for mechanical stimulation, were seeded with bovine SMCs and incubated under pulsatile flow for 8 weeks. Next, bovine ECs were seeded onto the lumen and exposed to constant flow for 3 days. The significance of this approach was evident, for they were the first group to achieve a burst strength and collagen content close to that of native arteries using a synthetic scaffold, as well as physiological responses to vasoactive compounds such as endothelin-1 and prostaglandin. Hoerstrup et al. [64] used poly(glycolic acid) (PGA) scaffolds and coated PGA-tubes with poly-4-hydroxy-butyrate (P4HB) to increase the degradation time. Scaffolds were sequentially seeded with ovine myofibroblasts and ECs. After 14 days of maturation under pulsatile flow, grafts possessed supraphysiological burst strengths and were used to replace the pulmonary artery in lambs. After an evaluation period of 100 weeks, grafts properly remodeled during animal growth, remained open, and showed no signs of thrombosis or calcification. Although encouraging, burst strengths that are achieved in vitro are insufficient for the arterial circulation.

Seifalian et al. have developed a novel poly(carbonate-urea)urethane (CPU), which has a special honeycomb structure that can maintain compliances similar to native arteries. CPU is not degradable and has already been approved for use as an AV conduit in hemodialysis patients, whereas it is also undergoing clinical trials as a permanent synthetic vascular bypass graft. Recently, inspired by the superior ability of CPU to attach ECs, they attempted to extend the use of this scaffold to the field of TEBVs by sequential seeding of human umbilical SMCs and ECs. They demonstrated support of SMC layer formation and the positive influence of SMCs on EC retention, as well as the increase of these properties when preconditioned under increasing pulsatile flow conditions. Iwasaki et al. [72], finally, used a modification of the successful approach from Cytograft (see below), although they did use a biomaterial for support. Sheets of PGA were seeded

with porcine SMCs and fibroblasts, while PCL sheets were seeded with porcine SMCs as well. Using a silicone mandrel, they sequentially rolled sheets of SMC-seeded PGA, SMC-seeded PCL, and fibroblast-seeded PGA around the mandrel, with static culture periods of varying length in between. Finally, the silicone support was removed and the luminal area was seeded with porcine ECs, and the construct was cultured for two more days in static medium, after which the tubes were mounted in an incubator capable of dynamic stimulation. After a 2 week dynamic culture period with gradually increasing pulsatile pressure and flow, constructs showed a remarkable resemblance to native arteries, not only in gross appearance, but also more importantly in mechanical properties. Stress–strain curves were almost identical for native and engineered vessels, while ultimate strength, strain, and elastic moduli in the elastin and collagen region of the stress–strain curves was also strikingly similar. Their report describes the maturation of TEBVs in vitro into thick tissues with native-like visco-elastic behavior. Although in vivo experiments have not been undertaken yet and similar effects with human tissue need to be shown, their concept appears to be promising.

15.4.3.3 Tissue Engineered Blood Vessels in the Clinical Setting

The first clinical replacement of a blood vessel using a TEBV was performed in Japan by Shin'oka et al. [131, 181], who developed a 50:50 PLLA-PCL copolymer scaffold reinforced with PLLA and seeded it with a mixture of autologous bone marrow cells, isolated from the patient at the time of surgery. After seeding of the scaffolds, the constructs were incubated for only 2–4 h until implantation. Ten days after seeding the scaffold and ex vivo culture, a graft was implanted to replace the patient's pulmonary artery. To date, several tens of children have been treated, without any complications such as occlusion or aneurysm formation. Although obtained results were very impressive, it remains to be seen whether their technology is applicable to a diverse patient population. For one, they only used grafts in the pulmonary circulation with much lower strength requirements compared with arterial grafts. Furthermore, it is not unthinkable that the healing potential in terms of re-endothelialization in children is much higher than in adults, for example, because of a healthier EPC population. Nevertheless, their results did for the first time indicate the true likelihood of future regular clinical application of TEBVs.

In stark contrast to the most widely-investigated approach of cell-seeded biomaterial scaffolds, the TEBV concept that is actually closest to clinical acceptance does not use a biomaterial scaffold. Only autologous cells are employed. In 1998, a so-called cell-sheet approach was reported to result in strong, functional vessels [104]. The technique involves obtaining dermal fibroblasts from a skin biopsy, growing them as sheets in vitro for 6 weeks, after which these cell sheets are rolled around a temporal support mandrel. Multiple layers can be combined this way, and after another 10-week maturation period, the individual plies are fused and constructs possess burst strengths in excess of 3,000 mmHg [104]. A few days before implantation, vessels are seeded with autologous ECs, either harvested from a peripheral vein or derived from EPCs obtained by venopuncture. This concept was shown to have layer-like structures resembling native vessels with sufficient mechanical properties (compliance >2.0%/mmHg-2) and is currently being further developed by Cytograft Tissue Engineering. Clinical trials have been initiated, in which these grafts are used as AV shunts in hemodialysis patients, and initial results are highly encouraging [103]. For one patient, the graft remained patent without complications for more than 13 months, at which time the patient received a kidney transplant. Furthermore, three other patients were reported to have completely functional grafts at 5 months postimplantation [103]. Interestingly, several compliance measurements at 5 months showed increases in compliance values without concomitant dilatation [103], suggesting remodeling and possible formation of elastic components, a desirable effect.

15.5 Clinical Applications

All described state of the art synthetic graft and TEBV strategies clearly offer great potential to replace or optimize current vascular repair methodologies. However, depending on the approach, different limitations present themselves, which will determine the ultimate chance of success for a particular application. Hence, one can clearly see the importance of defining a target application carefully and outlining the issues at hand, as well as the boundary conditions for successful clinical application that may not be obvious at first.

Applications for vascular grafts include:

1. Vascular grafts to replace or bypass atherosclerotic vessels
2. AV grafts for hemodialysis

Both applications possess their respective set of issues associated with them, albeit with large overlaps. Vascular grafts for small-diameter artery replacement are typically taken from the patient's vasculature, a fairly successful procedure and the current standard of care. However, besides the clearly suboptimal therapy of removing a functional blood vessel from another location, a significant portion of natural grafts suffer from restenosis or aneurysm formation. In addition, the underlying cardiovascular disease is usually systemic and healthy replacement vessels may not be available. The use of vascular synthetic grafts as highlighted in Sect. 15.2 has been highly successful in large-diameter vessels, but when used for small-diameter vessels, they usually suffer from occlusion secondary to thrombosis, and NIH and are therefore typically not used. AV shunts or grafts have similar problems associated with them, with the added issue of repeated needle puncture. Although the TEBV approach is enticing and theoretically offers the best solution to replace damaged vessels, it seems unlikely that off-the-shelf availability will be achieved, rendering this approach unsuitable for all emergency procedures. It is therefore difficult to justify vascular research solely focusing on TEBV technologies. Nevertheless, nonemergency bypass patients as well as the often ignored renal failure patient population that requires AV grafts offer the ability to plan ahead of time, enhancing the clinical feasibility of the TEBV approach. Moreover, for surgeries in infants, the TEBV ability to remodel is desirable to prevent reinterventions at a later age. For patients in need of emergency procedures, however, the biohybrid approach to improve synthetic grafts is more appropriate, since off-the-shelf availability is expected to be achieved more easily.

15.6 Expert Opinion and Limitations

15.6.1 *Biohybrid Strategies for Synthetic Grafts*

From the previous paragraphs it has become clear that a vast amount of research on the modification of synthetic grafts to inhibit thrombosis and NIH exists, with many of the incorporated drugs showing promising results, including animal and even some clinical studies. However, several limitations that apply to virtually all of the above methods remain. One of the major drawbacks is the risk for adverse effects to drugs, for instance the development of thrombocytopenia, which may be caused by any of the mentioned anti-platelet drugs. Heparin, for example, is known to cause this rare, but dangerous condition. Regarding anti-NIH drugs, even though positive results have been described for stents that eluted anti-proliferative drugs, recently it was reported that those drugs negatively affect endothelialization, which may cause late thrombosis. The negative effect of paclitaxel and sirolimus on ECs may be inherent to the drugs' nature, since they nonspecifically inhibit cell cycle progression. A way around these drawbacks may be the use of more selective drugs. Examples include dipyridamole, imatinib mesylate [53, 126], and tranilast [85], which have been reported to all prevent PDGF from exerting its effect on SMCs (see Table 15.2), without negatively affecting re-endothelialization. These therapies have however not yet been available clinically for local delivery.

Another issue with all these therapies is the appropriate dosing regime. The optimum duration of treatment, which depends mainly on the duration in which the grafts are at an increased risk for thrombosis and NIH development, may be hard to identify. In hemodialysis grafts, for example, repeated needle puncture imposes an ongoing stimulus for both thrombosis and the development of NIH. In coronary and peripheral bypass grafts, as well as angioplasty (with or without stenting) procedures, the risk level is transient. Indeed, it is currently surmised by many scientists that for long-term graft survival, it may suffice to have a therapy last long enough to overcome early thrombosis and the initial inflammatory response to prevent long-term NIH complications. Most scientists are therefore mainly focusing on matching the temporal profile of depot drug release to the biologic sequence of events that characterize the vascular injury process, using a variety of approaches, which include nanoparticles [41, 92, 114, 227] or polymer gels as the carrier [94, 174]. In spite of the current "early inflammatory and thrombosis prevention" theory, preventive therapies would ideally be continuous, the most obvious obstacle to the therapies discussed above as most approaches use limited depot formulations.

Theoretically, sustained release from a replenishable depot or the inclusion of compounds that catalyze

Table 15.2 Compounds under investigation or potential candidates for use in vascular repair to prevent thrombus formation and/or neo-intimal hyperplasia (NIH)

Anti-thrombosis agent	Mechanism of action	References
Anti-coagulant		
Heparin	Stimulates antithrombin III, which inhibits thrombin	[25, 37, 39, 61, 116, 123, 193]
Thrombomodulin	Binds to thrombin, thereby inactivating it	[112, 119, 192, 231, 233]
TFPI	Inhibits formation of thrombin from prothrombin by inhibiting factor VIII upstream	[159, 170, 196, 197]
Hirudin	Directly inhibits thrombin	[58]
Sodium citrate	Chelates calcium, which is a cofactor in the coagulation cascade	[236]
Warfarin	Inhibits vitamin K and thereby prevents formation of thrombin from prothrombin	N/A
Anti-platelet		
NO	Increases cGMP levels, which is a secondary messenger that inhibits platelet aggregation	[42, 44, 68, 135, 146, 157]
ADPase	Degrades ADP, which activate neighboring platelets for aggregation	[206]
Prostacyclins	Increases cAMP levels in the cell, which inhibits platelet activation	[23, 51, 58]
Aspirin	Blocks the COX pathway	[74, 164, 171]
Clopidogrel	Blocks binding of agonists to the P2Y12 receptor, preventing platelet activation	N/A
Dipyridamole	Inhibits phosphodiesterases, leading to increasing cGMP, inhibiting platelet activation	[2]
Abciximab	Blocks the GPIIb/IIIa receptor, which prevents platelet aggregation	[89]
Fibrinolytic		
tPA	Stimulates the conversion of plasminogen to plasmin, which inhibits fibrin formation	[51]
Urokinase	Stimulates the conversion of plasminogen to plasmin, which inhibits fibrin formation	N/A
Anti-NIH agent		
Paclitaxel	Halts cell division by preventing microtubule dissembly	[92, 109, 114, 130, 132]
Sirolimus	Blocks the response to IL-2, blocking lymphocyte activation and release of mitogens	[21, 168]
Imatinib mesylate	Blocks the PDGF-receptor, preventing the mitogenic effect of PDGF	[53, 126]
NO	Increases cGMP levels in SMCs, a secondary messenger inhibiting SMC proliferation	[82, 141, 152]
C-type natriuretic peptide	cGMP induction, possibly by NO induction, which inhibits SMC proliferation	[175]
Tranilast	Blocks the mitogenic effects of TGF and PDGF, restores NO production, inhibits collagen production	[85]
Dipyridamole	Inhibits PDGF and FGF-induced SMC proliferation	[100, 188]

N/A indicates that up till now, no reports have been published on the use of that particular agent for local delivery in vascular repair

anti-NIH/thrombosis reactions in vivo may accomplish (semi)continuous effects. Certain research groups have already showed this vision in projects aimed at engrafting molecules that utilize circulating blood compounds, most notably in efforts aimed at the local generation of NO. One may also envision immobilizing molecules on the grafts surface that may react with circulating exogenous compounds to generate the substance of choice, whose blood levels can then be controlled by regulating administration of the compound. An example that has already been explored is the immobilization of copper-ions on polymer grafts [68]. Copper-ions are known to catalyze the release of NO from circulating nitrosothiols, most notably GSNO, and their immobilization on a polymer-graft may provide a method to continuously generate NO. Although preliminary, Meyerhoff et al. obtained very exciting results with a copper-cyclen complex bound to PUs [68]. Their polymer successfully generated an NO surface flux from sheep whole blood in vitro, which clearly indicates the feasibility of this approach. Leaching of the copper-ions from the polymer remains a concern, thereby producing NO only temporarily [179]. Although NO thus shows great potential and a consensus on the benefit of prolonged local NO bioavailability exists, an open question remains that what NO levels and for what period of time is required for effective prevention of thrombosis and NIH. An initial burst release to overcome the inflammatory response to grafts followed by sustained NO release at lower levels is necessary until total endothelialization has been achieved and the risk for restenosis has diminished. However, the NO levels released need to fall within a therapeutic window, since excessive NO release has a toxic effect. More research is needed to determine NO threshold levels for therapeutic effectiveness.

15.6.2 Stimulation of EC Monolayer Formation

As pointed out in Sect. 15.3.3, the ongoing search for alternative cell sources to achieve a stable, confluent EC (mimicking) monolayer formation has shifted toward utilization of host cell populations, of which EPCs seem to offer the best opportunity. However, currently there are several vital issues that need to be addressed. First and foremost, our current understanding as to the exact nature of circulating EPC populations is still limited. There is currently no consensus on the combination of cell-surface expressed markers that cells need to express to identify them as EPCs, while a variety of cell populations with different cell marker expression profiles have been shown to express EC-like behavior. Moreover, it has not yet been established under what circumstances EPC (or EC-like) populations may differentiate toward ECs, whether that differentiation is stable and whether secretion of endothelial factors is similar to ECs. Therefore, more fundamental research to elucidate the exact nature of EPCs is needed.

Even when the successful adhesion and retention of ECs (or EC-like cells) is achieved on synthetic grafts, an issue that is often overlooked is the function of the new cell monolayer relative to native blood vessels. The phenotype of ECs in vivo is controlled by mechanical and chemical cues derived from the underlying substrate, peripheral cells, signaling molecules, and blood flow. When ECs are seeded and cultured on a synthetic substrate, initially these cues are dissimilar to those present in vivo. The focus so far of most studies on achieving EC confluence, although justified in the sense of diminishing the main concern of inherent surface thrombogenicity of the tested grafts, leaves this question wide open. A more systematic approach must be implemented to elucidate the question of function.

15.6.3 Gene Therapy

As promising as localized gene delivery may seem to be, there are several issues that may hamper its applicability. First and foremost, safety concerns are present with the use of viral gene delivery modalities, since they are commonly associated with the inherent risk of endogenous viral recombination [242], oncogenic effects, or unexpected immune responses [235]. For retroviral vectors, there exists the added disadvantage of their poor uptake into nonproliferating cells, which are abundant in vascular tissue. Another practical concern of viral vectors is the difficulty associated with large-scale production of the genetic material. Finally, the public opinion on viral vectors has become increasingly skeptical and critical, mainly because of the unexpected death of a patient in a recent clinical trial [46]. To circumvent the issues associated with viral vectors, nonviral approaches are becoming increasingly popular. Advantages include ease of fabrication and limited toxicity and immunogenicity, but

the intrinsic transfection efficiency and duration of effect is usually low compared with viral vectors. A separate issue with localized gene delivery is the timing of delivery. The discrepancy between the instantaneous response to injury upon graft transplantation and the 12–24 h lag time between gene activation and protein production raises questions about limitations to ultimate efficacy inherent to this approach. A possible solution to this may be a combinatory approach were genetic modulation is supplemented with protein delivery for the initial period.

Although most transgene methods serve the purpose of increased expression of target proteins, excessive protein activity can result in deleterious effects. For example, VEGF, although useful for endothelialization and angiogenesis, may have pro-NIH effects if overexpressed [113]. For NOS, another popular target, overexpression could lead to potentially toxic local levels of NO. Therefore, to target NIH and endothelialization in bypass grafts, it may be more appropriate to use methodologies that interfere with protein expression instead like the local delivery of antisense ODNs [136] or small interfering RNA [13].

The current knowledgebase on circulating progenitor cells, although constantly expanding, indicates that a significant cell fraction may have the ability to differentiate toward an endothelial phenotype, if properly stimulated. One might therefore envision delivery of DNA on the luminal side of grafts that encode for factors to direct progenitor cells toward an endothelial lineage.

Considering the distinct architecture of blood vessels and the local aspects of NIH and endothelialization, it is a logical step to move to more spatial control of genetic engineering to increase efficacy of a particular method. For instance, one might be able to use a multi-vector approach, where vectors that specifically block the expression of mitogenic factors are localized inside a graft (e.g., by impregnation), while vectors that upregulate factors beneficial for endothelialization may be localized on the luminal area using a coating material embedding the vector.

The field of gene delivery, although offering a broad spectrum of opportunities for vascular repair strategies, is still in its infancy and intensification of in vitro and preclinical research is needed to clarify exact molecular mechanisms and efficacies, exemplified by the failure of the recent PREVENT trials [176]. Moreover, surprisingly, it seems that the use of genetic engineering for existing permanent grafts is still largely unexplored. Besides focusing on the improvement of vein grafts, as advocated recently [1], increasing our efforts on synthetic grafts could be equally rewarding, if not more. Notwithstanding some disappointing results, likely due to current caveats in our knowledgebase, genetic engineering has clearly proven its potential and it seems to be only a matter of time before a genetic approach will be clinically available.

15.6.4 Tissue Engineering of Small-Diameter Blood Vessels

After several decades of extraordinary investments in the improvement of available graft materials, principally polyethylene terepthalate (PET) and extended polytetrafluoroethylene (PTFE) and, to a lesser extent, PUs, the new concept of completely tissue-engineered blood vessels (TEBVs), gained widespread popularity. Hailed as the concept of the future, the TEBV principle has been investigated vigorously for the past 20 years. However, the shift in paradigm from permanent synthetic grafts via biodegradable (or bio-eliminable) grafts up till completely tissue-engineered vessels may have been accompanied by an overly positive outlook. Recently, in a review by Baguneid et al. [12], it was stated again that TEBVs will most likely be the ultimate solution for vascular repair, mainly based on the premise of further advances in stem cell technology. Although indeed completely biological, "living" vessels made out of autologous (stem) cells may theoretically offer the best solution, they do not address vital aspects like off-the-shelf availability for emergency procedures. TEBVs require a significant time for vessel maturation, making this approach unsuitable for the often instantaneous need for bypass surgery in emergency situations. Even if healthcare practice would implement periodic screening for heart conditions, thereby accommodating TEBV creation as a precaution, a number of issues remain. Accurate prediction of which vessel will need to be replaced seems unlikely. Also, to minimize NIH (discussed in more detail in Sect. 15.1), the diameter of the TEBV should match that of the host vessel. Need for "shelved" TEBVs with various diameters would therefore be necessary. Furthermore, technical issues regarding the shelf life of such a biological, living TEBV are not trivial, as they will likely require similar storage conditions as those of transplant organs. Therefore, although the TEBV concept is theoretically and biologically enticing, it is hardly a panacea for vascular repair and

significant hurdles must be overcome to enable widespread use. Nonetheless, clinical trials on TEBV concepts are currently taking place and it may be surmised that we are getting closer to a tissue engineering solution for widespread acceptance in vascular surgery. However, the above-mentioned clinical trials face their respective issues toward clinical acceptance and commercialization. The PLLA-PCL concept by Shin'oka, for example, has not yet shown any promise for the replacement of vessels in high-pressure circulations. Cytografts cell-sheet based TEBV takes approximately half a year to fully mature, making this a costly approach and unsuitable for emergency situations. Although they argue that for a large part of patients in need of a graft, delaying surgeries to create a TEBV is possible and does not necessarily result in increased myocardial infarction of mortality rates [102], it remains to be seen whether patients and surgeons are willing to take that risk, especially if the use of vein grafts is an acceptable option. At the very least, they will convincingly need to prove that the potential benefit of their TEBV outweighs any risks due to a delayed surgery. Furthermore, they have acknowledged that overcoming reimbursement challenges of this costly approach is likely only feasible if 4–6 year postimplantation results prove significant improvements in efficacy and quality of life over the use of native vein [102]. Nevertheless, to date this approach is the closest to clinical use.

15.7 Five-Year Perspective

The concepts and design criteria for biohybrid tissue engineering of vascular grafts have been laid out and more efforts should focus on clinical translation [12, 63, 79, 172, 208, 220, 238]. One of the main problems lies in the fact that neotissue formation using cells from animal origin is vastly different compared with the use of human cells, complicating the translation of results from animal trials. Neotissue formation processes remain largely misunderstood and more in-depth studies on vascular biology focusing on that particular aspect of TEBV technology are necessary in the next years. A logical step, since, if we want to optimize the conditions for cells to generate the correct tissue in a timely manner, we need to understand fundamental aspects of how and why the cells react to specific environmental cues.

The true clinical potential for EC seeding technologies will likely be elucidated by a multicenter clinical trial currently in progress, led by Dr. Zilla. After several decades of huge efforts, this could very well be the last chance for this approach. As for more complex TEBVs, so far, the field has had the advantage of being both life-saving and applicable to a large patient population, resulting in widespread support and massive public and private investments over the years. However, the stagnation of TEBV concepts in making their mark by advancement toward the clinical environment has led to waning investor interest. To enjoy continued investments, in the next few years, irrespective of the TEBV approach used, an increasing number of second-generation concepts that achieve results with prospects of profitability will be needed. Their true clinical potential will only become clear after significant evaluations and particularly the cytograft TEBV undergoing trials will give a good indication of the capacity of these grafts. However, the issue of a universally applicable and readily available cell source for mass production of these products will likely linger and could hinder further developments. Current gene therapy concepts theoretically could make a large impact on all vascular repair approaches, including the issue of cell sources, although the next decade likely needs to be focused on an extension of our knowledgebase. Especially an increase in our efforts on the largely unexplored applicability to synthetic grafts could be highly rewarding and notwithstanding some earlier disappointing results, it seems only a matter of time before additional genetically active grafts will be clinically available.

15.8 Summary and Conclusion

In the end, the goal for all scientists pursuing improved vascular repair is to modulate phenomena after vascular interventions to the extent that repetitive interventions are no longer necessary. Whether this task is achieved by means of a synthetic graft that "tricks" the body into treating it as a normal blood vessel or by a TEBV that actually becomes one is basically irrelevant. Of relevance is sustained revascularization and maintenance of tissue function. Selectivity of molecules and proteins can generally not assumed to be strictly defined, and the effect of this quasi-selectivity on anticipated interactions and modulations in vivo is ill-defined. For example, the immobilization on the polymer surface of molecules

known to recruit and adhere to circulating endothelial (progenitor) cells may also attract other, undesirable plasma constituents such as platelets, leading to increased risks of thrombosis. Likewise, the release of drugs to inhibit SMC overproliferation or platelet adherence may also prevent reendothelialization of the polymers surface, increasing chances of adverse events at a later stage. The task at hand may therefore demand combination strategies for a more elegant solution that invokes pleiotropic effects.

Suggested Readings

Zilla P, Greisler H. Tissue engineering of vascular prosthetic grafts. 1st ed. Austin: Landes Bioscience; 1999.

The perfect vascular graft: A hypothetical discussion of what it is and how one may go about producing it. In spite of ongoing biomaterials research, synthetic small diameter vascular prostheses still perform significantly worse than autologous vein grafts. The main reason for this disappointing situation lies in the incompleteness of healing of current grafts in humans. Even after decades of implantation, surfaces remain free of endothelium. By exploring some basic biological areas and introducing the background of clinical and pathological failures and demands, this book shall serve as a comprehensive guide, furthering the research initiatives of academic vascular surgeons, scientists, and industry researchers.

Isenberg BC, Williams C, Tranquillo RT. Small-diameter artificial arteries engineered in vitro. Circ Res. 2006;98:25–35.

Although the need for a functional arterial replacement is clear, the lower blood flow velocities of small-diameter arteries such as the coronary artery have led to the failure of synthetic materials that are successful for large-diameter grafts. Although autologous vessels remain the standard for small diameter grafts, many patients do not have a vessel suitable for use because of vascular disease, amputation, or previous harvest. As a result, tissue engineering has emerged as a promising approach to address the shortcomings of current therapies. Investigators have explored the use of arterial tissue cells or differentiated stem cells combined with various types of natural and synthetic scaffolds to make tubular constructs and subject them to chemical and/or mechanical stimulation in an attempt to develop a functional small-diameter arterial replacement graft with various degrees of success. Here, we review the progress in all these major facets of the field.

Gorbet MB, Sefton MV. Biomaterial-associated thrombosis: roles of coagulation factors, complement, platelets and leukocytes. Biomaterials. 2004;25(26):5681–703.

Our failure to produce truly nonthrombogenic materials may reflect a failure to fully understand the mechanisms of biomaterial-associated thrombosis. The community has focused on minimizing coagulation or minimizing platelet adhesion and activation. We have infrequently considered the interactions between the two, although we are generally familiar with these interactions. However, we have rarely considered in the context of biomaterial-associated thrombosis the other major players in blood: complement and leukocytes. Biomaterials are known agonists of complement and leukocyte activation, but this is frequently studied only in the context of inflammation. For us, thrombosis is a special case of inflammation. Here we summarize current perspectives on all four of these components in thrombosis and with biomaterials and cardiovascular devices. We also briefly highlight a few features of biomaterial-associated thrombosis that are not often considered in the biomaterials literature:

- The importance of tissue factor and the extrinsic coagulation system.
- Complement activation as a prelude to platelet activation and its role in thrombosis.
- The role of leukocytes in thrombin formation.
- The differing time scales of these contributions.

Olsson P, Sanchez J, Mollnes TE, et al. On the blood compatibility of end-point immobilized heparin. J Biomater Sci Polym Ed. 2000;11(11):1261–73.

In the case of blood, contact with foreign materials induces activation of several host defense mechanisms, the most obvious being the haemostatic mechanism, with such consequences as thrombus formation, occlusion of medical devices and embolization. Systemic anticoagulation, usually with heparin, to counteract complications related to coagulation unfortunately increases the risk of uncontrolled bleeding, and does not exclude triggering of other defense systems. As a result of the intricate interaction between systems in blood and the vascular endothelium, a systemic (“whole body”) inflammatory condition may evolve, which can lead to multiorgan dysfunction [72]. Efforts to improve the performance of biomaterials for applications in contact with blood have followed two main approaches: (a) development of inert surfaces, which generally should

not interfere with mechanisms in blood to avoid adverse defense reactions; and (b) so-called bioactive surfaces, which are intended to actively support natural control mechanisms to prevent unwanted and uncontrolled responses of the host to the foreign material. Examples of the latter category are biomaterials surfaces modified by heparin coupling.

Rissanen TT, Ylä-Herttuala S. Current status of cardiovascular gene therapy. Mol Ther. 2007;15(7):1233–47.

Gene transfer for the therapeutic modulation of cardiovascular diseases is an expanding area of gene therapy. During the last decade, several approaches have been designed for the treatment of hyperlipidemias, postangioplasty restenosis, hypertension, and heart failure, and for protection of vascular by-pass grafts and promotion of therapeutic angiogenesis. Adenoviruses (Ads) and adeno-associated viruses (AAVs) are currently the most efficient vectors for delivering therapeutic genes into the cardiovascular system. Gene transfer using local gene delivery techniques have been shown to be superior to less-targeted intra-arterial or intra-venous applications. To date, no gene therapy drugs have been approved for clinical use in cardiovascular applications. In preclinical studies of therapeutic angiogenesis, various growth factors such as vascular endothelial growth factors (VEGFs) and FGFs have shown positive results. Gene therapy also appears to have potential clinical applications in improving the patency of vascular grafts and in treating heart failure. Postangioplasty restenosis, hypertension, and hyperlipidemias (excluding homozygotic familial hypercholesterolemia) can usually be managed satisfactorily by conventional approaches, and are therefore less favored areas for gene therapy. The development of technologies that can ensure long-term, targeted, and regulated gene transfer, and a careful selection of target patient populations, will be very important for the progress of cardiovascular gene therapy in clinical applications.

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Heart and Cardiovascular Engineering

16

Axel Haverich and Mathias Wilhelmi

16.1 Cardiac Engineering

End-stage organ failure and tissue loss represent the most devastating and costly problems in medicine. More than 8 million surgical procedures – reconstructive surgical techniques, the substitution by various implants, and tissue or organ transplantation – are associated with more than US $400 billion annually in the United States alone. However, although many treatment options, i.e., tissue and organ transplantation programs have significantly improved patient outcomes over last years, both these substitutional and reconstructive options are imperfect solutions, limited by a number of factors.

1. The increasing lack of donor organs and associated second line risks due to lifelong immunosuppressive therapy
2. The shortage of donor tissues, i.e., autologous and allogeneic small vessel grafts and donor-site morbidity, e.g., arteriosclerosis or varicosis
3. An increased risk for infectious and thromboembolic complications following the implantation of mechanical devices and alloplastic grafts due to an increased intrinsic thrombogenicity and the alloplastic nature of these grafts.

Thus, seeking for a method "*designed and constructed to meet the needs of each individual patient*" [133] the idea of tissue engineering as an interdisciplinary field that applies the principles and methods of engineering and the life sciences toward the development of biological substitutes that restore, maintain, and/or improve tissue function [117, 132, 133] was born.

Methods can be divided into three major approaches:

1. Guided Tissue Regeneration: engineered matrices – mainly acellularized or decellularized prior to implantation – are implanted into a recipient organism for in vivo cellular (re)seeding, maturation, and restoration. *Example*: a decellularized blood vessel or heart valve is implanted into a recipient organism and then regenerates under in vivo conditions in corpore.
2. Selective Cellular Transfer: general or local injection of cell preparations (*of autologous, allogeneic, or xenogeneic origin*) to elevate the concentration of one specific cell type or to provide a defined cell-mix within the targeted tissue area. *Example*: systemical or local injection of a stem cell suspension into areas of myocardial infarction to induce regenerative processes and improve function.
3. Tissue Engineering in the classical sense: in vitro isolated, expanded, and differentiated cells are seeded in vitro onto or into an engineered matrix scaffolds to form living tissue components or even a solid organ. *Example*: endothelial cells are seeded on the luminal surface of a decelullarized vessel graft to generate a bioartificial vascular prosthesis.

Out of these concepts the latter one represents the most commonly used approach. Using different cell-matrix combinations, many (*cardiovascular*) tissue structures have already been generated and larger 3D-tissue structures, e.g., bioartificial myocardium grown in vitro are beginning to take shape [29]. However, many obstacles and challenges, i.e., regarding specific tissue requirements of cardiovascular structures remain. As such isolated cells tend to form appropriate tissue

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structures in vitro only under favorable conditions, and they require a template to guide their organization into a proper architecture. Therefore, many basic physiological requirements have to be transferred into the experimental setting of all active laboratories. In this sense, some of the most fundamental problems involve the mass transfer of oxygen and other nutrients and the process of (neo)vascularization of living tissues in 3D structures. In addition, microfabrication technologies to generate custom-made alloplastic matrix scaffolds, the identification of required mechanical stimuli of proper tissue generation, and the establishment of in vitro bioreactor systems to mimic all these physical conditions have to be developed de novo. And finally, further knowledge regarding stem cell biology has to be generated *and* transferred to tissue engineering technologies.

The concept of "*Cardiovascular Tissue Engineering*" obviously comprises the generation of cardiac and vascular tissue structures, such as the myocardium, heart valves, and blood vessels. However, because of organ-specific cellular functions, it also describes the application of cellular preparations (*see above* "selective cellular transfer") to treat arrhythmic disorders or myocardial infarction. The following sections give an impression about basic aspects of "*Cardiovascular Tissue Engineering*" and highlight some areas of current research.

16.1.1 Heart Valves

In the 1960s, Ross and Barret-Boyes were the first who used biological, allogeneic human heart valve prostheses in the clinical setting [6, 98]. In contrast to mechanical prostheses, the lack of distracting "click" noises and oral anticoagulation following the implantation of these biological valves to avoid thrombembolic complications made these valves increasingly attractive. However, soon it was noticed that 8–10 years following implantation ongoing degenerative changes occurred, which finally led to complete destruction and thus, necessitated redo operations. Especially two factors seem to play major roles for this phenomenon: (a) immunological reactions in the sense of subliminal tissue rejection [140, 141], which seem to be induced by the antigenicity of resident allogeneic cells; and (b) the method of preservation/fixation of these tissues with glutaraldehyde. Initially, this latter agent was used to reduce the immunogeneity of tissues via crosslink of collagen fibers to prolong its durability. However, it became clear that it increases the risk for calcification, potentially amplifies immunological reactions, and inhibits processes of in vivo regeneration [84]. Trying to avoid the immunological influence of resident cellular components Gulbins reseeded cryo-preserved human allografts without prior decellularization in vitro with autologous endothelial cells and implanted these prostheses in animals – with moderate success [44]. Therefore, a working group around Wilson picked up these data and established a multistep decellularization process, which is based on the use of hypo- and hypertonic solutions, detergents, and enzymes to remove all cellular components of allogeneic canine heart valve prostheses [143]. Following 1 month after implantation of these valves in the pulmonary position of dogs, the valves were macroscopically intact and gave no hints to inflammatory reactions or other immunological side effects. Other working groups who used similar in vitro decellularization protocols prior to implantation reported on comparable good results. Thus, the first commercially available decellularized and cryopreserved heart valve prosthesis came up. However, although it could be shown that decellularized valves exhibit reduced immunogenicity in comparison with native control groups [6], Simon warned that the application of these decellularized valves may lead to accelerated destruction, especially when used in infants [115]. The presumptive reason for this phenomenon was an elevated activity of the infant immunosystem in combination with a physiologically increased calcium metabolism at this age.

Another concept that can be taken as a logical consequence of the previously described method was the autologous endothelial reseeding of decellularized allogeneic and xenogeneic heart valve prostheses. Following very promising data in animal models, first results in human patients are available now (*xenogeneic heart valves reseeded with autologous human endothelial cells*) [21] (Fig. 16.1). Another approach of autologous endothelial reseeding of decellularized heart valve prostheses could be realized in Hannover in close cooperation with the University of Chisinau (Republic of Moldavia). In front of the consideration that decellularized, i.e., xenogeneic matrix scaffolds may still induce immunological reactions due to interspecies differences, human allografts decellularized by

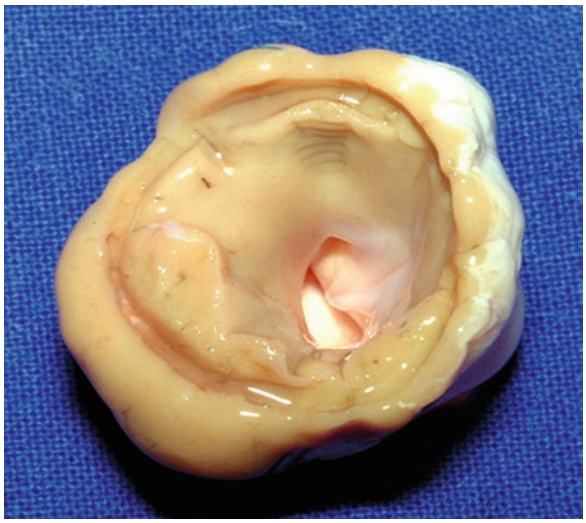

Fig. 16.1 Xenogeneic pulmonary valve (*porcine tissue implanted into sheep*) reseeded with autologous OVINE endothelial cells (*own working group*)

using an elaborated protocol were reseeded by autologous endothelial cells obtained as mononuclear cells isolated from individual blood probes. Positive stains for the von Willebrand factor, CD31 (PECAM-1) and flk-1 as observed in monolayers of cells cultivated and differentiated on the luminal surface of the scaffolds in a dynamic bioreactor system indicated the endothelial nature of these cells. Reseeded valves were implanted in pulmonary position of two pediatric patients (age 13 and 11 years) with congenital pulmonary valve failure. Postoperatively, a mild pulmonary regurgitation was documented in both children. On the basis of regular echocardiographic investigations, hemodynamic parameters and cardiac morphology changed in 3.5 years as follows: increase of the PV annulus diameter (18–22.5 mm and 22–26 mm, respectively), decrease of valve regurgitation (trivial/mild and trivial, respectively), one decrease (16–9 mmHg) and one increase (8–9.5 mmHg) of the mean transvalvular gradient, one remaining (26 mm) and one decreasing (32–28 mm) right ventricular end-diastolic diameter. The body surface area increased (1.07–1.42 m^2 and 1.07–1.46 m^2, respectively) and no signs of valve degeneration were observed in both patients now 7 years after the procedure. Thus, it could be shown that the tissue engineering of heart valves using autologous endothelial progenitor cells is a feasible and safe method at least for pulmonary valve replacement. Tissue engineered valves have the potential to remodel and grow according to the somatic growth of a child [13, 14] (Fig. 16.2).

An alternative approach followed since 1994 especially by the US surgeons John Mayer and Joseph Vacanti is the use of synthetic matrix scaffolds and the development of heart valves on the basis of polymers polyglycolic acid (PGA) and polylactid (PLA), respectively [150]. Following reseeding of these matrices with autologous arterial vessel wall-specific cells, they generated pulmonary valves and pulmonary artery segments and tested them in sheep [110, 111, 113]. The initially experienced disadvantage of very high rigidity of the polymer scaffold led to the development of new polymers and copolymers, e.g.,

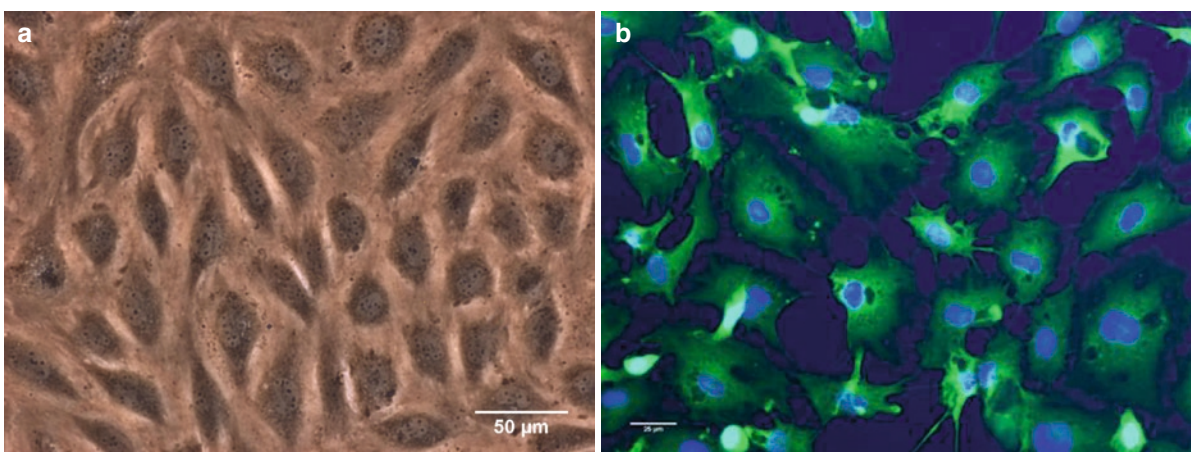

Fig. 16.2 (**a**) Endothelial progenitor cells (EPC) in culture dish, native – no staining, original magnification 200×. (**b**) EPC in culture dish, DAPI/anti e-NOS double staining, original magnification 200×

polyhydroxyoctanoate, poly-4-hydroxybutyrate, and PGA [47, 58, 109, 118]. However, till now none of these scaffold materials reached the clinical stage of development.

As of the year 2009, early successful attempts at pulmonary valve replacement have been performed. Regarding aortic and mitral valve replacement using Tissue engineering concepts, a long journey can be seen ahead of us.

16.1.2 Myocardial Tissue

According to the typical three-layer architecture of blood vessels, the heart in principle represents the largest "vascular" structure of the body. Although it seems to be very simple to mimic this tissue, bioartificial generation of this organ or parts thereof is still one of the most challenging tasks in biomedical science. The first, probably accidental event to develop a 3D myocardial tissue dates back to the early 1950s, when Moscana et al. isolated embryonic chicken heart muscle cells and cultivated them under continuous rotation in an in vitro setting. After 18 h, cells spontaneously formed speriod, 3D- and approximately 200 cells comprising aggregates, which exhibited spontaneous contractile activity [75]. Many other working groups picked up this promising model and observed that the cellular aggregates functionally resembled native cardiac tissue much more than any other 2D cellular monolayers before [70]. Thus, this early observation demonstrated that embryonic cardiac cells grow even under in vitro culture conditions and tend to form spontaneous cellular aggregates.

Following longer times of in vitro culture as well as the use of higher cell counts and densities, it could be observed that there was initial spontaneous contracting and the cellular monolayers adhering to cultures dishes loosened and subsequently floated in the culture medium, since these tissues were no longer exposed to mechanical stresses. However, contractions ceased and the monolayers retracted. For a long time, this phenomenon was explained by insufficient culture conditions, and many working groups did not follow this experiment anymore. However, Shimizu et al. regarded this phenomenon as a chance to harvest and staple the floating monolayers and thus to generate a 3D-tissue structure without the need for an additional matrix scaffold [107]. According to the observation that free floating and thus mechanical none loaded myocardial monolayers cease their contractile activity over time, Vandenburgh et al. covered cultured muscle cells with type-1 collagen to further mechanically load and stimulate these cells [134]. They observed that this method led to improved differentiation of myocytes so that this basic principle was transferable to other, none muscular cell types as well. The resulting data revealed that a 3D-matrix architecture led to improved cellular differentiation of all kinds of evaluated cells and tissue generation. Beside cellular composition and differentiation, living tissues are characterized by the orientation of resident cells. In this sense especially two factors seem to be of special interest: (a) mechanical stimuli and (b) the geometric orientation of matrix components, which seem to function as a kind of guidance. Therefore, working groups around Vandenburgh and Terracio constructed electronically controlled bioreactors in which stretching forces were applied to skeletal and cardiac muscle cell cultures. In their experiments, they could show that mechanical stimuli exhibit positive effects on the differentiation and orientation of muscle cells [15, 127]. Under conditions of continuous cyclic stress consisting of distension and relaxation skeletal muscle cell cultures formed longitudinal orientated 3D-muscle fibers, which began to form tendons as well [135]. Other working groups could show the essential importance of matrix scaffolds on tissue growth and cellular differentiation. One important example for this observation was that cardiomyocytes obtained from newborn rats, which were seeded on a rill-shaped collagen type-1 matrix grew along these structures [116]. Besides known and previously mentioned mechanical influences, other parameters such as strong magnetic fields could be identified as factors influencing the orientation of fibroblasts and smooth muscle cells as well [43, 128, 129].

Another approach to bioartificially generate cardiac tissue structures resulted from the search for an improved in vitro model to induce and investigate contractile forces, as well as the influences of genetic, pharmacologic, and mechanical stimuli. Initially developed to evaluate embryonic fibroblasts [57], cardiomyocytes were cultivated in a collagen-gel matrix [15, 45]. The particular characteristic of this experimental setting by Eschenhagen et al. was that the cell containing matrix was positioned between two Velcro covered glass rods, which were positioned rectangular to each other.

Spontaneous muscle activities and forces generated by the cellular aggregates adhering to these glass rods thus could be identified, measured, and documented [27]. Cardiomyocytes obtained from newborn rats, which were embedded in collagen type-1 gel actually showed no growth, no differentiation, and thus, no tissue generation [119] before the addition of further extracellular matrix components (Matrigel) [147]. Furthermore, generated contractile forces could be further increased (3×) just by application of cyclic distension forces [30].

Two other, basically different approaches were based on PGA matrices in combination with cell cultures [11, 62] and the seeding of fetal rat cardiomyocytes cultivated for 7 days in vitro within a liquid gelantine matrix, respectively [67]. Both these cell matrix composites exhibited spontaneous contractile activities both in vivo and in vitro. However, following implantation in areas of myocardial infarction only very few of these cardiomyocytes could be detected histologically. Leor used an alginate-based matrix, seeded this scaffold with fetal cardiac cells, and implanted it into rat hearts, too [64]. Interestingly, he observed distinctive vascularization within these constructs (*inflammatory?*), but a real integration into the surrounding recipient's myocardium could not be observed either.

The probably most important conceptional advantage of tissue engineering approaches in the classical sense and in contrast to liquid gel matrices would be solid scaffolds already preformed (=*biological*) or synthetic materials to be shaped individually so that almost every geometric form can be achieved. However, the most important disadvantages of this concept are an only limited diffusion capacity, the limited mechanical compliance, and a potential risk for the release of toxic substances as well as the disability of physiological growth in the case of synthetic materials. More and above, it was observed that cardiac myocytes are able to survive a certain time within these matrices and as mentioned earlier, and exhibit spontaneous contractile activity. However, they do not integrate into the surrounding recipient's tissue and may not form superordinate, coherent, and contractile myocardial tissue.

The development of new materials, the molecular optimization of surfaces, and the generation of microstructures enhancing the spreading of cells within these scaffolds may help to solve all the problems mentioned above. Therefore, a working group around Vunjak-Novakovic tried to realize a kind of hybrid concept. They combined collagen sponges with new born rat cardiomyocytes suspended in Matrigel and stimulated these constructs electrically [90]. This concept led to the formation of cardiac muscle structures with enhanced tissue morphology, contractile function, and specific molecular marker expression. Thus, by inducing myocardial differentiation, electrical stimulation seems to have the same or at least a very similar effect as mechanical stimuli mentioned above [30, 148]. Whether the electric activity per se acts as a stimulus for induction of differentiation of tissue formation or whether the resulting contractile activity of the tissue initiates muscular differentiation is still not clear.

Another approach of tissue engineering techniques without the use of matrix scaffolds was described by Shimizu et al. They used a thermosensitive surface coated culture flask on which cells of all kinds can be grown at 37°C as in any other known culture flask. However, via change of the temperature (room temperature), the cells begin to detach [107]. Thus, confluent monolayers of cardiac myocytes can be detached under definite conditions and subsequently piled up so that 3D-connected cellular layers/tissue patches (up to 50–75 μm thickness) result. The advantages of this technique are the simplicity of the method and the independence from the use of potentially immunogenic, pathogenic, or even toxic matrix scaffolds. The disadvantages comprise limitations with regard to geometric shapes, fragility, and thus, only limited mechanical load capacity. Meanwhile, various variations of this method and by using certain kinds of matrix scaffolds have been described, e.g., commercially available collagen sponges, fibronectin polystyrene beats, or collagen fibers in combination with cellular suspensions [1, 55].

In summary, during the last years, different techniques to realize the generation of spontaneous contractile 3D-cardiac tissue have been developed. Essential parameters for the in vitro generation of cardiac tissues can be summarized as: (1) the presence of biological matrix components, such as collagen type-1, -4, or laminin; (2) mechanical and/or electrical stimulation; (3) the presence of all cardiac-specific cell types (not only cardiomyocytes); and (4) a spontaneously occurring or electrically inducible contractile activity.

One of the most essential and still unsolved problems of all scientific approaches is the limitation of the

maximal achievable tissue thickness, which bases on an only limited diffusion capacity for nutrients and oxygen. Studies evaluating the process of angiogenesis in tumors have shown that in the absence of capillary vessels and perfusion the thickness of living tissues is restricted to a maximum of 2–4 mm [32]. One crucial parameter in this regard is the individual and tissue-specific metabolic activity and the local concentration of cells, respectively. Contractile cardiac tissue contains a very high density of cellular components and thus exhibits a very high metabolic activity. Depending on the stage of development, a human heart contains between 2,400 and 3,300 capillaries/mm^2 area [93]. On the other side, it is known that embryonic rat hearts and adult frog hearts are completely avascular and are fed only by diffusion. The reason for this phenomenon lays in the structure of these tissues, which are characterized by a wide spread trabecular system composed of muscular struts [94, 122]. Regarding strategies for the bioartificial generation of tissues means that a physiological vascularization or an intensive trabecular system with fibers not thicker than 50–75 μm has to be present. Some working groups tried to increase the transport capacity for oxygen and other nutrients by using specialized bioreactor systems and/or oxygen carriers, e.g., peroxyfluorocarbone [12, 91]. Actually it was possible to induce a positive effect on the cellular density as well as metabolic activity [12], subsequently cardiac tissue with a clinical relevant tissue thickness of up to 500 μm could be generated [91].

Other strategies were based on data observed at newborn rat hearts. Here it was tried to generate single tissue struts, which were then woven to each other so that the resulting network was completely perfused and allowed for diffusion through the whole tissue structure. Another approach tried to integrate bioartificially generated cardiac muscle constructs into native vascularized tissues (with or without additional pharmacological stimulation of angiogenetic processes). Although it was observed that all these artificially generated heart muscle tissues exhibited a fast (re-)vascularization following implantation and that hypoxic periods during and early after implantation seemed to be of only minor importance with regard to tissue fade, some essential questions could not be answered: (1) is the preexisting vascularization sufficient for long-term survival; (2) how many of the initially implanted cardiomyocytes survive over time; (3) do these implanted cells functionally and structurally integrate into the recipient myocardium; and (4) does the implantation of those constructs really lead to an augmented cardiac function? In context of some in vitro studies, such as Langendorff models, it was observed that the cardiac function actually improved, higher pressure values were obtained [67], and the fractional shortening was increased [64]. However, these effects were overall not very pronounced and could be obtained in a similar way by the injection of different and, i.e., non-cardiomyocytic cells. In direct comparison of cellular suspensions and 3D-bioartificially generated tissue grafts and the advantage of latter is not clear at present.

16.1.2.1 Matrix Scaffolds

As already mentioned earlier, the optimal matrix scaffold for the bioartificial generation of cardiac tissue is still unknown. The reasons for this fact are manifold and may be characterized as follows: (1) the necessity of high mechanical stability with coexistent high compliance; (2) the helical and interweaved fiber structure of the cardiac muscle, which are mandatory for optimal pump function; (3) the guarantee of a sufficient supply with oxygen and nutrients. Today it is not clear, if it ever will be technically possible to generate materials, which completely mimic the complex structure of a living heart. Even if it should be possible, e.g., on basis of modern nanotechnologies, it has to be guaranteed that quantitatively enough nishes are present within those matrices, so that specific cells can be nested within the scaffolds, connect with each other, and build up a full functioning electrical and mechanical syncytium. Whether strategies developed in Doris Taylor's laboratory to decellularize and reseed an entire organ will proof successful has to be awaited. At least, a matrix closest to native would be provided.

16.1.2.2 Cell Sources

Another conceptional issue regarding the bioartificial generation of cardiac tissue refers to specific cellular components and sources to isolate or generate such cells, respectively. It is estimated that an adult human heart comprises 5×10^9 cardiomyocytes within the left ventricle alone [8]. This means that approximately 40 million cardiomyocytes are present within 1 g of

cardiac tissue or in other words that it is nearly impossible to harvest a sufficient cell count to generate a complete bioartificial heart, e.g., via cardiac biopsies. Scientific approaches with focus on stem cell research and adult stem cell research might represent a possible solution for this problem. Today it is known that all cells necessary to generate autologous cardiac tissues can be differentiated from (adult) stem cells, which can be harvested from, e.g., bone marrow aspirates [69, 85], peripheral blood probes [2, 5], cord blood [16], or fatty tissue [149]. However, it has to be awaited if these cells are really quantitatively and qualitatively sufficient to fulfill both scientific hopes and clinical needs.

In summary, over the last 10 years, many ideas regarding the bioartificial generation of cardiac tissues have been born. However, it still has to be awaited whether it is possible to establish a real therapeutic alternative in the sense of a regenerative approach. More and above, such tissue engineering concepts would strongly compete with prospective developments of mechanical assistant devices, pharmacological approaches, and xenogeneic transplantation.

16.1.3 Cardiac Cell Therapy

Over the last 20 years, many advances in the therapy of ischemic cardiac diseases and resulting heart failure could be made. Nevertheless, coronary artery diseases and associated complications still represent the most common cause of morbidity and mortality in western industrial countries [33]. Besides the commonly known therapeutic options comprising pharmacological substances, percutaneous catheter techniques, the surgical application of coronary bypasses, organ transplantation, or the implantation of electromechanical devices, e.g., cardiac assistant- and pacing devices, another very interesting therapeutic strategy came up during last years. On the basis of the consideration that impaired contractile function of damaged cardiac tissue largely bases on reduced counts of vital cardiomyocytes, it has been aimed to transfer current knowledge obtained from stem cell research to the heart and thus try to nest newly differentiated and expanded cardiac cells into damaged areas of the myocardium. In contrast to previous therapeutic strategies, here it is intended not only to limit damage, but also to achieve a restitutio ad integrum. Currently evaluated cells comprise: (1) cardiac myocytes, (2) skeletal muscle cells, and (3) pluripotent adult stem cells.

16.1.3.1 Skeletal Muscle Cells

Skeletal myoblasts are resident cells within striated musculature. The physiological stimulus to activate these mainly resting cells is muscular damage, which then leads to cellular proliferation. In contrast to stem cells obtained from bone marrow, which are pluripotent, skeletal stem cells exhibit a kind of myocytic predifferentiation and thus are no “real” stem cells any more. However, in front of its autologous origin, the simple isolation of these cells from muscular biopsies and the ability to proliferate in vitro, they represented the ideal cell type for initial clinical trials [72]. Actually it was noted that left ventricular function improved following injection of preprocessed cellular suspensions into left ventricular ischemic areas [125]. However, a major disadvantage was that these cells kept their striated muscular characteristics and therefore did not differentiate into cardiac muscle cells (see Sect. 16.2.2). More and above, they exhibited spontaneous, nonsynchronized muscular/electrical activity without functional and electrical integration into the surrounding native myocardium [72]. Thus, in view of this electrophysiological nonintegration and the appearance of arrhythmias, it was necessary to implant cardioverter devices into all participants of this study [76].

16.1.3.2 Bone Marrow Stem Cells

As described for skeletal myoblasts, stem cells obtained from bone marrow led to improved left ventricular function when injected into ischemic areas in various clinical studies. Skeletal muscle cells differ from stem cells originating from bone marrow in that the latter do not represent one single specific cell type but a mixture of various cells. The portion of real stem cells comprise mesenchymic cells with the potential to differentiate in nearly all kinds of cells, progenitor cells of muscular structures, and progenitor cells of hematopoetic cell lines. Therefore, the resulting question is what kind of cell fits best in which situation? Interestingly, few studies reported on the ability of bone marrow stem cells to differentiate into cardiomyocytes [73]. Although negative side effects such as pronounced calcifications were

noticed in injection areas [145], data obtained from animal experiments were regarded so essential that clinical trials, e.g., the Duesseldorf studies by Strauer or the TOPCARE-AMI trial were initiated [3]. Isolated from bone marrow or peripheral blood probes of individual patients suffering from acute myocardial ischemia, cells were processed and – if a recanalization of the infarction related artery could be achieved – reinjected into this coronary artery. Although most of these studies were not randomized and not single- or double-blinded, an improved left ventricular ejection fraction was observed and was still present (to a lesser extend) 1 year after cell injection. Differences between stem cells taken from peripheral blood probes and those processed from bone marrow could not be detected. A subsequently randomized study (BOOST) in which stem cell suspensions were injected into areas of acute myocardial ischemia revealed an improved left ventricular function, too [144]. More and above, and in comparison with control, the incidence of unrequested side effects was comparable between groups.

A major disadvantage of such stem cell therapies is that only small numbers of cells can be isolated from bone marrow aspirates as well as from peripheral blood probes. Some smaller studies therefore evaluated the effect of granulocyte-colony-stimulating factor (G-CSF) on stem cell density/count within the peripheral blood [52]. Besides improved cardiac function and angiogenesis, an increase in the incidence in stent restenosis could also be observed. And most disturbingly, G-CSF was associated with an increase in morbidity and mortality related to cardiac ischemia, whereas a cardiac improvement was not verifiable [46].

16.1.3.3 Cellular Therapies in Cardiac Arrhythmias

An optimal cardiac function depends on a synchronized mechanical contraction of all regions of the heart and an activity adopted frequency. The structural basis for this demand is the hirachic organization and electrical specialization of the cardiac conductive system, which depends on differential expression of ionic channels within the various compartments [105]. The primary electric excitement is generated within the sinus node and then transmitted to the atrioventricular node and via the His-Purkinje fibers to the right and left ventricle, respectively.

Cardiac arrhythmias are defined as pathological variations of the normal heart rate and/or normal patterns of the conductive system. The clinical range of possible symptoms reaches from harmless palpitations to sudden cardiac death. Currently available therapeutic strategies to treat arrhythmias can be grouped into three categories: (1) pharmacological options, (2) surgical or catheter-based methods to dissect or ablade arrhythmogenic foci, and (3) the implantation of cardioverter or pacing devices.

Pharmacological approaches are increasingly under debate because of their less specific and only focal effect, the limited efficacy, and a high incidence of dangerous side effects, e.g., pro-arrhythmic effects [23]. New techniques of radiofrequency ablation led to a revolution in the field of clinical electrophysiology. Now many cardiac arrhythmias can be treated curatively and do not require life-long pharmacological therapy any more. Especially superventricular, but also some ventricular arrhythmias can be treated very effectively by these methods [103]. Implantable cardioverter and pacing devices represent state-of-the-art options in the treatment of low rate arrhythmias, whereas cardioverter systems are especially indicated for the palliative therapy of life-threatening tachycardias [4, 77]. The disadvantages of these devices comprise life-long intermittent surgical interventions and a high risk for associated complications. Although many of the above-mentioned methods represent state-of-the-art therapies, certain kinds of arrhythmias still cannot be treated sufficiently. Thus, alternative strategies have to be identified. Improved myocardial perfusion and contractility in chronic ischemic heart disease on the basis of new developments in the field of molecular- and cellular biology as well as on tissue engineering may represent prospective options in this context. In theory, cell therapeutic approaches may act in three different ways: (1) the replacement of nonfunctioning or absent cells of the conductive system, (2) the modification and genetic modification of electrophysiological relevant cellular structures, and (3) the induction of local secretion/release of specific recombinant proteins from the adjacent myocard. Dysfunction and loss of cellular structures at critical points of the conductive system may lead to an inefficient electric impulse induction/conduction and thus necessitate the implantation of pacing devices. A cell-based alternative therapy aiming to restitute the conductive system might use specific cardiac cell populations with specialized

conductive-, pacing- function, etc. Further steps on the way to realize such an idea comprise: (1) the identification of specific cells, (2) the in vitro characterization (functional and structural) of these cells, (3) the development and establishment of strategies and techniques to transfer and position these cells at certain points within the impaired conductive system, and (4) to develop methods and techniques, which guarantee that transferred cells survive and integrate (structural and functional) into the native conduction system.

The major obstacle on the way to realize this approach is to identify a source for human cardiomyocytes. The possible but due to legal and ethic restrictions, only theoretical solution is the use of human embryonic stem cell lines [35, 54]. The idea to isolate and differentiate various subtypes of human cardiomyocytes, e.g., such with pacing function, ventricular Purkinje fiber similar cells, etc., [79] has important impact with regard to regenerative cell therapeutic approaches in the clinical setting. The essential prerequisite of a complete functional integration of such cells into the conductive system could already been shown in studies using human embryonic stem cells [53]. In addition, some studies revealed that not only cardiac myocytes but also other cell types such as fibroblasts [28, 34, 95] connect via gap junctions with recipient cardiomyocytes and thus interact electrically with these cells. In contrast to systemic gene therapeutic techniques (not described here), the selective genetic manipulation of cellular grafts is characterized by many advantages. Advantages such as a higher efficacy and more exact control of the transfection process ex vivo, the ability to evaluate phenotypic characteristics of certain cells prior to transplantation and the possibility to induce long-term effects [78].

In summary, an increasing knowledge and development in the fields of pathophysiology and molecular- and cellular biology give hope that gene and, i.e., cell therapeutic approaches further increase the specificity and effectivity of therapeutic options in the treatment of cardiac arrhythmias [36].

16.2 Blood Vessel Engineering

The primary aim of all vascular interventions is to restore an impaired or destructed blood circulation. The spectrum of possible techniques reaches from local reconstructive approaches, e.g., thrombendarteriectomy to complete substitution by different kinds of vascular prostheses. On the basis of the materials used, synthetic (=*alloplastic*), biological (=*autologous, allogeneic or xenogeneic*) or hybrid vascular grafts, combining biological and synthetic materials, can be distinguished.

16.2.1 Biological Vessel Grafts

As early as at the beginning of the eighteenth century, autologous vessel grafts were used for substitutional or reconstructive vascular interventions [10, 40, 80]. However, allogeneic vascular grafts were not used until the 1940s. The clinical application of these prostheses mainly based on works of Charles Hufnagel [49] and Robert Gross [41, 42]. Harvested mainly in the context of autopsies, these grafts were sterilized by cobalt radiation and predominately used for aortic replacement. In the 1950s, the first tissue banks were established and allogeneic vascular grafts were used to replace nearly any diseased central or peripheral artery. However, following 3–5 years after implantation severe calcifications visible on X-rays pointed out ongoing degenerative processes and finally led to complete graft loss [22, 86, 87]. More importantly the clinical demand for such grafts was much higher than their availability [17, 123].

Today it is believed that the antigenic properties of allogeneic prostheses in the sense of histocompatibility differences are responsible for immunological responses and their resulting tissue rejection. At least in theory, although it is possible to modify immunological differences between donor tissue and the recipient immunosystem, e.g., via various methods of tissue preservation or low dose immunosuppressive therapy, the clinical application of allogeneic grafts is limited to special cases such as infections today. In contrast, autologous vessel grafts, e.g., the greater saphenous vein, are still the first choice for reconstructive and substitutional interventions, especially in small and middle caliber vessels. Alloplastic materials such as Dacron and expanded polytetrafluoroethylene (ePTFE) are primary used for reconstructive interventions at large caliber vessels such as the aorta and its side branches. The clinical restriction of alloplastic vascular grafts to mainly large vessel areas is

explained by the clinical observation that autologous vessel grafts such as the greater saphenous vein still reveals much better patency rates. However, in contrast to the assumption that the greater saphenous vein may represent a universally applicable vessel graft, it should be noticed that this vessel is not available in every patient because of prior surgical interventions, varicosis, or deep vein thrombosis. Furthermore, it belongs to the venous and thus low pressure part of the cardiovascular system, predisposing ectatic and degenerative deformations when exposed to arterial/higher blood pressure load. Other autologous venous grafts, e.g., the femoral vein or those obtained from the upper extremity exhibit the same structural disadvantages and are reported to be even less qualified than the greater saphenous vein. Autologous arteries, too, are not available in every patient and, i.e., not in greater length so that bypass grafts, e.g., at the lower extremity are difficult to realize.

Another milestone in the history of biological grafts goes back to Rosenberg et al., who tried to reduce the immunogenicity of bovine carotid artery grafts by impregnation with dialdehyde starch [96, 97]. Induced crosslinkings between extracellular matrix proteins of these vessels should weaken immunological responses of the recipient, while preserving graft structure/shape and prolonging its storage time. However, soon it became clear that dialdehyde starch was a bad choice, since all grafts underwent severe calcification and degradation. Nevertheless, soon another chemical agent – glutaraldehyde – came up and was found to be a much better choice. Many different tissues were impregnated very successfully with this agent and thus became available from the shelf.

In 1972, Irving Dardik evaluated glutaraldehyde preserved human umbilical veins as an alternative biological bypass material, i.e., for bypasses at the lower extremity [18]. However, as already postulated by Kunlin in 1949, these grafts failed long-term and again the greater saphenous vein was found to be the most suitable graft in this anatomic area [59].

The first vascular prosthesis completely generated on the basis of biological materials goes back to Weinberg and Bell [139]. They seeded smooth muscle cells in vitro on a collagen gel, which mimicked the lamina media, added fibroblasts to the outer surface of this construct, and thus generated a bioartificial adventitia. Subsequently and following 2 weeks of in vitro culture, endothelial cells were added to the luminal surface to serve as an artificial lamina interna. Scanning electromicroscopic evaluations revealed closed endothelial monolayers on the luminal surface of these grafts, which stained positive for von Willebrand factor. However, although histological data were very promising, biomechanical tests revealed that this constructs had only very limited structural stability so that Dacron nets had to be wrapped around the grafts to allow at least physiological pressure loads [139]. L'Heureux picked up this principal method, but changed some culture conditions. Then he noticed that it was possible to positively influence graft stability and pressure tolerance/burst strength [24, 65].

Another approach was described by Campbell et al. who implanted silastic tubings into the peritoneal cavity of rats. After 2 weeks they observed fibroblasts and mesothelial cells to seed on the outer surface these tubings. The resulting tubular tissue sheet then was dissected and everted so that the previously outer mesothelial cells subsequently built up the inner surface and thus mimicked the lamina interna. In animal models, these bioartificial vessel grafts showed physiological reactivity toward vasoactive agents and were patent for up to 4 months [9]. A working group around Huynh used small intestinal submucosa and bovine type-I collagen to generate a new kind of vascular prosthesis. After removal of all cellular components via hypotonic solutions, they tested autologous, allogeneic, and xenogeneic grafts in large animal models and reported on excellent patency rates. In the histological analysis, they found that these primary decellularized implanted grafts were spontaneously reseeded in vivo and thus obviously underwent regenerative processes [50]. However, in experiments using smaller animals such as rats, these small caliber vessel grafts were found to be occluded very early due to thrombus formation [104].

16.2.2 Alloplastic Vessel Grafts

In 1952, Voorhees et al. developed an alloplastic material called Vinyon "N" [138]. Vascular conduits made out of this material and tested in animal models revealed good function for at least a short time. However, in further characterization and evaluation, it became clear that it was not possible to autoclave this material without severe deformation and pronounced

shrinking. Nevertheless, these initial data led many scientific groups to think about alloplastic materials and soon polyester (=*Dacron*) was discovered and evaluated to serve as a vascular prosthesis. A new era in vascular surgery was heralded and soon the clinical application of these prostheses spread all over the world. Data obtained from these early experiences established the basis for all other approaches in the field and a new medical speciality – *medical material engineering* – was established. Besides medical and surgical competence, this early development in alloplastic vascular replacement therapy reflects the high qualification of weavers and loom technicians because many of the fundamental technical skills necessary for the industrial production of these new prostheses were taken from the textile industry. Following the establishment of basic technical methods in the production of woven prostheses, it was tried to optimize material properties, e.g., compliance, manageability, etc. In the first generation, prostheses were very porose and therefore had to be preclotted with blood prior to implantation. Furthermore, they were very stiff so that kinks and stenoses occurred when they were implanted in a too steep angle. Lester Sauvage therefore developed a crimped prosthesis to avoid those kinking-related stenoses [101]. A further development in this sense was the application of external rings and an outer velour coverage for better integration of the prostheses into the surrounding tissue.

In 1954, Shumaker and King discovered Nylon as a new material [112] and Edwards and Tapp constructed a new prosthesis made out of this material [25]. However, in 1958 Harris observed that 100 days following implantation of Nylon into the aorta, these prostheses lost their structural stability, whereas Teflon and Dacron did not reveal such structural changes. Thus, the era of Nylon was terminated before it really began. In the 1970s another new material became available. It was a variant of Teflon, named ePTFE. The microstructure of this material initially discovered by Ben Eiseman is characterized by microscopic small knots and transversal fibers running between them. The resulting internodal spaces contain nothing else than air, so that up to 80% of the prosthesial wall are simply made of air. The practical meaning of this characteristic is that these prostheses are primary blood tight and thus do no not require preclotting prior to implantation. Deduced from further studies aiming to increase the impermeability of vascular prostheses collagen, albumin, and gelantine impregnations were discovered to be useful tools. Importantly, it was observed that these modifications were not associated with loss of biomechanic stability or other characteristics relevant for the clinical application. Other imaginable and in part already realized developments and surface modifications comprise the binding of various effectors, e.g., fibrinolytic- or antibiotic substances, anticoagulants, or other effectors to minimize damaging influences [37, 38, 61, 81, 99, 100].

16.2.3 Hybrid Vessel Grafts

The basic idea for the generation of hybrid constructs combining biological and synthetic materials was to profit from positive attributes of both these material groups. An early approach aiming to realize such a concept was the subcutaneous implantation of polyethylene, polyvinyl, or silastic mandrains covered by a polyester net. The consideration was that fibroblasts seed on these alloplastic nets and a de novo synthesized sheet would occur following 6–8 weeks. After the removal of the mandrain, the remaining collagenous tubular structure could be anastomosed at both ends with a native artery and thus be used as an arterial substitute. Actually, Sparks demonstrated that it was possible to generate such a tubular structure but reported on degenerative changes in the sense of aneurysma formation and resulting graft loss long-term [120].

The application of alloplastic vascular prostheses with an inner diameter smaller than 5 mm was and still is often combined with early thrombotic occlusion and thus graft failure. Thus, one of the most important clinical issues in cardiovascular surgery was and is to identify materials to generate vascular grafts ≤5 mm. In early experiences it was tried to reduce the intrinsic thrombogenicity of alloplastic grafts by seeding them with autologous endothelial cells (Fig. 16.3). One of the first successful isolations and subsequently seedings of venous endothelial cells on the surface of a synthetic vascular graft was reported in 1978 [142]. Miwa and Matsuda developed prosthesis on the basis of polyurethane with an artificial lamina basalis composed of collagen type-I and dermatosulfate on which an endothelial monolayer was transferred. Following in vitro culture, they implanted this construct in dogs

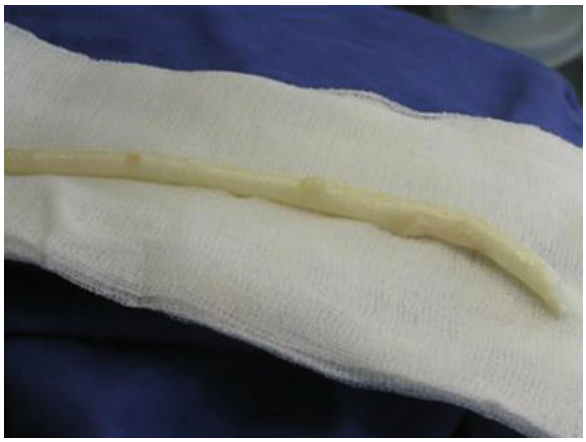

Fig. 16.3 Bioartificially generated vessel graft (matrix: ovine Art. Carotis) reseeded with autologous endothelial cells obtained from peripheral blood probes

and observed a primary patency rate of 75% without addition of anticoagulative therapy [74]. Deutsch reported on a study with a 9 year patency rate of 65% in the above the knee application of endothelial seeded ePTFE prostheses, and thus comparable results to those obtained with venous bypass grafts. Nonseeded prostheses revealed only a patency rate of 16% [20]. The good patency rates of seeded grafts, however, could not be achieved when used for coronary revascularization [63]. It was hypothesized that an increased intrinsic thrombogenicity might be responsible for this phenomenon so that polyurethane matrices seeded with endothelial cells were coincubated with heparin and RGD groups to increase endothelial adherence. Actually a 75% increase in endothelial surface coverage was observed. However, at least in theory the intrinsic thrombogenicity of the resulting vitalized grafts should be reduced, it is not clear yet. Possibly, other remodeling processes will be negatively influenced by the nonresorbable graft material [106]. An alternative to overcome this potential disadvantage might be the use of resorbable materials and thus those which degenerate over time and in parallel to the bioartificial in vivo generation of living grafts. However, one essential prerequisite in this regard is the initially implanted biodegradable material to be biomechanically stable enough to withstand physiological hemodynamic stress load. Also, the bioartificially generated tissue has to develop in parallel to material degradation with subsequently biomechanical properties similar to those of a native vessel.

Over the last years, many polymeres (degradable and nondegradable) were evaluated, but most of them were found to be very thrombogenic, induced foreign body reactions, led to the formation of aneurysms, or were resorbed too fast. Niklason therefore tried to combine biodegradable polymeres with living cells [83]. PGA polymeres were seeded with smooth muscle cells obtained from bovine aortas and cultivated under pulsatile flow conditions in vitro. After 8 weeks, endothelial cells were added and in the histological analysis 50% of all extracellular matrix components were identified as elastin and collagen proteins. Furthermore, scanning electromicroscopic analyzes revealed homogeneous cellular monolayers on the luminal surface, which stained positive for CD31 (PECAM-1). In animal models, these prostheses were patent for up to 8 weeks and withstood a blood pressure load of more than 2,000 mmHg. Presently many working groups try to identify, synthesize, and characterize further biodegradable materials, which exhibit optimized properties for the realization of hybrid concepts [82].

In summary, over the last years it became clear that the most important issues regarding the (bio)artificial generation of vascular grafts relate to the following characteristics.

1. Durability
2. Susceptibility for infectious complications
3. Thrombogenicity
4. Antigenetic properties

Whether prospective gene therapeutic approaches that aim to modulate the intrinsic thrombogenicity via induction of endothelial cell growth and antithrombotic properties or modified surgical techniques and methods will help to increase patency rates is still unclear [48, 60], but till now only very few positive effects were observed [19, 31, 68, 73, 114, 124, 131].

16.2.4 Conclusion

During the last decade, tissue engineering techniques have made significant progress and emerged to a seriously taken and promising superordinate concept, which already found its way into clinical practice of cardiovascular medicine. However, at a time characterized by rapid development with hundreds of working groups following myriad scientific ideas in the field of

cardiovascular tissue engineering alone, it seems to be impossible to give a conclusion, rather than an interim report. Today the anciently ultimate goal to replace diseased tissue structures, e.g., heart valves is almost passed and the second step – the generation of larger 3D-structures with primary connection to the vasculature, such as bioartificial myocardium – comes into focus. Although there are numberless areas of current active research, future directions may be characterized by especially three issues: (1) the development and adoption of dynamic in vitro systems mimicking physiological mechanical stimuli, (2) the investigation of microfabrication technologies to generate microstructured matrix scaffolds to realize a 3D-tissue architecture with primary vascularization, and (3) the selective isolation, contamination free expansion and differentiation of multipotent and primarily undifferentiated stem cells for matrix (re-) vitalization. Notwithstanding the current limited clinical application of tissue engineering in cardiovascular medicine, the prospect of its further use in broadening clinical application can be clearly foreseen. Both the high chronic failure rate of prosthetic/mechanical implants and the general direction in medicine toward personalized therapies will inforce and reinforce these concepts.

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Tissue Engineering of Organs: Eye/Retina

17

Stephen Redenti, Henry Klassen, and Michael Young

17.1 Introduction

The loss of retinal neurons due to degenerative diseases such as retinitis pigmentosa and age-related macular degeneration affects millions of people and remains a daunting and untreatable medical problem. Current strategies target disease prevention or delay of progression. For patients who have already lost significant numbers of photoreceptors, there is no restorative treatment; however, the potential to replace photoreceptor cells using stem or progenitor cell transplantation holds great therapeutic potential. Recent studies have demonstrated that retinal progenitor cells (RPCs) can restore modest levels of visual responsiveness in the degenerated mouse retina. Major remaining obstacles include the need for improvements in the efficiency of cell delivery, degree of donor cell survival, and final differentiation of grafted cells. Retinal tissue engineering offers the advantages of increasing cell survival and directing stem or progenitor cell differentiation toward a photoreceptor state. While the experimental transplantation of retinal tissue into the ocular environment has been investigated since the early twentieth century, attempts to generate new tissue de novo has only been approached more recently, in concert with technical advancements in polymer design capabilities. Here we will review research involving progenitor cell selection, the use of exogenous, soluble factors to guide cell differentiation in vitro, and the combined use of cells and polymers for retinal tissue engineering. We will also address design considerations for the creation of biomimetic polymeric substrates and the generation of retinal tissue equivalents in culture. The guidance of cells toward a photoreceptor-specific fate remains a significant challenge and will likely require multipathway guidance cues in the form of both exogenous molecules and physical substrate cues to mirror endogenous microenvironments and more accurately recapitulate normal development. Efforts to generate a practical paradigm for retinal tissue engineering are focusing on the isolation and expansion of optimal cell populations, directed differentiation, efficient delivery, and functional integration into the degenerated host retina.

17.2 Aim of the Discipline

17.2.1 Restoring Damaged Retina Using Embryonic Retinal Tissue

The first attempts to replace retinal tissue avoided the intrinsic challenges of directing cell differentiation by transplanting intact embryonic retinal sheets with developed cell morphologies and synaptic processes. While this approach offered the advantage of delivering morphologically defined retinal cell types, a major obstacle proved to be directing the connectivity of the transplanted tissue toward the host. Although there were neurites that could be seen extending from transplanted tissue, synaptic connections with the host were minimal unless the tissue was transplanted in the vicinity of the retinal target regions of the brainstem. These early studies did, however, reveal that the transplantation of allogeneic neural retinal tissue to either the ocular anterior chamber,

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vitreous cavity, or retina could result in long-term cell survival, with an absence of severe immune complications [7, 41] Progress was made with the use of retinal aggregates that were shown to survive and reconstitute or replace host retina when they were positioned with their photoreceptor outer segments (OS) in contact with host retinal pigment epithelium [12]. More recently, a study has re-evaluated the transplantation of full-thickness embryonic retina into adult rats and a moderate level of synaptic connectivity was observed [44]. While this study provided anatomic evidence of synaptic connectivity between transplanted and host retina, functional improvement remains to be established. The same group also transplanted 10–15 week old human embryonic retina with attached RPE into advanced (20/200 or worse) retinitis pigmentosa or age-related macular degeneration patients and reported absence of immune rejection and mild benefit to vision which might be due to neurotrophic effects [33]. A fundamental problem with this approach is that the ganglion layer of transplanted full-thickness retina is placed in contact with the residual photoreceptor layer, creating a double retina with no obvious path for normal functional synaptic connectivity. A more feasible approach might be the transplantation of isolated photoreceptor sheets with processes that are in the correct position to synapse with adjacent host bipolar cells [12]. The use of photoreceptor sheets will, however, present additional challenges. Benefits may be limited if photoreceptors neurites are severed and fail to re-extend synaptic processes. For this reason, ongoing full sheet transplant studies require additional investigations demonstrating functional synaptic connectivity between donor and host. It is also worth considering some of the drawbacks inherent in the use of fetal retina as a tissue source for therapeutic purposes. First, as retinal photoreceptor development occurs in the second trimester, obtaining donor material would be a rare event at best. Secondly, this tissue cannot be expanded in vitro, leaving the problem of insufficient transplantation material unresolved.

17.2.2 In Vitro Engineering of Retinal Cell Types

Several different types of cells have been investigated as potential sources of photoreceptors for transplantation and, in addition, researchers have identified many soluble factors involved in photoreceptor fate specification (Table 17.1). However, unlike many neuronal fate choices, which are reproducible, terminal photoreceptor fate decision appears to involve stochastic elements, and even precisely timed intracellular and extracellular signals can result in indeterminate outcomes [17]. An additional challenge lies in the fact that the earlier the developmental origin of a selected cell type, the greater the number of factors and the time interval likely required for successful differentiation toward a photoreceptor fate. A noteworthy example involved a 3-week, sequential differentiation paradigm to direct human embryonic stem cells (hES) first to forebrain neural and then to retinal progenitor fate. Cells were sequentially exposed to a number of factors including noggin (an inhibitor of the BMP pathway), Dkk-1 (an antagonist of the Wnt/β catenin pathway), and insulin growth factor-1 (a director of eye fate) [37]. Stepwise differentiation is used frequently by investigators to recapitulate in vitro the temporal chemical signaling and stage-by-stage retinal neural development occurring naturally in vivo (Fig. 17.1). While this study was significant as one of the first to derive photoreceptor precursors from ES cells, it must be noted that the percentage of cells expressing opsins was quite low (0.01%). Another study, using mouse embryonic stem (mES) cells, resulted in the generation of approximately 36% recoverin and rhodopsin positive photoreceptor-progenitor cells. Unlike the previous study, these mES cells were cocultured with embryonic retina [14]. While media enriched by embryonic retina is known to direct progenitor cell fate, the specific soluble factors involved in differentiation have not yet been fully characterized. More recently, an important cross-species study was carried out in which mouse, monkey, and human ES cell lines were directed first to a neural retinal progenitor state via exposure to Dkk1, LeftyA (an inhibitor of nodal signaling), and activin (an eye fate transcription factor) and then toward a photoreceptor precursor state via a Notch pathway inhibitor [31]. These cells were further directed to express opsin using the synergistic effect of DAPT (γ-secretase inhibitor), FGF (opsin expression agonist), sonic hedgehog, taurine, and retinoic acid (rod photoreceptor inducers). A significant component of this cross-species study was that photoreceptor progenitors were produced in the absence of xenogenic-contaminants, a critical consideration for clinical applications.

Table 1. Original Cell Type/Culture Treatment/Photo receptor Marker Expression

mouse, es, cells	notch signal inhibitor, fibroblast, growth factors, sonic hedghog, taurine, retinoic acid.	in vitro: rhodopsin
monkey, es,	feeder and serum free suspension, Wnt,	
human, es,	Nodal inhibitors add: retinoic acid taurine.	in vitro: rhodopsin Osakada et al. 2008
human, es,	noggin insulin growth factor-1 Dickkopf-1.	in vitro: 0 01% rhodopsin Lambda et al. 2006
mouse, es,	serum free suspension culture, Dickkopf-1, Left yA, serum, activin.	in vitro: 36% rhodopsin Ikeda et al. 2005
human, cord blood	selection of lineage-negative cells	in vivo: rhodopsin Koike- Kiriyama et al. 2007
rat, bone marrow	selection of mononuclear cells	in vivo: rhodopsin Tomita et al 2002
mouse, brain	DMEM/F12, epidermal growth factor, fibroblast growth factor	in vivo: nf200, calretinin, Mizumoto et al. 2002
rat, adult brain	DMEM/F12, N2, FBS	in vivo: nf-200, GAP-43 Guo et al. 2003
mouse postnatal retina	FBS	in vitro: rhodopsin vivo: recoverin, opsin Klassen et al. 2004, Maclaren et al. 2006
pig embryonic retina	FBS, CNTF	in vitro: recoverin, rhodopsin in vivo: transducin, rhodopsin, receoverin Klassen et al. 2007
human embryonic retina	retinoic acid, thyroid hormone	in vitro: 30% recoverin, PNA, opsin Kelly et al. 1995

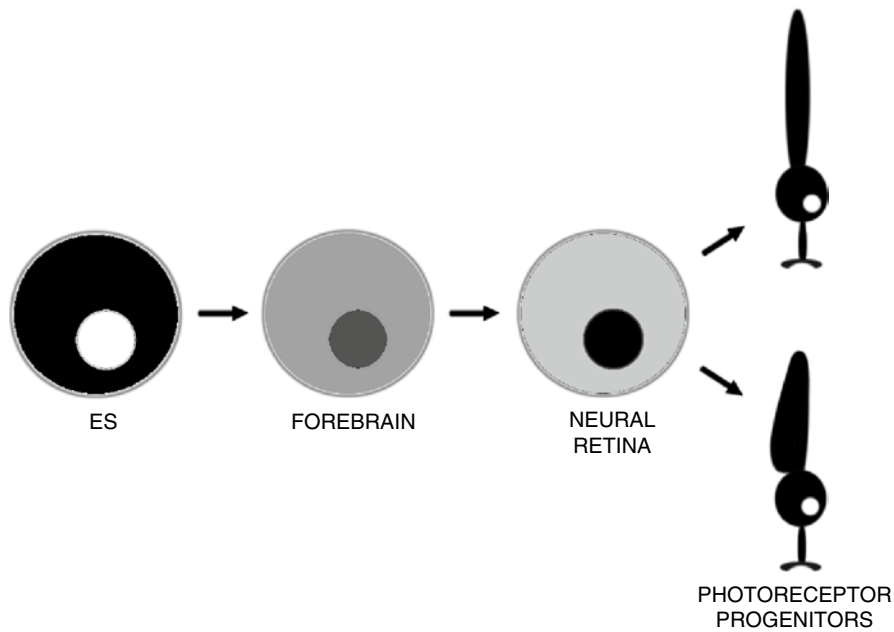

Fig. 17.1 Engineering photoreceptors by recapitulation of development. A standard paradigm for derivation of photoreceptor progenitors from embryonic stem cells (ES) involves stage-by-stage differentiation by exposure to selected factors to inhibit or agonize intrinsic signaling pathways. Using this strategy the fate of a cell population is directed to increasing states of fate restriction from totipotent ES to pluripotent neural forebrain cells, multipotent retinal neural cells, and finally to photoreceptor progenitors

Factors used to drive ES-derived or in vitro established RPCs toward photoreceptor fate have also been shown to drive RPCs isolated from postnatal retina toward photoreceptor fate. A well-characterized factor capable of directing photoreceptor-specific fate is the amino acid taurine, which is found in the developing retina and interacts with a subtype of glycine receptors expressed by RPCs [1, 37, 40]. The oxidized form of vitamin A, retinoic acid (RA), is also present as an active fate determinant in retinal development and its receptor RAR is expressed on RPCs late into development [37]. In vitro exposure of both ES-derived RPCs

and RPCs isolated from postnatal retina to taurine and RA influences gene transcription and results in increased photoreceptor phenotypes and opsin expression [1, 31, 37]. These studies offer promising results that demonstrate the ability to generate photoreceptor precursors from cells isolated from both early and late development. While further research is required to engineer pure photoreceptor progenitor populations from ES cells in vitro, cells isolated from the immature retina currently remain the cell type of choice for reproducibly achieving photoreceptor replacement in mammals.

17.2.3 Transplantation of Cells Isolated from the Postnatal Retina of the Mouse

In recent years, several laboratories have transplanted retinal progenitor or precursor cells (collectively referred to here as RPCs) isolated from a range of species and developmental ages. Much progress has been made with transplantations of homologous RPCs into healthy and diseased retina. The success of RPC transplantation is often assessed by the expression of photoreceptor-associated proteins such as crx, nrl, recoverin, and opsin, as well as by the degree of morphological and functional connectivity between transplanted RPCs and the host retina. Based on these criteria, it has been reported that the optimal retinal cells for transplantation in the mouse are isolated between postnatal days 1–4 [2, 21, 28]. Postnatal day one (P1) is the peak of rod photoreceptor histogenesis and a high percentage of cells isolated at this stage are already fated to differentiate into mature rod photoreceptors [1]. In 2004, we [21] demonstrated for the first time that the subretinal injection of in vitro-expanded P1 mouse RPCs could result in morpholigical integration of donor cells and partial rescue of light-mediated behavior in retinal degeneration mice. A number of years later, other researchers isolated primary postnatal day 4 (P4) retinal precursors from transgenic mice containing a fluorescent reporter gene for neural-leucine zipper (nrl) expression. Nrl is an early, photoreceptor fate determining transcription factor and is expressed shortly after exit from cell-cycle [28]. The authors FACS-sorted freshly isolated retinal cells for nrl-positive cells. Subretinal injection of this specific population also resulted in the integration of cells into the outer nuclear layer and partial preservation of visual responsiveness based on a number of measures. The ability of unexpanded P4 RPCs to integrate into the retina and differentiate toward photoreceptor-specific fate has been attributed to the fact that, while the soma of outer retina are in place at P0 during normal development, active process extension occurs during P4, followed by terminal synapse formation at P7 [45]. In addition, the transplantation of cells expressing photoreceptor precursor markers such as nrl increases the probability of differentiation into the cell type of interest. Unfortunately a significant drawback of this study is that the nrl-positive cells are postmitotic and therefore not expandable prior to transplantation. While these findings have been important to demonstrate proof of principle, the fundamental problem of identifying an in vitro developmental paradigm to differentiate culture-expanded RPCs remains to be solved. An additional obstacle to be addressed is identifying a reliable technique to increase the integration of delivered cells. In the study using postmitotic precursor cells, less than 0.5% of injection-delivered RPCs migrated into the host retina. To facilitate differentiation and integration, a growing number of bioengineering studies are demonstrating that in addition to soluble factors, a range of polymer substrates are capable of directing progenitor cell differentiation as determined by changes in cell morphology and gene expression, as well as improving cell delivery during transplantation [24, 30, 34, 51, 56].

17.3 State of the art

17.3.1 Polymer and RPC Composites for Retinal Transplantation

Current ocular tissue engineering strategies, which involve the combining of stem or progenitor cells with substrate materials, began to emerge in the early 1990s. Since that time, it is becoming increasingly clear that a multipronged approach to differentiation is essential. In designing a comprehensive differentiation paradigm, one also needs to consider the complex cell–cell contacts and mechanical cues derived from cell-substrate interactions that occur in vivo. To this end, a growing number of bioengineering labs have

demonstrated that a range of polymer substrates are capable of directing RPC morphology and gene expression (Fig. 17.2) [24, 30, 34, 51, 56].

It has been shown that cells alone are less capable of complete differentiation when compared to cell-polymer composites. When cells are grown under appropriate conditions on a three-dimensional scaffold, they can recapitulate complex tissue structure and function [9, 36]. Biodegradable polymers are useful for providing temporary scaffolds that dissolve as the tissue forms, thus avoiding long-term foreign body responses. A number of biodegradable polymers have been shown to be biocompatible in the eye including poly(caprolactone) and poly(lactic-co-glycolic acid) [53]. Poly(lactic acid) and poly(glycolic acid) have been approved by the FDA for a number of applications, and shown to be biocompatible in the CNS [5]. Furthermore, they may be functionalized by surface modification and have been widely used for sustained delivery of a number of growth factors [5, 6].

We were involved in one of the first retinal tissue engineering studies. In this instance, a poly (L-lactic acid) and poly(lactic-co-glycolic acid) (PLLA/PLGA) were fabricated using a solid-liquid phase separation technique that resulted in a scaffold with approximate 90% porosity. The resulting PLLA/PLGA biodegradable scaffold was seeded with postnatal day 1 mouse RPCs and transplanted into the subretinal space of allorecipients [56]. RPCs transplanted on PLLA/PLGA scaffolds showed a 14-fold greater survival rate in comparison to RPCs delivered via bolus injection. Additionally, RPCs cultured on PLLA/PLGA expressed markers of differentiation toward mature neural retinal cell fates.

One drawback of the above study was the thickness of the polymer substrate, making it difficult to position within the confines of the subretinal space. To improve on this, an ultra-thin (~6 μm) poly(methyl methacrylate) (PMMA) scaffold was designed [51]. The new PMMA scaffold was micro-machined to contain ~20 μm diameter through-pores for the retention of RPCs. Porous PMMA was shown to provide significantly greater RPC adhesion when compared to nonporous controls. PMMA-RPC composites transplanted into the subretinal space of mice caused no observable pathology to surrounding tissue, and within weeks allowed for the migration of high numbers of new cells into the host retina.

Building on these earlier studies, a new polymer scaffold has recently been analyzed for application in the retina. Based on the effectiveness of ultra-thin PMMA, further studies evaluated a template-synthesized biodegradable nanowire surfaced poly (caprolactone) (PCL) [52]. The nanowires were vertically oriented, 3–5 μm long, and 200 nm thick to mimic the native extracellular matrix environment. The total thickness of this PCL scaffold was 10 μm and RPCs responded to the topology by extending membrane protrusions, altering cell shape, and upregulating a number of photoreceptor genes [34]. Following transplantation, new cells migrated into the host retina and expressed photoreceptor proteins.

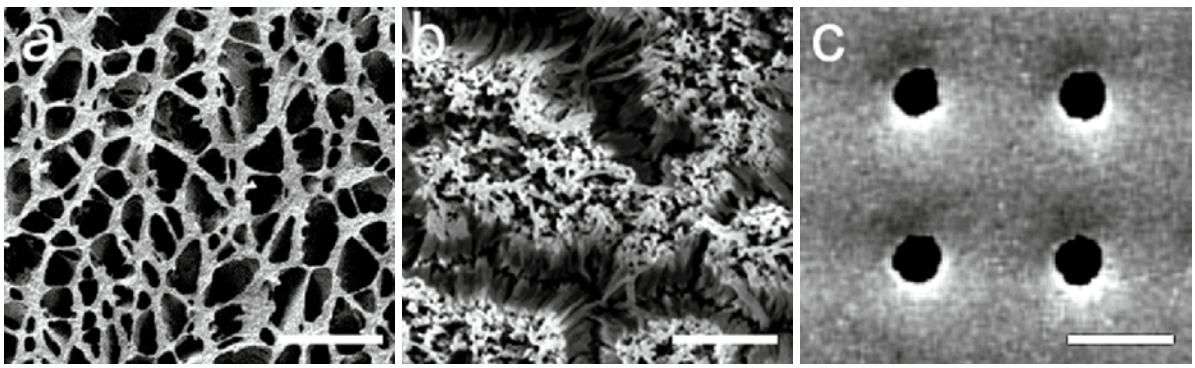

Fig. 17.2 Evolution of polymer scaffold topology for RPC differentiation and delivery. (**a**) The initial polymer scaffold evaluated for delivery of RPCs to the subretinal space was a highly porous (PLLA/PLGA) fabricated using solid-liquid phase separation scale, 50 mm. This polymer showed a 14-fold greater survival rate of transplanted RPCs in comparison to bolus injection. (**b**) To mimic extracellular matrix cues, poly(capro lactone) was template-synthsized to produce a surface of 2.5 mm long, 200 nm diameter wires scale, 5 mm. RPC in response to this nanowire topology upregulated expression of mature neuronal markers. (**c**) To facilitate transplantation via a syringe poly(glycerol sebacate) was microfabricated to be scrollable with 50 mm diameter pores to enhance cell-retention during transplantation scale, 100 mm

To further improve polymer transplantability, researchers most recently designed a scrollable and biodegradable scaffold for RPC delivery to the subretinal space via cannula injection [30]. This novel microfabricated polymer, poly(glycerol sebacate) (PGS) scaffold, was shown to possess several useful properties including biocompatibility, elasticity, porosity, and a microtopology conducive to mouse RPC differentiation. In vitro proliferation assays revealed that PGS held up to 86,610 (±9,993) RPCs per square millimeter, which were retained through simulated transplantations. RPCs adherent to PGS differentiated toward mature phenotypes as evidenced by changes in mRNA, protein levels, and enhanced sensitivity to glutamate. Transplanted composites demonstrated long-term RPC survival and migrated cells exhibited mature marker expression in host retina. As with the initial PLLA/PLGA-RPC composite, significantly higher numbers of RPC migrated into host retina when compared to delivery via bolus injection.

17.3.2 In Vitro Multilayer Film and Reaggregation Approaches

- In addition to the polymer scaffold approaches described above for RPC differentiation and delivery, a number of in vitro studies have begun to evaluate novel substrates and three-dimensional retinal reconstruction approaches. A recent study incorporated interphotoreceptor matrix (IPM) isolated from porcine eye and fibroblast growth factor (bFGF) into a multilayered polyelectrolyte film [54]. Films were composed of layers of endogenous extracellular matrix molecules such as poly (L-lysine)/ chondroitin sulfate, poly(L-lysine)/poly(styrenesulfonate) or poly(L-lysine)/hyaluronic acid, attempting to imitate the retinal extracellular matrix. When adult photoreceptor viability was analyzed on the various film composites, poly(L-lysine)/chondroitin sulfate with adsorbed bFGF and IPM was shown to be optimal for photoreceptor survival, maintaining viable cells for up to 7 days in culture. RPCs were not analyzed in this study.

In an attempt to reconstruct retinal layers from dispersed embryonic retina, a number of labs have used reaggregation approaches [25, 39]. In these studies, whole embryonic retina were dissociated and then reaggregated into spheres by either constant mechanical rotation or growth in a motion-free bioreactor with continuously circulating media. Mechanical rotation led to reaggregation of dispersed embryonic chick retinal cells into spheres which developed into highly organized histotypical spheres. These spheres eventually developed full retinal laminar architecture after several days of culturing in the presence of retinal pigment epithelium (RPE) or RPE conditioned medium [25]. Similar results were found using dissociated P3 gerbil retina, reaggreated within a microfluidic circulation culture system. As in vitro differentiation methods are improved, reaggregation strategies may be useful for engineering fully laminated retina derived from a renewable cell source.

17.4 Clinical Application

Because of the nascent stage of retinal neural tissue engineering there have not been any clinical applications in humans, to date. Looking forward, however, it seems likely that treatments involving a tissue engineering approach will find applicability in a broad range of conditions involving loss of retinal neurons. Relevant diseases affecting the retinal photoreceptor layer specifically include retinitis pigmentosa, age-related macular degeneration, retinal detachment, and some forms of diabetic retinopathy. In addition there is an increasing incidence of battlefield-related retinal injury, for instance from lasers. In cases where the primary disease or injury has been resolved and a patient has lost vision due to photoreceptor loss, the transplantation of engineered retinal tissue may ultimately represent a viable therapeutic option.

Currently, a number of studies are evaluating the use of polymers to deliver neuroprotective drugs to the retina. One such example is a biodegradable microsphere which slowly releases glial-derived growth factor (GDNF) in concert with the degradation of its constituent polymers. The strategy of using biodegradable polymers for drug delivery to the retina offers the advantage of sustained activity without requiring repeated intravitreal injections. These microspheres are being investigated as a potential neuroprotective treatment for damaged ganglion cell axons forming the optic nerve in the treatment of glaucoma (Fig. 17.3) [16, 61]. Another aspect of polymer-mediated glaucoma treatment involves the use of polymer tubes carrying cells and extracellular matrix

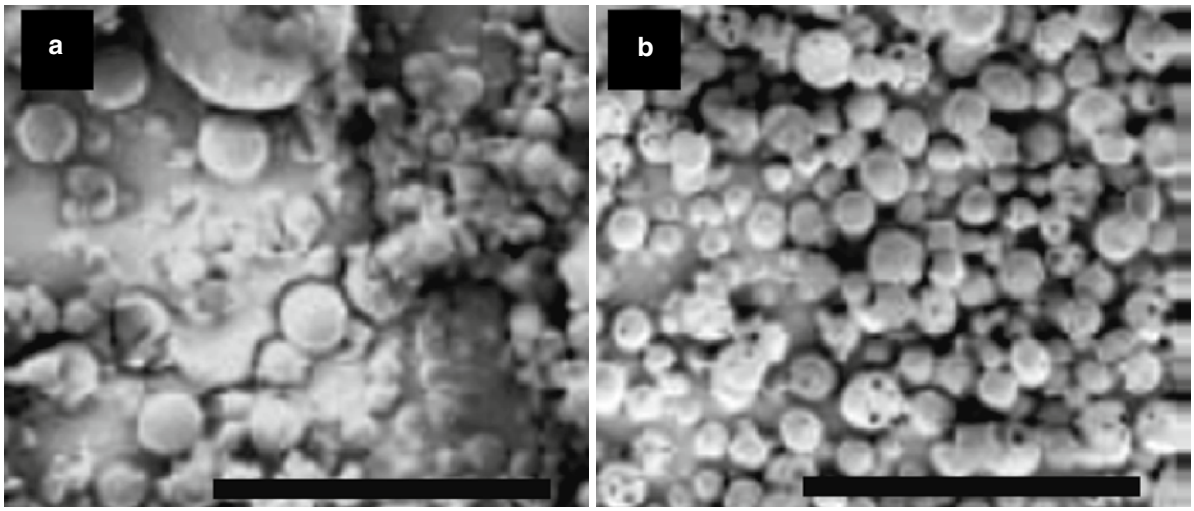

Fig. 17.3 *Biodegradable GDNF-loaded PLGA microspheres.* (**a**) Slow releasing GDNF-loaded PLGA microspheres are produced by spontaneous emulsion with an average diameter of 10 μm. scale:100 μm. (**b**) Micorspheres degrade through hydrolysis and loose mass while consequently releasing GDNF from intrasphere pockets, scale: 200 μm. Microspheres injected into the vitreous of a mouse model of spontaneous glaucoma released GDNF for 71 days and exerted a neuroprotective effect on ganglion cells

components to serve as synthetic guides for axons to bridge the severed nerve gap and to release neurotrophic factors to overcome inhibitory signals [13, 32]. These polymer-based studies show promise in suppressing the inhibitory environment of the CNS to allow for optic nerve regeneration.

Polymers that form cage-like structures have also been used to protect transplanted genetically engineered cells from host immune responses. A related technique involves the uses of nondegradable constructs to encapsulate cells, allowing for the release of ciliary neurotrophic (CNTF), a photoreceptor neuroprotectant [46]. In a phase 1 clinical trial, encapsulated CNTF-releasing cells were surgically implanted in the vitreous of retinitis pigmentosa patients and results indicated a two to three line benefit in visual acuity over control eyes. The results of ongoing encapsulation studies support the use of polymer caged cells for sustained delivery of neuroprotective proteins to diseased retina.

Polymers scaffolds have also been used as a cell delivery vehicle for transplantation of RPE cells. The autologous transplantation of RPE cells from peripheral retina to the macula has been shown to rescue photoreceptors in some situations [18]. The structure of the RPE is a simple monolayer of cells tightly joined by gap-junctional proteins and successful transplantation does not require synaptic connectivity, a critical factor in neural cell replacement strategies. RPE cells are in constant contact with photoreceptor OS and are essential for normal photoreceptor retinoic acid cycle, phagocytosis of OS disk membranes, and rod and cone survival. Transplantation of adult or stem cell-derived RPE cells into the subretinal space using biodegradable polymers could have practical and relatively near-term application in age-related macular degeneration, which is characterized by damaged RPE and Bruch's membrane [27].

17.5 Five-Year Perspective

Although the above mentioned polymer scaffolds have been shown to drive RPC differentiation to varying degrees, a wide variety of polymer-based biomimetic cues remains to be explored. For example, the endogenous matrix elasticity of CNS tissue is known to be between 100 and 1,000 Pa and stem cells cultured on substrates with elasticity in this range exhibit more neuronal morphologies [8, 11, 43]. Cells have been shown to engage with - and respond to - the extracellular matrix via surface integrins and mechano-sensory systems that involve nonmuscle myosins and cytoskeletal elements to modify cell morphology and gene expression [8, 26]. Interestingly, stem and progenitor cells cultured on stiffer, less elastic substrates appear

to have a tendency to differentiate into glial type cells. While polymers require a basic rigidity to maintain shape during surgical transplantation, a surface elasticity close to that of the endogenous developing nervous system may aid photoreceptor fate specification.

Additional physical cues derived from biology which can be incorporated into polymer substrates include both naturally-derived and synthetic extracellular matrix surface components. Natural matrix components, which have been evaluated in vitro to guide stem cell differentiation, include laminin, collagen, fibronectin, and matrigel (a combination of laminin, collagen, and heparan sulfate). An advantage of natural fibers is the intrinsic motifs that can bind cell receptors and thereby direct cellular behavior. Of these, fibronectin has been shown to direct stem cells toward a neural phenotype with longer axonal or dendritic processes [26]. Synthetic matrix fibers, which are reliably nonimmunogenic and can be produced with predictable mechanical strength, include self-assembling peptides, which can be synthesized using motifs from ECM proteins [26, 47]. Neural progenitors cultured on ECM motif-modeled synthetic fibers have been shown to migrate and differentiate into viable neuron and glia [10].

The encapsulation of both neuroprotective and neurotrophic factors into polymers for use in retinal tissue engineering will also likely be valuable. Potential candidates include factors present in both the developing and adult retina, such as GDNF, nerve growth factor, pigment epithelial derived growth factor, and CNTF, to name a few. These factors are normally released from either Muller or retinal pigment epithelial cells and developing retinal neurons express the appropriate receptors for binding and activation of downstream signaling pathways. Also, these molecules have been evaluated in stem and progenitor cell culture and shown to influence cell fate and increase cell survival [55]. Additional studies are required to identify optimal release parameters including timing and concentration.

Alternate cell sources for application in retinal tissue engineering may include adult ciliary margin zone cells, bone marrow or cord blood, and adult stem cells transformed from the patient's somatic cells. While ciliary margin cells have shown neural potential in retinal transplantation, a major challenge is that a very low percentage of these cells in the marginal zone possess true stemness and a large number of cells are required to develop a population of potential therapeutic significance [58]. Bone marrow and cord blood-derived stem cells have also been transplanted in the retina with reported expression of photoreceptor proteins, although directed neural differentiation has been challenging [22, 57]. A new and exciting source of stem cells which may be used for retinal repair are adult somatic cells that have been transformed into a pluripotent state via the introduction of the stem cell genes Oct3/4, Sox2, Klf4, and c-Myc [50]. These induced pluripotent stem cells (iPS) can be both patient and disease specific. For a disease like retinitis pigmentosa with a known gene mutation, the patient's iPS cells can be engineered with the wild-type gene, developed into photoreceptor fate via soluble factor and polymer-based cues, and transplanted for anatomic and functional restoration.

In addition to ongoing evaluations of new therapeutic cell populations, a number of labs are continuing the characterization of enzymes and extracellular matrix cues in retinal development and disease that can be manipulated to enhance the integration of transplanted de novo tissue.

17.6 Limitations/Critical View

17.6.1 Creating Optimal Retinal Environmental Conditions for Transplantation

It has been established by several laboratories that the receptivity of the host retinal environment is essential for the integration of significant numbers of cells. The retinal environment of younger animals is characterized by ongoing developmental plasticity allowing for a greater level of transplanted cell migration in comparison to mature animals [42]. The developing retina is permissive for new cell integration in part due to the presence of mitotic progenitors, growth factors, and an immature outer limiting membrane (OLM) [4, 59, 63]. In mouse, the OLM begins to form at P5 and matures near P21 and is formed by occluding junctions between Muller cell processes and photoreceptor inner segments [62]. In mature retina, the OLM isolates the neural retina from both the circulation of the choriocapillaris and the active phagocytosis of photoreceptor OS by retinal pigment epithelial cells (Fig. 17.4a). It has been shown that the disruption of the OLM using a Muller cell

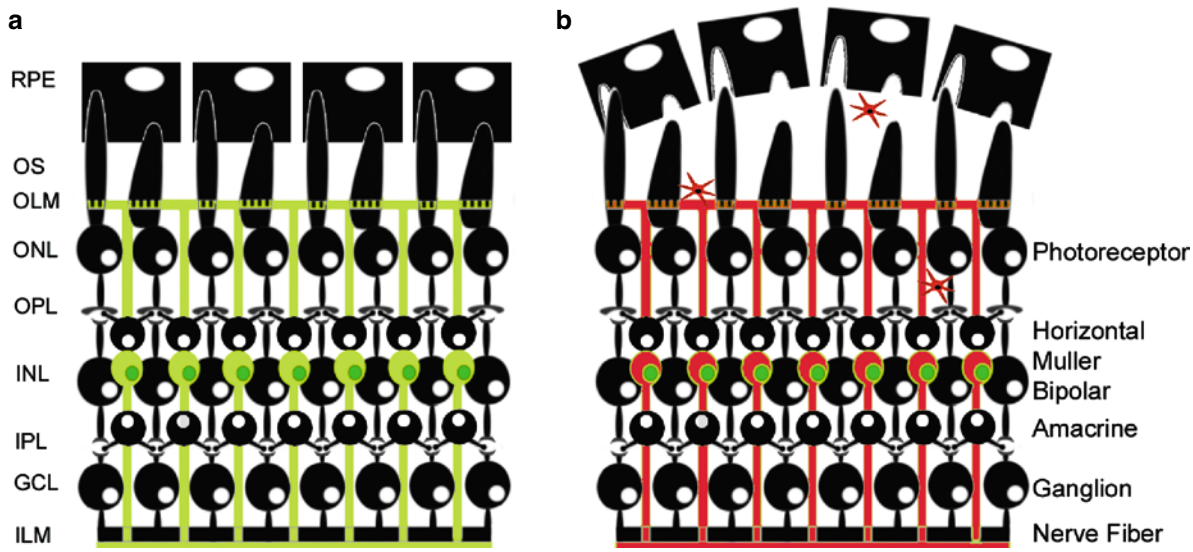

Fig. 17.4 *Healthy and reactive adult retina and outer limiting membrane.* (**a**) Under normal conditions the adult retinal lamina including outer nuclear (ONL), outer plexiform (OPL), inner nuclear (INL), inner plexiform (IPL), ganglion cell (GCL), and nerve fiber layers are protected by Muller cells. Muller cells are resident retinal radial glia, which form the protective and insulating outer and inner limiting membranes (OLM, ILM). (**b**) In disease states or following photoreceptor detachment from the retinal pigment epithelial (RPE) layer, Muller cells become hypertrophic and upregulate GFAP and vimentin expression, contributing to glial scarring. In addition, resident astroglia become activated and contribute to scarring, which inhibits new cell migration into host retina. Studies attempting to facilitate new cell migration into host retina are attempting to disrupt the OLM, degrade glial hypertrophy-associated ECM components, and suppress reactive astroglia

toxin, Dl-alpha-aminoadipic acid (AAA), significantly increases the migration of transplanted, freshly isolated P4 RPCs into host retina. This process is reversible and shortly following disruption via AAA, the integrity of the OLM is reestablished. While temporary disruption of the OLM remains a potential means of facilitating new RPC migration into host retina, the use of AAA is unlikely to be used as a therapeutic agent due to the potential to induce outer nuclear layer cell death, with suppression of the ERG response [38, 62]. The identification of a less toxic alternative to AAA may allow OLM disruption to be assessed clinically.

Muller cells also present an obstacle to new cell migration by contributing to a transplantation-induced inflammatory barrier. Following subretinal injection, both Muller cells and astrocytes become reactive and hypertrophic, upregulating expression of glial fibrillary acid protein (GFAP) and vimentin which are associated with glial hypertrophy and consequential inhibition of transplanted cell migration into host retina (Fig. 17.4b) [20]. It has been demonstrated that the retinal environment of mice deficient in GFAP and vimentin does not exhibit reactive gliosis and is permissive for transplanted RPC migration. A similar study has approached the problem of gliosis by targeting activated retinal microglia and chondritin sulfate proteoglycan (CSPG) accumulation at the site of subretinal cell injection in the Royal College of Surgeon (RCS) rat [48]. In healthy retina, microglia serve a macrophagic function and CSPGs are extracellular matrix molecules which guide microglia and are also known to inhibit axonal sprouting and regeneration [15]. In degenerating RCS retina, there are higher than normal baseline levels of microglia and CSPGs deposits, as well as more severe activation following transplantation. In this model, the combined inhibition of microglia activation using immunosuppressants and the degradation of CSPGs using chondroitinase allowed for the robust migration of stem cells across host retinal layers [15]. Additional studies evaluating degradation of gliotic barriers have identified a role for matrix-metalloproteinase (MMP) in creating a permissive environment for RPC transplantation [49, 64]. MMP2 is an endogenous proteolytic enzyme that degrades extracellular matrix proteins including CSPGs [65]. In the presence of both active-MMP2 and its stimulators, concavalin A and 17β-estradiol, enhanced RPC migration into host retina has been demonstrated [49]. An interesting subsequent

study identified that matrix degradation in degenerative retina correlates to the activation of MMP2 expression by Muller cells, which are stimulated by transplanted RPCs [65]. This study revealed a mechanism intrinsic to RPCs that modifies the host retinal extracellular matrix and may involve their known secretion of both neurotrophic and neuroprotective factors. Further evaluation of modifiable factors in host retinal environment will be invaluable for improving the delivery of engineered cells and tissue engineered constructs in transplantation paradigms.

17.7 Conclusion/Summary

A significant amount of research remains to be conducted in stem cell biology, polymer biochemistry, and their interactions during the formation of de novo retinal tissue. Whether the cell source is a population of genetically competent RPCs, ES cells, or patient specific iPS cells, greater insight into cellular development and epigenetic response properties is essential. It is also imperative that the selected cell population be expandable and capable of differentiation into functional photoreceptors. Of equal importance is the need for identification of optimal mechanical and biochemical polymer properties for use in cell fate direction and transplantation into the subretinal space. To restore healthy retinal physiology, polymers should be nonimmunogenic, biodegradable, porous, and ultra-thin. Additional factors to consider in improving the integration of transplanted retinal tissue include the recognition and modulation of host glial reactivity, treatment of the retina with enzymes that selectively degrade ECM components, transient disruptions of the OLM, and reduction of inflammatory responses via targeted delivery of specific agents. Development of patient- and disease-specific retinal tissues may further contribute to cell replacement strategies in the setting of retinal disease.

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Part III

Tissue Types

Engineering of Adipose Tissue

18

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18.1 Introduction

18.1.1 White Adipose Tissue

White adipose tissue (WAT) has traditionally been considered to be an organ of energy storage, thermal insulation, and mechanical support [108]. The discovery of leptin just over a decade ago has fundamentally altered our understanding of adipose tissue [165]. WAT is now recognized as a major endocrine and secretory organ and considered to be integrated into the overall physiological regulation of mammals [140]. Adipose-secreted products, adipokines, are involved in lipid metabolism, haemostasis, blood pressure regulation, appetite and energy balance, reproduction, glucose homeostasis, angiogenesis, immunity and inflammation [141].

WAT is composed of a largely uniform population of adipocytes [22]. Each mature adipocyte is enclosed by a basement membrane abundant in type IV collagen and laminin [78]. In addition to adipocytes, fibroblasts, macrophages, mast cells and fibroblast-like mesenchymal progenitor cells are present [167]. Together these non-adipocyte elements are frequently termed the stromal vascular fraction (SVF) of adipose tissue. WAT is also supplied by a rich capillary network [10].

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The earliest adipose precursor cells (adipoblasts) have been detected in morphological studies of foetal adipose tissue. They are small epithelioid cells with little perinuclear cytoplasm [53]. We observed that human adipose tissue-derived mesenchymal stem cells (ASC) and bone marrow-derived mesenchymal stem cells (BMSC) when implanted into a mouse chamber home to the microvascular niche and colocalize with the periendothelial pericytes suggesting that the pericyte is the adipose stem cell. Tang et al. have recently confirmed that pericytes of the microvasculature of adipose tissue give rise to white adipocytes [132]. Pericytes are smooth muscle-like cells that cover the endothelial cells of blood vessels. They express both preadipocyte markers such as PPAR-γ as well as pericyte markers [132].

Mouse embryonic preadipocyte cells lines, such as 3T3-L1, undergo spontaneous adipose differentiation on confluence in vitro [48]. They underpin much of our understanding of the molecular biology of adipocyte differentiation [11, 49, 122, 133]. Primary mesenchymal stem cells with adipogenic potential have been isolated from bone marrow [106], adipose tissue [167], muscle [3, 14] and foetal and adult blood [98, 168].

Adipogenesis occurs as a cascade of genetic events. Proteomic studies have established that changes in the expression of at least 300 proteins are involved in the structural and functional morphogenesis associated with adipogenesis [154]. Many of these changes occur at the transcriptional level with the altered expression of over 2,000 genes [51]. The key drivers of differentiation are members of two families of transcription factors, the CAAT/enhancer binding proteins (C/EPBs) and peroxisome proliferators-activated receptors (PPARs) [112]. The onset of adipogenesis is accompanied by a rapid expression of C/EPBβ and

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C/EPBδ that precede the induction of the master transcription factors C/EPBα and PPARγ [82, 138].

Whether adipocyte differentiation is initiated or not depends on a balance of opposing stimulatory and inhibitory factors acting on the preadipocyte [88]. Stimulators of adipogenesis include glucocorticoids [12, 155], cyclic-adenosine monophosphate (c-AMP) signalling activators (such as MIX) [115], fatty acids [44] and prostaglandins [4, 44], insulin-like growth factor-1 [48] and macrophage-colony stimulating factor [80, 143]. Inhibitors of adipogenesis include Wntb glycoprotein [113], transforming growth factor-β [85], tumour necrosis factor-α [101, 148] and inflammatory cytokines (including interleukin 1β (IL-1β), IL-6, IL-11, leukaemic inhibitory factor and interferon γ) [50, 102, 103].

In vitro an adipogenic cocktail typically includes a glucocorticoid agonist, an insulin-like growth factor receptor I agonist, an agent to raise intracellular Camp and often a PPAR-γ agonist. These overwhelm constitutively-expressed suppressor inhibition and drive preadipogenic differentiation. In vivo the regulation of adipogenesis is far more subtle and less well understood. It involves a complex interplay between autocrine, paracrine (from adipocytes, preadipocytes and other stromal vascular cells) and endocrine signals and the local extracellular environment [88].

18.1.2 The Clinical Need for Adipose Tissue Engineering Applications

Reconstruction of soft-tissue defects presents a major challenge in medical practice, particularly in the field of plastic and reconstructive surgery. Such defects can be broadly divided into congenital and acquired. Congenital defects include hemifacial microsomia, Romberg, Poland's syndrome and lipodystrophies. Acquired defects include those resulting from complex trauma and oncologic resection such as head and neck tumours and mastectomies. These deficits usually require large volume replacement. Cosmetic augmentation of cheek and chin and facial rejuvenation procedures for wrinkling require small volume replacements.

Current treatment strategies for large volume replacement include the use of synthetic materials and/or autologous tissue transfer. The latter usually requires complex procedures with vascularized tissue in the form of local or free flaps. The state of the art for small volume defects is free fat grafting devised by Coleman [23]. Biologically compatible fillers are also used to restore small volume deformities.

The outcomes of the above procedures are often less than satisfactory [66, 107]. Free tissue transfer for example for breast reconstruction following mastectomy is a common procedure. Donor site morbidity can lead to prolonged hospital stays and may delay planned adjuvant chemotherapy and radiotherapy. Free flap loss results in the creation of another donor site wound. Synthetic materials such as silicone breast implants stimulate local fibrosis in the surrounding capsule that contracts in time often needing revision surgery [124].

Free fat grafting remains unpredictable [121]. Free fat grafts resorb, or occasionally hypertrophy, in an idiosyncratic manner and patients may undergo multiple procedures in an attempt to successfully correct a single defect [137]. However, new techniques have been developed recently suggesting better long-term survival of the transplant. Regenerative cell-based strategies, such as those applying adipose derived stem cells, hold some promise for augmentation of small soft-tissue defects [96, 153]. Rigotti et al. applied repeated low-invasive computer-assisted injection of purified autologous lipoaspirates to resolve the late side effects of radiotherapy. The ultrastructure of target tissue was suggested to exhibit progressive regeneration, resulting favourable clinical outcomes with systematic improvement or remission of symptoms [110].

Given the above constraints, the logical solution for reconstruction of soft-tissue defects would be the generation of adipose tissue constructs by tissue engineering. The clinical impetus of adipose tissue engineering strategies is enormous. Currently, the discipline of adipose tissue engineering is still some way from meeting these aims, although progress has been steady over the last 15 years. This was enabled to a great extent by the increasing understanding of adipose tissue biology in health and disease states, the latter mostly related to obesity and metabolic disorders.

Body fat content and fat distribution differ between individuals and genders. Females have a higher percentage of body fat than men. In females fat is particularly found subcutaneously in the thigh and gluteal regions while in males it usually accumulates in the upper body and abdomen, both subcutaneously and intra-abdominally. This body distribution of fat is

particularly related to sex hormonal levels. Gender-related patterns of fat deposition become established during puberty, showing also significant familial associations [119].

The body has a natural ability to gain and loose fat. In polycystic ovary syndrome almost half of females are obese and many have a central distribution of body fat [36]. Pathological states caused by increased cortisol concentration are associated with central obesity and a localized hump on the back of the neck [16, 63]. Cortisone, in addition to affecting the body fat distribution, is known to cause lipoatrophy after steroid injection into soft tissue [99]. On the other hand, insulin injections cause lipohypertrophy [29]. Lymphoedema, characterized by enlargement of the legs and/or arms, is not only associated with lymph fluid retention but also with a progressive abnormal deposition of adipose tissue, termed lipedema [77], suggesting that lymph fluid may contain factors that induce adipogenesis [8].

Lipomas are benign soft-tissue tumours composed of normal adipose tissue. They can develop in virtually any part of the body; however, so far there is no explained pathogenesis and aetiology [7]. Post-traumatic pseudolipomas are fatty tissue masses that develop in areas that have been subjected to acute severe trauma [6]. Though their mechanism of development has been traditionally mechanically and anatomically based [114], it is possible they develop as a result of inflammatory triggers mimicking the development of an obese state in localized form [45].

18.2 Aims of the Discipline

The goal of adipose tissue engineering is the generation of autologous, vascularized, mature adipose tissue. It must be of sufficient volume to restore a given defect. It is also important that it has long term stability.

18.3 State of the Art

A tissue engineered construct is made up of three essential components: cells, extracellular matrix or scaffold and vascularization. These do not act in isolation but are interdependent and modulated by the in vivo environment [15]. Vascularization in particular is important for the achievement of clinically relevant construct volumes. Growth factors and cytokines may augment the growth potential of these components. In this section we review these crucial components of adipose tissue engineering, together with the various types of growth factors and cytokines that have been studied to date. We also introduce the concept of space and how it can be important in adipose tissue engineering and include a short review of the literature that lies behind the current inductive theory of adipose tissue development in vivo.

18.3.1 Cells

Cells utilized for adipose tissue engineering can be classified as autogenous or heterogeneous. They can also be divided into exogenous and endogenous. Exogenous cells are explanted, seeded and expanded on a scaffold ex vivo in bioreactors and then reimplanted [13, 46, 81]. Endogenous cells, given the appropriate cues, are recruited to the area of need and contribute to adipose tissue construct development in vivo. Cells can be divided into pluripotent mesenchymal stem cells (MSC), preadipocytes (3TS-L1s) or differentiated adipocytes. MSC include BMSC and ASC.

18.3.1.1 Mesenchymal Stem Cells

BMSC have several drawbacks for clinical use including invasiveness of harvest and morbidity of the procedure [166]. Though initial processing techniques were hampered with low yields [166], methods have emerged that permit automated expansion of human BMSC without loss of their pluripotent potential [135]. Further, the biological function of human BMSC does not appear to be affected by ageing [127] and under appropriate conditions in vitro over 80% of human BMSC differentiate into adipocytes [64]. Human BMSC have been induced to differentiate into adipocytes in a three-dimensional scaffold in vitro [47]. Recently, BMSCs were used successfully to generate adipose tissue when seeded on poly(ethylene glycol) based hydrogel and implanted in vivo [126].

Adipose tissue is an abundant source of ASC. Procedures for isolation of these cells from excised

fat or lipoaspirate are not difficult [5] and they can be easily differentiated into adipocytes when cultured in adipogenic media [166]. Adipose tissue constructs have been engineered using these cells [21, 83]. Human ASC have been expanded in the presence of basic fibroblast growth factor (bFGF) prior to seeding on scaffolds and implantation in vivo [142] and spinner flasks used to expand ASCs on macroporous poly(lactic-co-glycolic acid) (PLGA) microspheres. Expanding ASCs this way yielded significantly more adipose tissue than when ASC were cultured on plates, trypsinized and then loaded on microspheres [67].

18.3.1.2 Preadipocytes and Adipocytes

Preadipocytes are committed adipose precursor cells in different stages of differentiation that eventually give rise to adipocytes [72]. They have been seeded on freeze-dried collagen and PLGA scaffolds and implanted in vivo with some success [40, 74, 105, 150]. When cultured in perfused rotating wall vessel bioreactors with microcarriers in a high aspect ratio, preadipocytes increased the size of adipose tissue aggregates [42]. When loaded onto amidated hyaluronan (HYADD3), preadipocytes generated adipose tissue 6 weeks following implantation [56]. Adipocyte formation is improved when preadipocyte seeded scaffolds are treated with adipogenic media ex vivo and differentiated prior to implantation [40, 52].

However, as opposed to preadipocytes, implantation of mature adipocyte containing scaffolds is likely to lead to increased cell loss at the construct's core. This is due to the fact that these cells have significantly higher metabolic demands than preadipocytes [149]. Also, mature adipocytes have lost their ability to proliferate [2]. Another advantage of using preadipocytes in preference to mature cells is that the former have decreased susceptibility to mechanical irritation [68].

In terms of clinical application the above techniques will probably be limited by the time required to expand the cell population to a desired size as well as a two stage procedure to harvest the cells and reimplant the cell seeded scaffold. The alternative strategy of in vivo adipose tissue engineering uses the body as a "bioreactor" that recruits endogenous cells to the area where adipose tissue needs to be formed. Kawaguchi et al. studied early events in their model and identified fibroblast-like cells that invaded the construct's matrix, acquired lipid-filled cytoplasmic vacuoles and at 5 weeks formed mature adipocytes [69, 139]. These cells can possibly represent the same population of adipose precursor cells isolated from WAT [139]. Neoangiogenesis precedes adipogenesis [139] and microvascular pericytes -smooth muscle-like cells that cover the endothelial cells of blood vessels- have adipogenic potential [38]. It has been recently shown that pericytes of the microvasculature of adipose tissue give rise to white adipocytes [132].

18.3.1.3 Inflammatory Cells

Implantation of scaffold materials in vivo inevitably results in acute and chronic host inflammatory responses. Recruitment of inflammatory cells such as neutrophils and macrophages to the area of interest takes place [55, 136]. The mechanisms by which scaffold materials influence the host tissue responses, and how the latter influence the tissue engineered product is still poorly understood. Studies on the participation of macrophages and their subtypes in scaffold degradation and remodelling are emerging [9, 146]. However, in the field of adipose tissue engineering, this area still has to be explored and exploited.

18.3.2 Extracellular Matrix

An adequate scaffold for adipose tissue engineering should:

- Allow cellular penetration into the construct,
- Allow sufficient preadipocyte survival, proliferation and differentiation,
- Encourage neo-angiogenesis to minimize cellular ischaemia,
- Ideally maintain its stability and 3D architecture until it is replaced by mature adipose tissue,
- Degrade after replacement by tissue without eliciting a harmful inflammatory response [57, 134].

To date no scaffold material has all the above conductive or inductive criteria to adipose tissue engineering. They can be classified as synthetic, biological and biohybrids (combination of synthetic and biological

materials); or biopolymers (solid state), hydrogels and carrier microspheres.

18.3.2.1 Biopolymers

Solid state scaffolds that have been utilized to date include collagen-1, PLGA, polyglycolic acid (PGA), hyaluran and polytetrafluoroethylene (PTFE). These materials serve as 3D carriers for ex vivo seeding and expansion of preadipocytes prior to in vivo implantation. Scaffolds without seeded cells do not have substantial adipogenic activity [40, 105, 150, 151].

Collagen scaffolds seeded with human preadipocytes and subsequently implanted in mice showed initial successes [150, 151]. Bovine collagen scaffolds contain irregular pore sizes with many pores "closed" to the ingrowth of cells. Development of collagen scaffold with more regular pore sizes by directional solidification and subsequent freeze-drying led to more reproducible results. Preadipocytes filled collagen pore spaces and had the ability to differentiate into mature adipocytes. However, the main problem was insufficient penetration of the scaffold (96). Type 1 collagen in an in vivo mouse model of tissue engineering showed minimal connective tissue and angiogenic ingrowth [26].

Fibronectin-coated PTFE resulted in attachment of preadipocytes and full differentiation of preadipocytes to mature fat cells [76]. However, although PTFE is a nondegradable scaffold and therefore likely to resist structural collapse better than biodegradable scaffolds, it will remain as a persistent foreign body when implanted in vivo. Also, foreign body-type reactions to the construct can lead to construct failure [156].

Preadipocytes seeded on PLGA discs implanted in vivo resulted in mature adipocytes and neovascularization in the short term (2 and 5 weeks and peaked at 2 months). However, long term studies showed complete resorption of the newly formed adipose tissue (5–12 months) [105]. PGA, in contrast to PLGA, did not allow in vivo differentiation of preadipocytes. However, prolonged culture of 3T3-L1 cells in PGA scaffolds resulted in uniform occupation of the scaffold by mature adipocytes at 3 weeks. Blank scaffolds and undifferentiated-preadipocyte loaded scaffolds did not produce adipose tissue [40].

Hyaluronic acid is present in the extracellular matrix of many tissues and has a supportive role on progenitor cell development [120]. Esterified hyaluronan-derived sponges to slow down the degradation process yielded mature adipose tissue formation at 3 and 6 weeks post-implantation [151]. Preadipocyte-loaded hyaluronan sponges with a pore size of 400 μm showed good vascularization, cell spreading, vascularization and construct stability, though no mature adipocytes were identified [58].

18.3.2.2 Hydrogels

Hydrogels, as opposed to biopolymers, have minimal structural rigidity and deform easily. They have been found to be useful in adipose tissue engineering both in vitro and in vivo [15, 69, 128, 139, 152]. Matrigel™ is an effective scaffold for adipose tissue engineering. It is a basement membrane extract of the murine Engelbreth-Holm-Swarm (EHS) sarcoma tumour. Therefore it can only be utilized in animal experiments. It is constituted mainly of laminin-1, collagen-IV, entactin and perlecan (product data sheet, BD Biosciences, NJ, USA). When supplemented with heparin sulphate and FGF-2 it has significant adipogenic potential. This property of Matrigel lies mainly in its ability to bind and present FGF-2 which has adipogenic potential in vivo [69, 74, 128, 157]. Laminin-1, also derived from EHS sarcoma in the form of a viscous fluid after 4 weeks post-implantation in vivo, yielded a construct about two thirds the size of that obtained from Matrigel™ and 35% of the tissue was vascularized adipose tissue [26].

Fibrin is another hydrogel that has adipogenic potential in vivo. Dome-shaped PLGA structures provided mechanical support to fibrin matrix with preadipocytes. This resulted in a volume-stable adipose tissue over 6 weeks. FGF-2-enriched fibrin glue yielded the most adipose tissue [18]. ASCs extracted from human liposuction aspirate when suspended in fibrin glue and implanted in athymic mice resulted in mature adipose tissue after 12 weeks [164]. Fibrin glue for adipose tissue engineering can possibly be extracted from the patients' own blood, thus avoiding foreign body reactions [159].

Myogel™, an extracellular matrix hydrogel extracted from skeletal muscle, has been developed in our laboratories. It has been formed from mice, rats, pigs and humans. It has significant levels of laminin α4- and α2-subunits and collagen 1 as well as growth factors such as FGF-2, TNF-α, PDGF, TGF-β and

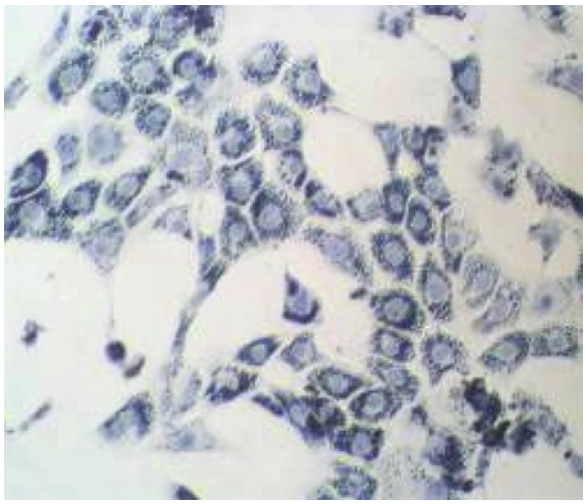

Fig. 18.1 Differentiating 3T3L-1 cells on Myogel. Lipid accumulating in cells stained with Sudan black

NGF. It has adipogenic potential both in vitro (Fig. 18.1) and in vivo (Fig. 18.2) [1]. Adipose derived hydrogels have also been extracted from subcutaneous fat of rats [145]. They also have adipogenic potential both in vitro and in vivo [145].

18.3.2.3 Microspheres

Microspheres have been utilized in experimental adipose tissue engineering for over 15 years [33]. They have been used in various forms including dextran [33, 34], gelatin [59, 73, 74, 90, 128, 147], PLGA [19–21, 67, 162] and poly(ethylene) glycol [161, 163] (Fig. 18.3). They can have several roles in this field.

They can act as a means of drug delivery. Growth factor-loaded microspheres have been used as a means of slow delivery and therefore the stability of growth factors needed for adipose tissue development in vivo. Fat grafts mixed with dextran beads pre-treated with FGF-2 maintained better overall graft form and had nearly complete weight maintenance when compared to fat grafts with FGF-2 alone in a rat facial model [34]. Human fat grafts mixed with platelet-derived growth factor(PDGF)-loaded gelatin microspheres were implanted in SCID mice. This resulted in weight maintenance and adipose tissue architecture when compared to fat grafts control, fat grafts with free PDGF and fat graft with blank microspheres [24]. Sustained delivery of FGF-2, insulin and insulin-like growth factor 1 bound to gelatin microspheres resulted in significant adipose tissue at 4 weeks in vivo [90]. Loaded FGF-2 microspheres in a Matrigel™ scaffold yielded significantly more tissue than the same dose of free FGF-2 [128].

Being very small particles and therefore having a great surface area, microspheres can be used as carriers for cells. FGF-2-loaded gelatin microspheres in combination with human preadipocytes and collagen as scaffold yielded more adipose tissue than when FGF-2 was not encapsulated in microspheres at 6 weeks [74]. Human ASC attached to and cultured on injectable microspheres fully differentiated into adipocytes after 8 weeks from implantation in nude mice [19].

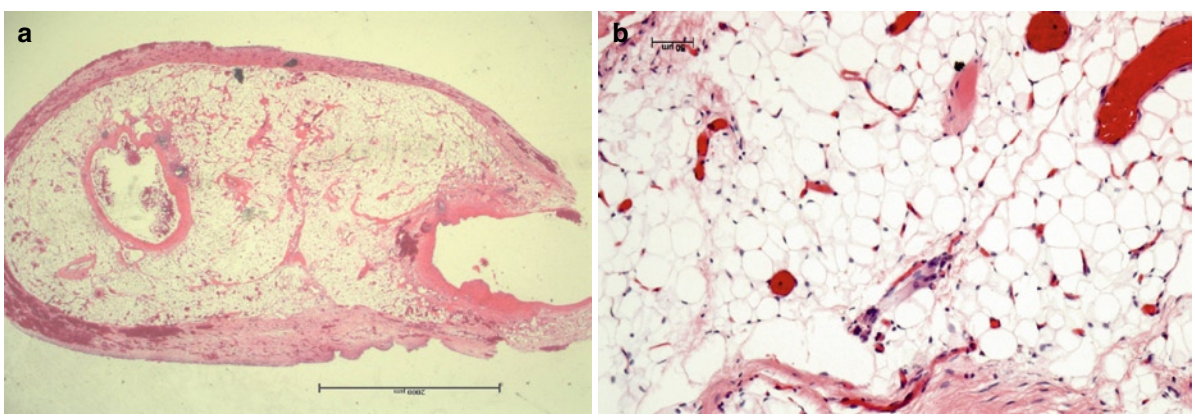

Fig. 18.2 Adipose tissue developing in a rat arteriovenous loop model at 10 weeks (stained with H&E)(**a**). Higher magnification showing normal white adipose tissue with new blood vessels (**b**)

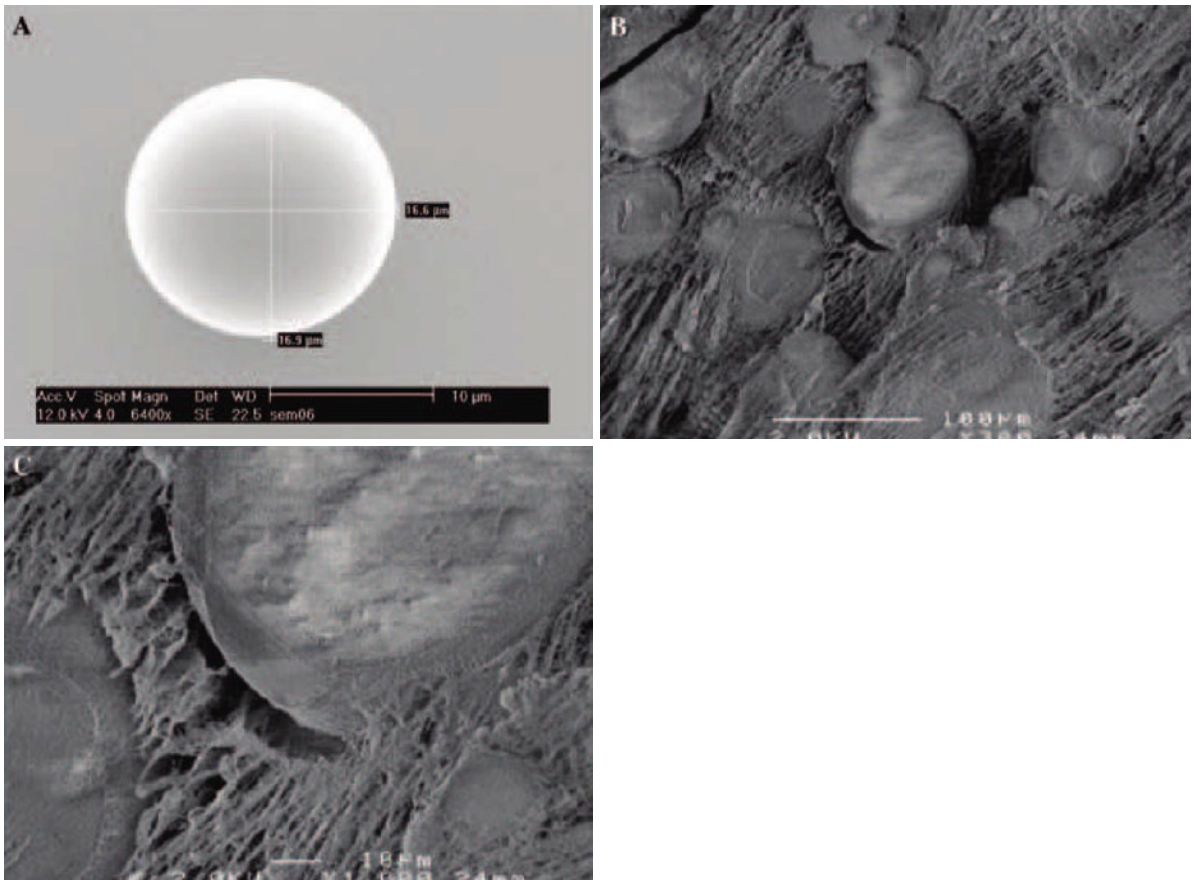

Fig. 18.3 Scanning electron micrographs of the gelatin microspheres. (**a**) Representative glutaraldehyde crosslinked gelatin microsphere showing smooth surface and spherical nature. Also depicted are cryo-scanning electron micrograph images of gelatin microspheres in collagen at lower magnification (**b**) and higher magnification (**c**), which illustrate the disperse embedding of the gelatin microspheres into the collagen scaffold (from Vashi et al. Tissue Engineering 12 (11) 3035–42)

18.3.3 Vascularization

The size of an adipose tissue construct will be determined by the extent to which it can be vascularized. Mature adipose tissue is highly vascular. Diffusion will only support normal cell function over a distance of 200 µm in tissue [41]. Adipose tissue constructs of substantial volumes must therefore be vascularized. There are two approaches to vascularization of 3D constructs, extrinsic and intrinsic vascularization. The former relies on the sprouting of microvessels from the surrounding recipient vascular bed into the construct tissue. This type of vascularization is commonly employed in the classical paradigm of tissue engineering.

This approach has been used to vascularize adipose tissue engineered constructs [40, 150, 151] and although it has had some success, there is delay in recipient blood vessel growth into the scaffold, resulting in limited blood perfusion and oxygen supply to implanted tissues. This makes it suitable for tissue thickness of approximately 1–3 mm only [89, 105].

In our laboratories, explorations with intrinsic vascularization of 3D tissue engineered constructs derived from experiences with prefabrication of tissue flaps and grafts [94]. Prefabrication is a technique of vascularizing tissues or grafts by implanting an arteriovenous loop or a vascular pedicle underneath or within the tissue. This results in spontaneous angiogenic sprouting from a vascular arteriovenous loop or pedicle and subsequent invasion of new blood vessel growth into the tissue graft. This establishes dependence of that tissue on the implanted blood supply and

allows it to be transferred as a separate living entity [35, 94]. This phenomenon forms the basis of the tissue engineering chambers that were designed in the rat, mouse and pig which have enabled us to produce volumes of vascularized 3D tissues especially fat which is several times larger than any current alternative techniques [86, 87, 93, 95, 130, 131].

The rat tissue engineering chamber is formed by creating an arteriovenous shunt between the right femoral artery and vein using an interposition vein graft from the left femoral vein. The shunt is then placed onto the base of a polycarbonate cylindrical chamber with an internal diameter of 1.4 cm, the lid closed and the construct sutured to the groin musculature with the aid of small holes on the base of the chamber (Fig. 18.4) [131]. This model is able to develop spontaneous de novo vascularised fibrous connective tissue which maintains its volume for at least 16 weeks (Fig. 18.5) [87]. Larger volumes of tissue can be created by increasing the chamber volume [60] and particularly by incorporating multiple perforations in the chamber wall [32, 129]. A perfused capillary network that remodels to generate arterioles, postcapillary venules and venules develops throughout the new tissue (Fig. 18.6) [87].

The mouse tissue engineering model utilizes the epigastric pedicle. The epigastric artery and vein in the groin are stripped of their surrounding fat but the vessels remain in continuity as a flow-through system and a 5 mm long piece of split silicone tubing is placed around the pedicle. The proximal end and the longitudinal split of the silicone tubing are sealed with bone wax. The chamber is then filled with matrix, the distal end wax sealed and the wound closed. Spontaneous angiogenesis occurs from this pedicle to vascularize the matrix (Fig. 18.7) [25]. Although the mouse model broadens experimental applications, the vascularization is not as robust as the rat model.

When a 5 mL volume of fat flap vascularized on an epigastric vascular pedicle was inserted into a perforated 80 mL chamber in a pig, the chamber completely filled with tissue by 6 weeks. The fat component had expanded to occupy approximately 30% of the volume of the new tissue. Its volume remained stable after it was removed from its chamber and left in situ for a further 12 weeks (Fig. 18.8).

There is a close relationship between angiogenesis and adipogenesis [54]. In Kawaguchi's Matrigel plug model of adipose tissue engineering, the immigration of preadipocyte-like cells was preceded by invading

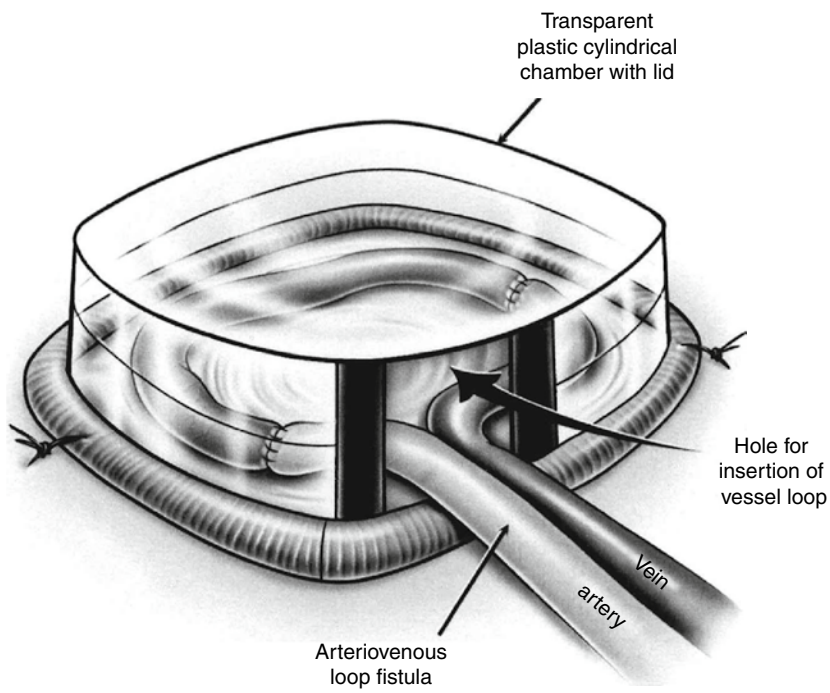

Fig. 18.4 The rat arteriovenous loop model with shunting between the right femoral artery and vein using an interposition vein graft

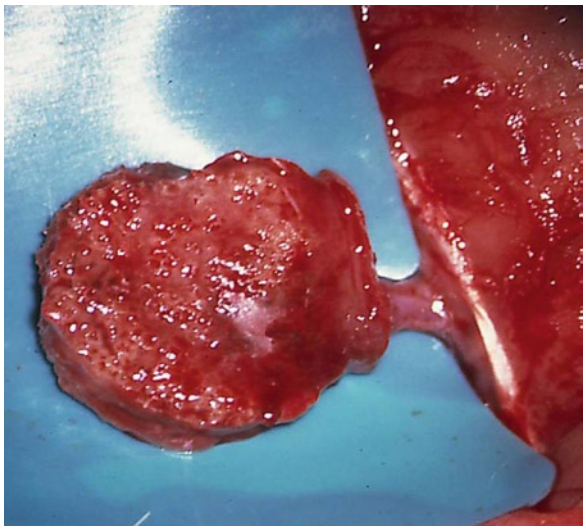

Fig. 18.5 Tissue construct at 4 weeks being harvested from the rat chamber

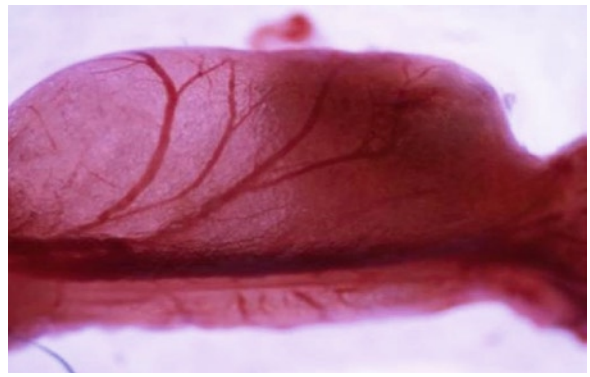

Fig. 18.7 Construct at 6 weeks harvested from the mouse tissue engineering chamber, showing new blood vessels developing from the epigastric pedicle

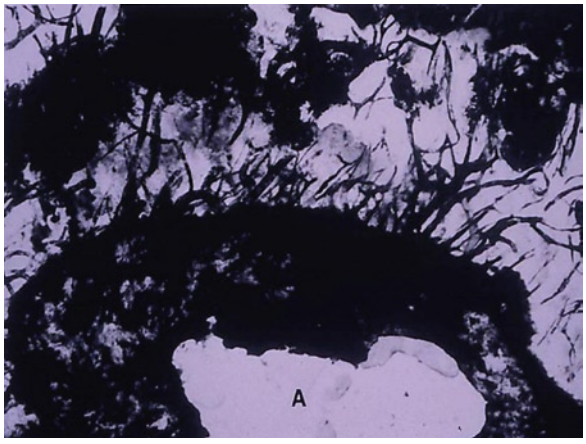

Fig. 18.6 India ink injection showing new blood vessels developing from the arteriovenous loop in the rat tissue engineering model

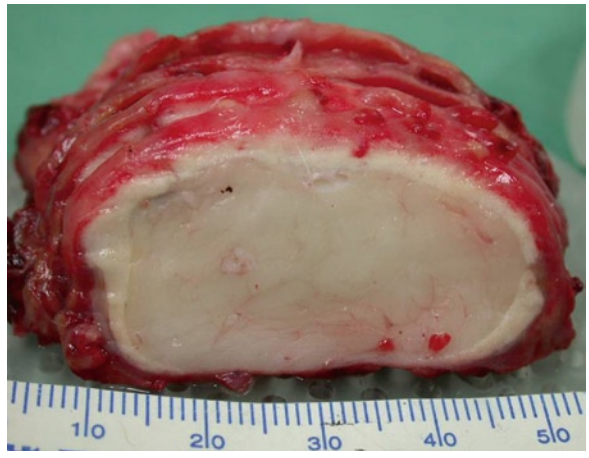

Fig. 18.8 Adipose tissue construct harvested from the pig chamber at 12 weeks (Findlay, Dolderer and Morrison et al.)

endothelial cells. Adipocyte formation was observed to occur in close proximity to new blood vessels [69, 139] suggesting a paracrine interaction between preadipocytes and microvascular endothelial cells (MVEC) [54]. Preadipocytes express vascular endothelial growth factor (VEGF) that induces angiogenesis via VEGF receptor-2 [43] while MVEC conditioned media induce preadipocytes to proliferate [61]. Vascular supply to fat can be rate limiting in the accumulation of adipose tissue [116]. As stated earlier, Tang et al. have shown that precursor cells that give rise to white adipocytes reside within the walls of the blood vessels that supply adipose tissue [132]. These cells are pericytes, smooth muscle-like cells that cover the endothelial cells of blood vessels. They express both preadipocyte markers such as PPAR-γ as well as pericyte markers [132].

In summary, given the close association between angiogenesis and adipogenesis, the intrinsic vascularization approach seems the logical pathway to develop large volumes of adipose tissue engineered constructs.

18.3.4 Growth Factors and Cytokines

Growth factors and cytokines are important in adipose tissue engineering. For instance, although Matrigel™ contains a trace of bFGF, on its own it is not adipogenic. However, in Kawaguchi's model, supplementation of Matrigel with 1 ng/mL and 1 μg/mL lead to threefold and fivefold increase in new adipose tissue weight at 5 weeks respectively [69]. Slow release of FGF-2 in gelatin microspheres in collagen matrix resulted in adipose tissue formation [147]. bFGF is an important angiogenic cytokine by promoting endothelial proliferation and capillary formation as well as stimulating proliferation and migration of mesenchymal cells [17, 117]. Angiogenesis is a prerequisite for de novo adipogenesis. When controlled release FGF-2 to induce angiogenesis, and insulin and insulin-like growth factor 1 to induce proliferation and differentiation of preadipocytes were bound to gelatin microspheres, significant adipose tissue developed at 4 weeks in vivo [90].

Vascular endothelial growth factor (VEGF) is a potent stimulator of endothelial proliferation and migration [39] while platelet-derived growth factor (PDGF)-PP is involved in pericyte recruitment around capillaries during angiogenesis [28]. The effect of applying triple growth factor was studied in the mouse tissue engineering chamber using FGF-2, VEGF and PDGF [111]. At 2 weeks angiogenesis was synergistically enhanced by VEGF and FGF-2. At 6 weeks sequential addition of growth factors increased the percent volume of adipose tissue, with a synergistic increase in adipose tissue in combination treatments [111].

Monocyte chemoattractant protein (MCP)-1, a potent macrophage chemoattractant, has been added to Matrigel in the mouse tissue engineering chamber (see below). In low doses it induced adipogenesis but at high levels it caused a pronounced inflammatory response, filling the chamber with pus [55]. CXC chemokine ligand 12 (CXCL12), previously celled stromal cell-derived factor-1, is an angiogenic chemotactic cytokine. In proof of principle experiments, it was shown that exogenous human endothelial precursor cells when systemically inoculated are recruited to AV loop chambers described above via CXCL12 introduced in the chambers [118].

18.3.5 The Concept of Space

For a tissue engineered construct to develop, it needs to have a space in which to grow. This is particularly true for soft tissues such as fat. Rigid chambers of the make described above are designed to protect the space in which tissue constructs develop from local compressive forces. They also facilitate moulding of tissue to a desired three-dimensional form as well as enable the easy identification and retrieval of constructs. They also allow delayed procedures on the developing tissue where chambers can be accessed, opened and closed again.

Increasing the volume of the chambers leads to greater volume of tissue produced [60]. The chambers do not need to be completely sealed to maintain the space. Perforated chambers, using a fat flap rat model with a dedicated pedicle, yielded greater volume of fat than closed chambers (Fig. 18.9) [32, 129]. The former may improve the supply of nutrients and increase angiogenic invasion from the surroundings that link with the intrinsic blood supply. This improved vascularization may stimulate adipose tissue growth [32].

Cells sense their physical environment and are able to migrate down a rigidity gradient from soft to hard substrates - a phenomenon called durotaxis [84]. Cells involved in adipogenesis may also migrate to the chamber space down a durotactic gradient. They are able to maintain their appropriate cell/cell and cell/extracellular matrix force relationship through a variety of molecules, cellular components and extracellular structures. These include cadherins, gap junctions, integrins, focal adhesions, ion channels, caveolae, surface receptors and cytoskeletal components including microfilaments, microtubules and intermediate filaments [31, 62]. When these forces change to a different modulus the cells through a process of mechanotransduction induce molecular signalling through the Roc/Rho pathways to change their phenotype and proliferate, migrate and differentiate or conversely cluster to form balls of cells and even apoptose [91, 160].

When cells migrate to the chamber space they may feel a change in the surrounding mechanical environment and react accordingly. For instance, in the perforated chamber described above, connective tissue invades from the exterior through the perforations in the chamber wall and forms guy-rope-like connections

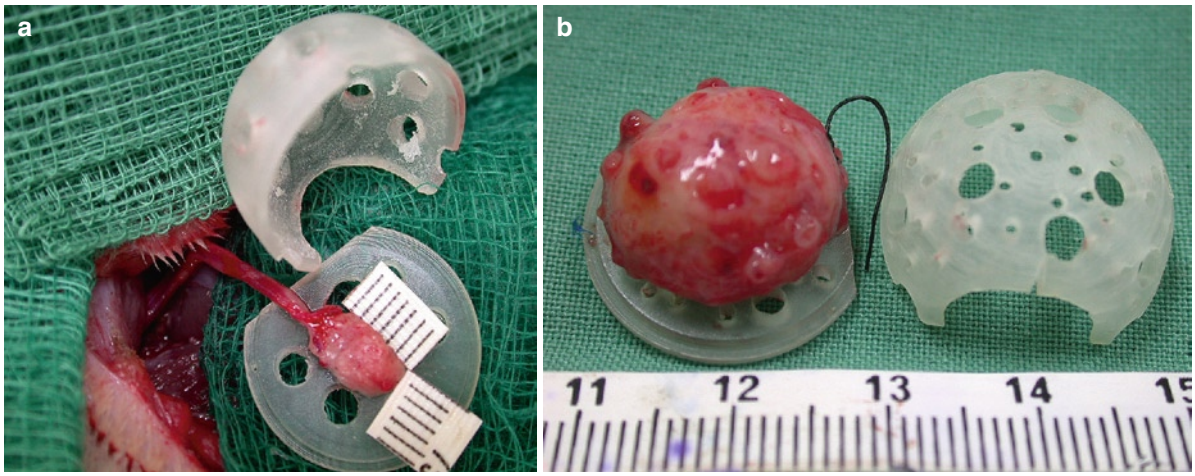

Fig. 18.9 Fat flap model being inserted in a perforated chamber in the rat groin (**a**). Tissue construct harvested at 6 weeks (**b**)

with the intrachamber tissue and exerts a tensional force. The change in forces exerted on the tissue may increase the growth capacity of the tissue in the perforated chamber. In the closed chamber, which has no such connections, the construct tends to retract, encapsulate and shut down the proliferative process [32]. Di Gennaro from our department has shown that using a novel modification of the rat chamber that applies negative pressure to its contents produces bigger and more vascular constructs. This is probably related to changes in mechanical transduction forces acting on the cells within the chamber (unpublished data). The BRAVA™ device described by Roger Khouri for fat augmentation of the breast employs an external vacuum device applied to the skin that distracts the tissues and incites tissue growth [71]. This device is probably creating a chamber-like potential space that in combination with the trauma induced, angiogenesis and inflammation is conducive to new adipose tissue development. Cellular and matrix injection into this environment may magnify and direct the growth potential of the response.

18.3.6 The Inductive Theory of Adipose Tissue Engineering In Vivo

Understanding the mechanisms underlying adipose tissue development is vital to improving adipose tissue engineering and its eventual clinical application. The technique of free fat grafting, in which autologous adipose tissue is harvested from a patient, processed and then re-implanted to correct small volume contour defects, remains unpredictable [121, 137].

The mouse tissue engineering model described above [25] has been mostly utilized to study adipose tissue generation in vivo. Open chambers containing Matrigel™ as matrix that had access to abutting epigastric fat at one end showed strong adipogenesis at 6 weeks. However, sealed chambers without any connection to the surrounding tissue except the epigastric pedicle did not generate adipose tissue [70]. Adipogenesis was restored in sealed chambers when a free fat graft was included, though the graft itself was usually found to be necrotic. It was concluded at that stage that contact with existing adipose tissue was necessary for Matrigel-induced neoadipose tissue formation and that the new adipose tissue in the chamber originated from the stromal-vascular fraction of the abutting fat pad or the free fat graft respectively [70].

However, further studies using human xenografted free fat grafts in chambers implanted in severe combined immunodeficient mice revealed that the generated fat was predominantly host-derived [125]. It was postulated that the fat graft acts as a stimulus for the recruitment of adipogenic stem cells directly from perivascular cells within the chamber or from the systemic circulation via the newly developed vascular network. These studies question the cell survival theory of autologous fat grafting from processed lipoaspirates and favour the host replacement theory where the fat grafts act as an inductive source for de novo adipogenesis.

Following these studies, different types of tissues were inserted into sealed chambers and all induced adipose tissue development within the chamber. These included skeletal muscle, liver and thymus (unpublished data). Adipose tissue was also formed at 6 weeks in the arteriovenous loop chamber using human and rat skeletal muscle as the inductive source [92].

Adipose tissue is a source of proinflammatory cytokines including tumour necrosis factor-α (TNF-α) and interleukin-6 amongst many others [37]. Also, WAT is progressively infiltrated by inflammatory cells with increasing obesity. Inflammatory cells such as macrophages, neutrophils and mast cells have been identified in the mouse chambers. To test whether inflammation is related to adipogenesis in vivo, the fat graft was replaced with an inflammagen called zymosan [55, 136]. The latter is a polysaccharide preparation of the cell wall of the yeast *Saccharomyces cerevisiae* and consists of insoluble particles of approximately 3 μm diameter [30]. It triggers an inflammatory reaction by activating inflammatory cells, such as macrophages and neutrophils [79, 144].

Zymosan, with Matrigel as matrix, exhibited adipogenesis in the chamber in an inverse dose-dependent manner. At 6 weeks, adipose tissue formation was greatest with lowest concentrations of zymosan and least with the highest. Aminoguanitidine, a relatively selective inhibitor of inducible nitric oxide synthase that attenuates zymosan-induced inflammation [27] inhibited the adipogenic response to zymosan [136]. It is postulated that zymosan-induced inflammation recruits resident and circulating populations of inflammatory cells and adipose precursor cells [136].

Adipose precursor cell proliferation in vivo may be driven by the inflammation created in the chamber as key inflammatory cytokines such as TNF-α and IL-6, are strongly mitogenic for preadipocytes in vitro. However, macrophage secreted factors and inflammatory cytokines directly inhibit differentiation in vitro (91). This may account for the reduced volume of adipose tissue obtained at higher concentrations. At lower zymosan doses inflammation may have resolved fast enough to allow differentiation of the recruited adipose precursor cells to proceed [136]. Also, MCP-1, a potent macrophage chemoattractant, has been used to study the role of inflammation in adipogenesis in the mouse chamber. Low levels of MCP-1 in the chamber created a low-grade inflamatory response which induced adipogenesis. However, in high doses it inhibited fat formation in the chamber, presumably for the same reasons mentioned above [55].

Inflammation-induced adipogenesis also occurred in contralateral chambers containing just Matrigel without zymosan. It is thought that the systemic inflammatory response of zymosan is able to traffic adipose precursor cells to the contralateral chamber [136].

Mast cells have recently been shown to play a major role in chronic inflammatory-type reactions [75]. Given this and the theory of inflammation-induced adipogenesis, we studied the role of mast cells in the mouse tissue engineering setting. Mast cells have been observed in chamber adipose constructs. Using mast cell-deficient mice we found that mast cells have an inhibitory role in adipose tissue development, though this role is relatively minor (unpublished data).

In summary, these studies have led to a better understanding of de novo adipose tissue formation in vivo. It is evident that an inflammatory inductive source is necessary for adipose tissue formation in this setting. Regulating inflammation processes may be important to optimize adipose tissue constructs.

18.4 Clinical Application

To date successful adipose tissue engineering is still in its experimental preclinical stage. The concerted effort of scientists from various interrelated fields of tissue engineering and clinicians, particularly in the speciality of plastic and reconstructive surgery, will hopefully lead to successful clinically applicable adipose tissue in the near future.

18.5 Expert Opinion

As alluded to in the introduction, adipose tissue engineering has a myriad of clinically relevant applications. The primary aim of this field is to develop strategies that are able to replace soft-tissue defects by the generation of mature adipose tissue. Over the last decade, adipose tissue biology, especially in the field of molecular biology has provided vital insight into the origin and fate of adipose precursor cells. Techniques of harvesting and expanding adipose precursor cells from subcutaneous fat have become

routine. The combination of scaffolds, stem cells and/or the addition of growth factor and cytokines and the way they behave in various animal models has been studied.

Though autologous fat grafting is clinically acceptable, it is unpredictable and often repeated injections are needed, most probably due to insufficient vascularization. This problem was addressed by in vivo models with an intrinsic blood supply. This resulted in much greater adipose tissue volume than by any other means.

18.6 Five-Year Perspective

Adipose tissue biology research in all its aspects from development to senescence and in normal and pathological states will continue to feed information to researchers in the exciting field of adipose tissue engineering. Strategies to generate autologous, stable, vascularized adipose tissue led to the generation of various in vivo models over the last decade [104]. These models have great potential in the continued search for the optimization of adipose tissue engineering for soft-tissue reconstruction. The next 5 years will probably see more applications in higher animals, such as pigs and sheep, in the process towards human clinical applications.

Many scaffold materials and their interaction with stem cells of various types have already been analysed in various combinations [100]. However, the ideal matrix material is yet to be developed. Collaboration with biomaterials researchers, including those in the innovative field of nanotechnology, will hopefully identify the ultimate matrix material. This will probably include an injectable matrix for small defects with or without stem cells. The use of cell- and/or growth factor/cytokine-loaded microspheres may be a possible form of injectable. For large volume defects a scaffold that maintains its mechanical properties until it is replaced by de novo tissue formation may be a possibility. Another alternative would be a custom-made biodegradable shell that holds the ideal adipogenic scaffold. This will avert the need for a two-stage procedure where a nonabsorbable rigid or semirigid chamber needs to be removed once adipose tissue has formed.

Given the close association between angiogenesis and adipogenesis and the high metabolic rate of mature adipocytes it is plausible that large volume replacements will need an intrinsic blood supply. This may need the surgical creation of an autologous blood supply with a vascular pedicle or custom made synthetic or biosynthetic vascular pedicle that is anastomosed to recipient vessels. Also the possibility of incorporation of angiogenic growth factors within the scaffold is not remote.

To date there is hardly any data on the mechanobiology of adipose tissue. Research into this field may identify methods of modulating forces on stem cells and adipose tissue cells in vitro using mechanical force bioreactors. This will lead to better methods of expanding adipose cells in vitro and possible applications of mechanical forces in vivo.

MSC are capable of differentiating into mesenchymal-linage cells (including adipocytes, osteoblasts and chondrocytes) as well as endothelial cells, neurons, astrocytes and epithelial cells [65, 106]. Human ASC are a potential source of MSC. Also, MSC have the unique ability to suppress immune responses [109, 123, 158]. They have been utilized with success in severe graft-vs.-host disease and may be effective in a variety of autoimmune diseases [97]. It is possible that adipose-derived MSC banks will be developed not only for adipose and other tissue engineering purposes but also for cell therapy of chronic inflammatory and autoimmune diseases.

18.7 Limitations

The major limitations to clinical applications of adipose tissue engineering strategies are currently the identification of a suitable matrix material, scaling and stability of engineered adipose tissue.

As mentioned above the ideal scaffold material that allows cellular penetration into the construct, encourages angiogenesis and allows adipose tissue development still has to be developed. More importantly this matrix has to be compatible with humans. Matrigel™ supplemented with FGF-2 is an adequate angiogenic and adipogenic matrix; however, for reasons already described it is not compatible with humans and therefore can only be used in animal experimentation. Myogel™ may prove to be an effective alternative for human applications.

The scaling of adipose tissue constructs is a significant challenge particularly for the replacement of large

volume losses, such as following mastectomy. Intrinsic vascularization has produced appreciable volume of adipose tissue in our pig model. However, modification of chamber design and possibly the addition of growth factors +/− stem cells still need to be analyzed to determine the possibility of development of larger mature vascularized adipose tissue constructs.

Once an adipose tissue construct has been formed there is still the issue of maintaining stability. We have shown that adipose tissue generated by an inflammatory stimulus remained stable for up to 24 weeks [136]. Longer term studies may be necessary to assess the long term viability of adipose tissue engineered constructs. Also, the adaptation of adipose tissue constructs in vivo to changes in body metabolism such as obesity and weight reduction, by exercise and diet for example, may need to be addressed in the future.

18.8 Conclusion

Adipose tissue engineering has evolved rapidly over the last decade. A variety of in vivo strategies have emerged that approach the central issue of generating stable, vascularized adipose tissue from novel perspectives. The successful recruitment of endogenous preadipocytes by in situ tissue engineering strategies is an exciting development which, should it reach clinical practice, promises to save patients multiple surgical procedures and health services the time, cost and complications of expanding preadipocyte populations ex vivo. The development of an ideal human compatible, angiogenic and adipogenic matrix would be a great advance towards implementing adipose tissue engineering at the bedside.

18.9 Suggested Readings with Abstracts

Kawaguchi N, Toriyama K, Nicodemou-Lena E, Inou K, Torii S, Kitagawa Y. De novo adipogenesis in mice at the site of injection of basement membrane and basic fibroblast growth factor. Proc natl Acad Sci USA. 1998;95(3):1062–6.

Autografting of fat pads has a long history in plastic and reconstructive surgery for augmentation of lost soft tissue. However, the results are disappointing because of absorption of the grafts with time. The fate of transplanted fat is linked to adipose precursor cells distributed widely in connective tissues. Adipocyte precursor cells can proliferate and mature into adipocytes even in the adult body depending on microenvironment. When reconstituted basement membrane, Matrigel, supplemented with more than 1 ng/mL bFGF was injected s.c. into 6-week-old mice, the neovascularization induced within 1 week was followed by migration of endogenous adipose precursor cells, and a clearly visible fat pad was formed. The pad grew until 3 weeks after the injection and persisted for at least 10 weeks. Such de novo adipogenesis was induced reproducibly by s.c. injection of Matrigel and bFGF over the chest, lateral abdomen or head. Adipogenesis could be induced even in ear cartilage or in muscle. Thus, our results demonstrated that an abundant population of adipose precursor cells is distributed widely in connective tissues of the adult body and that they migrate into the neovascularized plug of Matrigel for proliferation and maturation. These results suggest a technique of augmenting lost soft tissue in plastic and reconstructive surgery.

Tabata Y, Miyao M, Inamoto T, Ishii T, Hirano Y, Yamaoki Y, et al. De novo formation of adipose tissue by controlled release of basic fibroblast growth factor. Tissue Eng. 2000;6(3):279–89.

De novo adipogenesis at the implanted site of a basement membrane extract (Matrigel) was induced through controlled release of basic fibroblast growth factor (bFGF). bFGF was incorporated into biodegradable gelatin microspheres for its controlled release. When the mixture of Matrigel and bFGF-incorporated gelatin microspheres was implanted subcutaneously into the back of mice, a clearly visible fat pad was formed at the implanted site 6 weeks later. Histologic examination revealed that the de novo formation of adipose tissue accompanied with angiogenesis was observed in the implanted Matrigel at bFGF doses of 0.01, 0.1 and 1 μg/site, the lower and higher doses being less effective. The de novo formation induced by the bFGF-incorporated microspheres was significantly higher than that induced by free bFGF of the same dose. The mRNA of a lipogenesis marker protein, glycerophosphate dehydrogenase, was detected in the formed adipose tissues, biochemically indicating de novo adipogenesis. Free bFGF, the bFGF-incorporated gelatin microspheres, or Marigel alone and bFGF-free gelatin microspheres with or without Matrigel did not induce formation of adipose tissue. This de novo adipogenesis by mixture of Matrigel and

the bFGF-incorporated gelatin microspheres will provide a new initiative for tissue engineering of adipose tissue.

Mian R, Morrison WA, Hurley JV, Penington AJ, Romeo R, Tanaka Y, et al. Formation of new tissue from an arteriovenous loop in the absence of added extracellular matrix. Tissue Eng. 2000;6(6):595–603.

A major requirement for the microsurgical repair of contour defects of the skin, for example, following removal of a skin cancer on the face, is a mass of vascularized subcutaneous tissue. Such tissue can be generated in vivo using basic tissue engineering principles. In previous studies in our laboratory, we have used a model comprising an arteriovenous (AV) shunt loop sandwiched in artificial dermis, placed in a cylindrical plastic growth chamber, and inserted subcutaneously to grow new connective tissue progressively up to 4 weeks. To learn more about the basic growth characteristics with this model, the same AV shunt loop within a chamber without added extracellular matrix was inserted subcutaneously into the groins of rats for 2, 4 or 12 weeks ($n=5$ per group). There was a progressive increase in the mass and volume of tissue such that the chamber was two thirds full after 12 weeks. Histological examination showed that at 2 weeks there was evidence of fibroblast and vascular outgrowth from the AV shunt, with the formation of granulation tissue, surrounded by a mass of coagulated exudate. At 4 weeks the connective tissue deposition was more extensive, with a mass of more mature granulation tissue containing considerable collagen. By 12 weeks there was an extensive, well vascularized mass of mature fibrous tissue. The blood vessels and residual adventitia of the AV shunt were the likely source of growth factors and of the cells which populated the chamber with new maturing connective tissue. A patent AV shunt in an isolated chamber appears to be the minimal requirement for the generation of new vascularized tissue that is potentially suitable for microsurgical transplantation.

Cronin KJ, Messina A, Knight KR, Cooper-White JJ, Stevens GW, Penington AJ, et al. New murine model of spontaneous autologous tissue engineering, combining an arteriovenous pedicle with matrix materials. Plast Preconstr Surg. 2004;113(1):260–9.

The authors previously described a model of tissue engineering in rats that involves the insertion of a vascular pedicle and matrix material into a semi-rigid closed chamber, which is buried subcutaneously. The purpose of this study was to develop a comparable model in mice, which could enable genetic mutants to be used to more extensively study the mechanisms of the angiogenesis, matrix production and cellular migration and differentiation that occur in these models. A model that involves placing a split silicone tube around blood vessels in the mouse groin was developed and was demonstrated to successfully induce the formation of new vascularized tissue. Two vessel configurations, namely, a flow-through pedicle ($n=18$ for three time points) and a ligated vascular pedicle ($n=18$), were compared. The suitability of chambers constructed from either polycarbonate or silicone and the effects of incorporating either Matrigel equivalent ($n=18$) or poly(DL-lactic-co-glycolic acid) ($n=18$) on angiogenesis and tissue production were also tested. Empty chambers, chambers with vessels only, and chambers with matrix only served as control chambers. The results demonstrated that a flow-through type of vascular pedicle, rather than a ligated pedicle, was more reliable in terms of patency, angiogenesis, and tissue production, as were silicone chambers, compared with polycarbonate chambers. Marked angiogenesis occurred with both types of extracellular matrix scaffolds, and there was evidence that native cells could migrate into and survive within the added matrix, generating a vascularized three-dimensional construct. When Matrigel was used as the matrix, the chambers filled with adipose tissue, creating a highly vascularized fat flap. In some cases, new breast-like acini and duct tissue appeared within the fat. When poly(dl-lactic-co-glycolic acid) was used, the chambers filled with granulation and fibrous tissue but no fat or breast tissue was observed. No significant amount of tissue was generated in the control chambers. Operative times were short (25 min), and two chambers could be inserted into each mouse. In summary, the authors have developed an in vivo murine model for studying angiogenesis and tissue-engineering applications that is technically simple and quick to establish, has a high patency rate and is well tolerated by the animals.

Stillaert F, Findlay M. palmer J, Idrizi R, Cheang S, Messina A, Abberton K, Morrison W. Thompson EW Host rather than graft origin of Matrigel-induced adipose tissue in the murine tissue-engineering chamber Tissue Eng. 2007;13(9): 2291–300.

We have recently shown that Matrigel-filled chambers containing fibroblast growth factor-2 (FGF2) and placed around an epigastric pedicle in the mouse were

highly adipogenic. Contact of this construct with pre-existing tissue or a free adipose graft was required. To further investigate the mechanisms underpinning formation of new adipose tissue, we seeded these chambers with human adipose biopsies and human adipose-derived cell populations in severe combined immunodeficient mice and assessed the origin of the resultant adipose tissue after 6 weeks using species-specific probes. The tissues were negative for human-specific vimentin labelling, suggesting that the fat originates from the murine host rather than the human graft. This was supported by the strong presence of mouse-specific Cot-1 deoxyribonucleic acid labelling, and the absence of human Cot-1 labelling in the new fat. Even chambers seeded with FGF2/Matrigel containing cultured human stromal-vascular fraction (SVF) labelled strongly only for human vimentin in cells that did not have a mature adipocyte phenotype; the newly formed fat tissue was negative for human vimentin. These findings indicate that grafts placed in the chamber have an inductive function for neo-adipogenesis, rather than supplying adipocyte-precursor cells to generate the new fat tissue, and preliminary observations implicate the SVF in producing inductive factors. This surprising finding opens the door for refinement of current adipose tissue-engineering approaches.

Thomas GP, Hemmrich K, Abberton KM, McCombe D, Penington AJ, Thompson EW, et al. Zymosan-induced inflammation stimulates neo-adipogenesis. Int J Obes (Lond). 2008;32(2):239–48.

Objective: To investigate the potential of inflammation to induce new adipose tissue formation in the in vivo environment. *Methods and Results*: Using an established model of in vivo adipogenesis, a silicone chamber containing a Matrigel and fibroblast growth factor 2 (1 μg/mL) matrix was implanted into each groin of an adult male C57Bl6 mouse and vascularized with the inferior epigastric vessels. Sterile inflammation was induced in one of the two chambers by suspending Zymosan-A (ZA) (200–0.02 μg/mL) in the matrix at implantation. Adipose tissue formation was assessed at 6, 8, 12 and 24 weeks. ZA induced significant adipogenesis in an inverse dose-dependent manner ($P<0.001$). At 6 weeks adipose tissue formation was greatest with the lowest concentrations of ZA and least with the highest. Adipogenesis occurred both locally in the chamber containing ZA and in the ZA-free chamber in the contralateral groin of the same animal. ZA induced a systemic inflammatory response characterized by elevated serum tumour necrosis factor-alpha levels at early time points. Aminoguanidine (40 μg/mL) inhibited the adipogenic response to ZA-induced inflammation. Adipose tissue formed in response to ZA remained stable for 24 weeks, even when exposed to normal tissue environment. *Conclusions*: These results demonstrate that inflammation can drive neo-adipogenesis in vivo. This suggests the existence of a positive feedback mechanism in obesity, whereby the state of chronic, low-grade inflammation, characteristic of the condition, may promote further adipogenesis. The mobilization and recruitment of a circulating population of adipose precursor cells is likely to be implicated in this mechanism.

Patrick Jr CW, Zheng B, Johnston C, Reece GP. Long-term implantation of preadipocyte-seeded PLGA scaffolds. Tissue Eng. 2002;8(2):283–93.

Studies were performed in a long-term effort to develop clinically translatable, tissue engineered adipose constructs for reconstructive, correctional and cosmetic indications. Rat preadipocytes were harvested, isolated, expanded ex vivo and seeded within PLGA scaffolds. Preadipocyte-seeded and acellular (control) scaffolds were implanted for 1–12 months. Explanted scaffolds were stained with osmium tetroxide, processed and counterstained using H&E. Quantitative histomorphometric analysis was performed on all tissue sections to determine the amount of adipose tissue formed. Analyzes revealed maximum adipose formation at 2 months, followed by a decrease at 3 months, and complete absence of adipose and PLGA at 5–12 months. These results extend a previous short-term study (Tissue Engineering 1999;5:134) and demonstrate that adipose tissue can be formed in vivo using tissue engineering strategies. However, the long-term maintenance of adipose tissue remains elusive.

Choi YS, Park SN, Suh H. Adipose tissue engineering using mesenchymal stem cells attached to injectable PLGA spheres. Biomaterials. 2005;26(29):5855–63.

The reconstruction of soft-tissue defects remains a challenge in plastic and reconstructive surgery, and a real clinical need exists for an adequate solution. This study was undertaken in order to differentiate mesenchymal stem cells (MSCs) into adipocytes, and to then assess the possibility of constructing adipose tissue via the attachment of MSCs to injectable PLGA spheres. We also designed injectable PLGA spheres

for scar-free transplantation. In this study, MSCs and adipo-MSCs (MSCs cultured in adipogenic medium for 7 days) were attached to PLGA spheres and cultured for 7 days, followed by injection into nude mice for 2 weeks. As a result, the difference between lipid accumulation in adipo-MSCs at 1 and 7 days was much higher in vitro than in the MSCs. Two weeks after injection, a massive amount of new tissue was formed in the APLGA group, whereas only a small amount was formed in the MPLGA group. We verified that the newly formed tissue originated from the injected MSCs via GFP testing, and confirmed that the created tissue was actual adipose tissue by oil red O staining and Western blot (PPAR(gamma) and C/EBP(alpha) were expressed only in APLGA groups). Therefore, this study presents an efficient model of adipose tissue engineering using MSCs and injectable PLGA spheres.

Hemmrich K, von Heimburg D. Biomaterials for adipose tissue engineering. Expert Rev med Devices. 2006;3(5):635–45.

There is high clinical need for an adequate reconstruction of soft-tissue defects as found after tumour resections, deep burns or severe trauma. A promising solution for these defects is adipose tissue engineering, with adult stem cells of the adipose tissue, implanted on 3D biomaterials. These adipogenic precursor cells survive ischemia better than mature adipocytes and have the potency to proliferate and differentiate into fat cells after transplantation. They can be yielded from excised adipose tissue or liposuction material. When preadipocytes are seeded on carriers for the generation of adipose tissue, chemical composition, mechanical stability and 3D architecture of the construct are crucial factors. They ensure cellular penetration into the construct, sufficient proliferation on the material and full differentiation inside the construct after transplantation. In hydrogels, it is especially the use and combination of growth factors that determine the overall outcome of the applied biopolymer. Over recent years, in vivo trials in particular have allowed significant insights into the potential, the perspectives and also the current difficulties and draw-backs in adipose tissue engineering. This review focuses on the main strategies in adipose tissue regeneration, compares the various materials that have been used as carrier matrices so far and considers them in the light of challenges they have yet to meet.

Patrick CW. Breast tissue engineering. Annu Rev Biomed Eng. 2004;6:109–30.

Tissue engineering has the potential to redefine rehabilitation for the breast cancer patient by providing a translatable strategy that restores the postmastectomy breast mound while concomitantly obviating limitations realized with contemporary reconstructive surgery procedures. The engineering design goal is to provide sufficient volume of viable fat tissue based on a patient's own cells such that deficits in breast volume can be abrogated. To be sure, adipose tissue engineering is in its infancy, but tremendous strides have been made. Numerous studies attest to the feasibility of adipose tissue engineering. The field is now poised to challenge barriers to clinical translations that are germane to most tissue engineering applications, namely scale-up, large animal model development and vascularization. The innovative and rapid progress of adipose engineering to date, as well as opportunities for its future growth, is presented.

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Blood Substitutes

19

Andreas Pape and Oliver Habler

19.1 Introduction

Blood substitutes have been developed to replace the transfusion of banked blood. Actually, it is unlikely that one singular infusion fluid will ever replace all functions of whole blood. Indeed, "blood substitutes" merely substitute the O_2-carrying function of red blood cells (RBC), so these substances should rather be referred to as "oxygen carrying blood substitutes" or as "artificial oxygen carriers."

Oxygen carrying blood substitutes (OCBS) intend to increase arterial O_2-content (CaO_2) and thereby O_2-delivery to the tissues. Since the end of the nineteenth century, clinicians confronted with the treatment of acute blood loss and severe anemia have been seeking for a plasma expanding fluid, which would at the same time be able to replace the O_2-carrying function of lost RBCs [36]. In 1898, von Starck documented the first results of experimental injections of isolated hemoglobin in dogs [119]. In 1949, Amberson and coworkers were the first to report a transient stabilization of a 22-year-old woman presenting with hemorrhagic shock after severe postpartum hemorrhage. However, this patient developed severe pyrexia and finally died from acute renal failure on day 9 after delivery [3].

To replace allogeneic blood transfusions, safe and effective OCBS are today expected (1) to be at least as efficacious as banked RBCs regarding O_2-transport and tissue oxygenation and (2) to exert fewer side effects than allogeneic blood. Having such OCBS at disposal has always been of particular interest to military medical services: in the battlefield-scenario with a large number of wounded soldiers and extremely limited supply of homologous blood, infusion of OCBS appears to be an attractive resuscitation fluid for the initial treatment at the combat site [43, 123]. Additionally, civilian tragedies like large scale injuries, natural disasters, or terrorist attacks may acutely increase the need for banked blood by several dimensions. In these situations, OCBS might play a key role as an immediate treatment option [91]: OCBS are universally applicable without blood-group typing and crossmatching, which appears particularly advantageous in out-of-hospital scenarios.

OCBS may also represent an attractive transfusion-alternative in clinical routine, since costs of homologous blood products are continuously increasing [114], while allogeneic RBC-transfusions are – despite continuous advancement of quality in production and transfusion of allogeneic blood products – still associated with risks for the recipient: allergic reactions, transfusion-related lung injury (TRALI), accidental transfusion of incompatible blood ("clerical error" related to the mix-up of cross-matching specimen or blood product), and the transmission of viral and bacterial infections (Hepatitis, HIV, CMV, EBV) [69]. The resulting transfusion-related morbidity burdens public health systems with additional costs [92].

To reduce both, risks for the recipient and costs for public health systems, allogeneic blood transfusions should be avoided wherever possible. Due to their O_2-transporting properties, OCBS may improve tissue oxygenation in situations usually requiring the transfusion of homologous RBC (e.g., extreme normovolemic

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anemia with critically impaired O_2-transport capacity). To achieve this goal, a large number of OCBS have been developed in the last decades. Presently, the following types of OCBS are under clinical and experimental investigation:

1. Synthetically processed PFC
2. Hemoglobin based oxygen carriers (HBOC), i.e., solutions based on isolated human or bovine hemoglobin
3. Hb-containing liposomes mimicking the erythrocytic milieu as "artificial red cells" (liposome encapsulated hemoglobin, LEH)

While the efficacy of most OCBS regarding tissue oxygenation could be shown in various experimental and clinical studies, the risk potential of side effects is presently not completely under control. Due to their particulate nature, PFC-emulsions may only be infused in low doses to avoid overload and malfunction of phagocytic cells of the reticulo-endothelial system (RES).

In addition to their O_2-transport capacity, most HBOC possess strong vasoconstrictor properties, the impact of which on tissue perfusion and organ function is not yet completely understood. Regarding LEH, only data from experimental studies are available at present.

To date, only one HBOC and one PFC emulsion are approved for sale in South Africa, and in Russia and Mexico, respectively. Although a reduction in perioperative allogeneic RBC-transfusions could be demonstrated for most HBOC and PFC, the world-wide approval of OCBS and their implementation into the clinical routine are not foreseeable in the near future. Nevertheless, the development of safe and effective OCBS as an alternative to allogeneic RBC-transfusion is still a matter of high socio-economic significance [72].

19.2 Aim of the Discipline

Although safer than ever before, the transfusion of allogeneic RBC-concentrates is still associated with risks for the recipient, the most serious being immunosuppression, allergic reactions, TRALI, the accidental transfusion of incompatible blood ("clerical error" related to the mix-up of cross-matching specimen or blood product),and the transmission of viral and bacterial infections (Hepatitis, HIV, CMV, EBV). The incidence of these transfusion-related risks are summarized in Table 19.1.

Significant costs resulting from transfusion-related morbidity and from continuously rising costs of blood

Table 19.1 Incidences of potential risks associated with the transfusion of allogeneic RBCs

Risk factor		Incidence
Mistransfusion	Acute hemolytic reaction	1:6,000–1:33,000
	Delayed hemolytic reaction	1:2,000–1:11,000
Infections (viral)	HIV	1:20 Mio.
	Hepatitis A	1:1 Mio.
	Hepatitis B	1:63,000–1:320,000
	Hepatitis C	1:1.2 Mio.–1:11 Mio.
	CMV	1:10–1:30
	EBV	1:200
Infections (bacterial)	*Yersinia enterocolica*, *Serratia marcescens*, *Pseudomonas*, Enterobacteriacae	1:200,000–1:4.8 Mio.
Immunological	TRALI	1:4,000
	Alloimmunization	1:1,6000
	Immunosuppression	1:1
	Allergic transfusion reaction	1:2,000

products imply increasing economic strain for public health systems. Although the willingness to donate blood had temporarily increased after 11th Sep 2001 in the USA, the Food and Drug Administration (FDA) realizes a constantly decreasing rate of blood donation and therefore prognoses a shortage of four million units of banked blood by 2030. Occasionally, blood supply is subject to seasonal shortages during summer and winter holidays.

According to public health statistics, 43% of all donated blood products are transfused to patients aged 65 or older. Since this patient population is growing consistently with the common demographic development, the demand for banked blood will increase significantly within the next years. Due to the growing imbalance between decreasing availability and increasing demand, the costs of blood products are expected to double until 2030 [30, 114].

The expected cost-explosion in transfusion medicine underlines the socio-economic significance of alternatives to transfusion of allogeneic RBC in general and the development of safely and effectively applicable OCBS in particular. Once such an OCBS can be produced in a large scale, blood supply could be supplemented and public health systems could become more independent of blood donations.

Contrasting the maximal storage time of RBCs (maximum 49 days at 4°C), a long-term storage of OCBS under ambient conditions could be possible. The costly infectiological check-up could be dispensable and the application of OCBS would be void of immunological risks as well as risks associated with clerical error.

However, the introduction of OCBS into clinical routine requires the proof of efficacy in exactly those situations where normally RBC are transfused (i.e., extreme anemia with critical limitation of O_2-transport capacity). Moreover, the risk profile of OCBS should at least be comparable with the excellent quality standard of banked blood.

To lend themselves to large scale production, OCBS should be producible and available at reasonable cost. This implies that resources and raw materials necessary for the production (i.e., RBC and Hb) are available in abundant supply.

In the past decades, a considerable number of industrial companies and academic institutions have developed several OCBS intended for use in clinical routine. Many of them entered clinical phase I–III studies.

Table 19.2 Properties expected from the "ideal oxygen carrying blood substitute"

Enhancement of tissue oxygenation (even in ischemic tissues)
Effective at ambient conditions
In vivo half-life comparable with RBC
Universal compatibility (no need to type and crossmatch)
No transmission of diseases
Absence of immunosuppressive effects
Long shelf life at usual storage conditions
Availability in abundant supply
Producible at reasonable cost

Until now, however, no product has been able to meet the high standards of purity, potency, and safety as required by the U.S. FDA. Characteristics required from an "Ideal OCBS" are summarized in Table 19.2:

To develop an OCBS with these properties, its large-scale production, and its introduction into clinical routine still remain the major goals of the discipline.

19.3 State of the Art

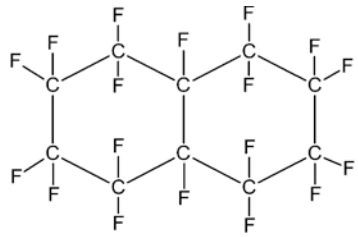
Presently, three types of OCBS are under preclinical and clinical investigation:

1. Synthetically processed PFC
2. HBOC, i.e., solutions based on isolated human or bovine hemoglobin
3. Hb-containing Liposomes mimicking the erythrocytic milieu as "artificial red cells" (LEH)

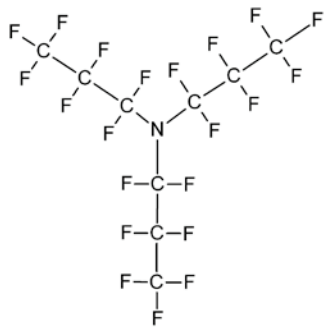
19.3.1 Perfluorocarbons

PFC are simply constructed molecules (MW 450–500 Da) derived from cyclic or straight-chain hydrocarbons with hydrogen atoms replaced by halogens (i.e., fluorine or bromide, cf. Fig. 19.1).

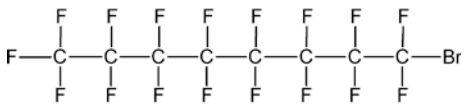
PFC are chemically and biologically inert, insoluble in water, and have therefore to be emulsified before i.v.-infusion. Their pharmacokinetic is characterized by two elimination steps: after intravenous administration, emulsion droplets (diameter: 0.1–0.3 μm) are



Perfluorodecalin ($C_{10}F_{18}$)



Perfluorotripropylamin ($C_9F_{21}N$)



Perfluoroocylbromid ($C_8F_{17}Br$)

Fig. 19.1 Chemical structure of several PFCs used for the manufacture of O_2-carrying PFC-formulations

rapidly (within few hours) taken up by the RES. Depending on the volume infused, the intravascular half life therefore amounts to about 5–9 h [98]. In a second elimination-phase (about 20 days), PFC are redistributed to the blood, transported to the lungs, and finally exhaled due to their high vapor pressure [36].

Potential toxicity of PFC predominantly results from overload of the RES in the case of overdosing. Clinically relevant consequences include hepatosplenomegaly, vacuolated macrophages, and compromised immunological function of the RES [24].

PFC are characterized by high gas-dissolving capacity (CO_2>O_2>N_2) and a linear relationship between arterial O_2 partial pressure and O_2-content, which fundamentally contrasts with the sigmoidal O_2-kinetics of Hb [98, 99]. Therefore, the presence of high arterial O_2 partial pressures – achieved by ventilation with supra-

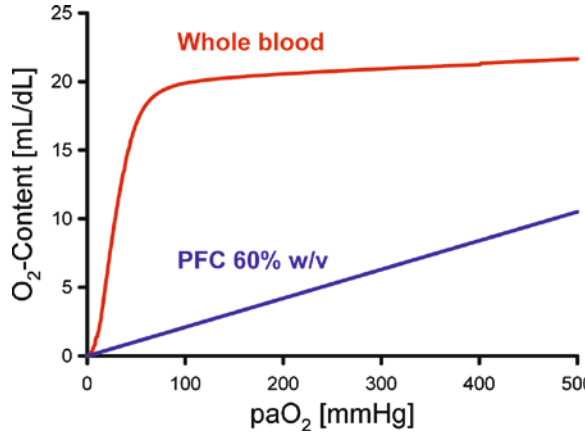


Fig. 19.2 Relationship between arterial O_2 partial pressure (paO_2) and O_2-content (CaO_2) in blood (supposed Hb-concentration: 15 g/dL, *red curve*) and a 60% (w/v) perfluorooctylbromide emulsion (Oxygent™), *blue curve*. Contrasting the sigmoidal O_2-dissociation kinetics of whole blood, the paO_2/CaO_2 relationship of PFC is linear

physiologic FiO_2, usually with pure oxygen – are required to maximize the amount of O_2 transported by PFC (Fig. 19.2) [33].

Although even at high O_2 partial pressures PFC transport a smaller amount of O_2 than do RBCs, the O_2-release to the tissues from PFC is almost complete so that the amount of O_2 released from PFC can even exceed the amount of O_2 released from Hb [81]: in the presence of a high pO_2 gradient between arterial blood and the tissues (e.g., tissue pO_2 40 mmHg, arterial pO_2 600 mmHg, resulting pO_2 gradient 560 mmHg), 100 g of a 60% perfluorooctobromyl-emulsion release 15 mL O_2 to the tissues (Fig. 19.3). The same amount of O_2 is provided by 450 mL whole blood with an Hb-concentration of 14 g/dL.

After intravenous infusion, PFC-droplets are exclusively distributed within the plasma compartment, thereby elevating plasma-solubility for O_2 and arterial O_2-content. Regarding O_2-delivery to the tissues, it is discussed that PFC also facilitates the release of Hb-transported O_2 by lowering the diffusion-barrier between the erythrocyte and the plasma ("facilitated diffusion") [38].

19.3.1.1 First-Generation PFC

The first PFC-emulsion experimentally and clinically investigated in a large scale was Fluosol-DA™ (Green

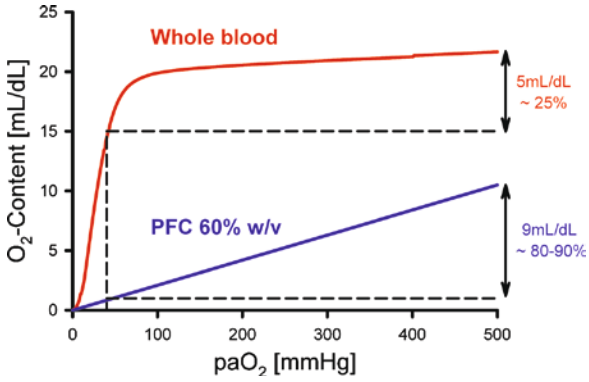


Fig. 19.3 O_2-extraction from native blood (supposed Hb-concentration 15 g/dL, *red curve*) and a 60% (w/v) perfluorooctylbromide emulsion (Oxygent™), *blue curve*. At a given tissue pO_2 of 40 mmHg, O_2-extraction from PFC is – in contrast to blood – almost complete (O_2-extraction rate 80–90% PFC vs. 25% blood)

Cross Corp., Osaka, Japan), a 20% emulsion consisting of perfluorodecaline, perfluorotripopylamin, and the synthetic emulsifier pluronic F-68™. This emulsion was chemically unstable (shelf life 8 h), and so the components had to be stored frozen and separated from each other.

In clinical studies performed from 1979 through 1988 in Japan, high dosages of Fluosol-DA™ (20 and 50 mL/kg) had been applied to about 650 patients [59, 60]. These investigators did not report adverse events for the majority of patients,, although other authors reported relevant side effects associated with the emulsifying agent (anaphylactic reactions, interference with neutrophil chemotaxis [117], activation of the complement system, and interference with pulmonary surfactant causing pulmonary hyperinflation [115]).

However, most studies investigating the efficacy of Fluosol-DA™ failed to demonstrate significant blood saving effect. A large part of these data have not been published until today. Nevertheless, by the end of the 1980s, all clinical studies investigating the i.v. infusion of Fluosol-DA™ in anemic patients had been suspended.

However, at least the short-term potential of Fluosol-DA™ to provide sufficient tissue oxygenation remained unchallenged. In 1989, the substance was therefore approved by the FDA for interventional cardiology as an additive O_2-transporter intended to improve myocardial oxygenation during percutaneous transluminal coronary angioplasty (PTCA) [48]. Due to low sale figures, the production of Fluosol-DA™ was finally suspended in 1993.

In 1996, perfluoromethyl-cyclohexylpiperidin (Perfortan™ 10%, Perftoran Corp., Pushchino, Russia) was approved by the Russian Ministry of Health for perioperative use [40]; the same substance was approved in Mexico in 2005 under the name PERFTEC™ (KEM Lab., Mexico) [124]. Basically, Perftoran™ consists of the same agent as Fluosol-DA™. However, the emulsifying substance was advanced to further reduce potential side effects. Particle size amounts to 0.07 µm, so that a part of the particles can leave the RES before degradation. Plasma half life is thereby increased.

19.3.1.2 Second-Generation PFC

A major consequence of the experience with Fluosol-DA™ was the need to develop a higher-concentrated emulsion with increased O_2-transport capacity. By the end of the 1980s, Alliance Pharmaceutical Corp. (San Diego, CA) achieved this goal with the introduction of Oxygent™. Oxygent™ is a 60% preparation of perfluorooctylbromide, which is emulsified with egg-yolk lecithine. Compared with the first-generation PFC Fluosol-DA™, Oxygent™ features fourfold O_2-transport capacity. In experimental studies, no activation of the complement system, no immunogenic potential, and no alterations of microcirculatory function were observed after i.v. administration of Oxygent™ [37].

To avoid an overload of the RES-macrophages with a consecutive immunosuppression, the manufacturers of PFC emulsions recommend strict dose limitations. The dose limit recommended for Oxygent™ is 0.9–2.7 g/kg.

In 145 volunteers and 30 surgical patients infused with 0.45–3 g/kg Oxygent™, no relevant adverse effects regarding hematology, coagulation or organ function (liver, kidney, lungs) was observed [46]. Frequently reported side-effects of mild to moderate severity included flu-like symptoms, primarily fever (1–2°C rise in temperature), chills, headache, nausea, and myalgia.

These symptoms were related to the clearance process by cells of the RES and were sensitive to prophylaxis with cyclooxygenase inhibitors and corticosteroids [36]. Moreover, a transient decrease of platelet count by approximately 15% was reported 3 days post-dosing, which completely returned to normal by day 7

Table 19.3 Physico-chemical characteristics and actual state of clinical research on PFC

PFC	Concentration (w/v)	Indication	Phase of clinical testing
Oxygent™	60	Reduction of perioperative transfusion rate	III, presently suspended
OxyFluor™	40	Reduction of gas-embolism during cardiopulmonary bypass	I, presently suspended
Perforan™	10	Reduction of perioperative transfusion rate	Approved 1996 in Russia and 2005 in Mexico

Oxygent™ = perfluorooctylbromide (Alliance Pharmaceutical Corp., USA). OxyFluor™ = perfluorodichlorooctan (HemaGen Inc., USA). Perftoran™ = perfluorodecaline/-paramethylcyclohexil piperidine (Perftoran Corp., Russia)

[98]. Both, the release of cytokines and the sequestration of thrombocytes can be almost eliminated by further development of emulsifying substances.

Aside from the above mentioned mild to moderate side-effects of PFC, an increased incidence of postoperative ileus was reported in a clinical phase III study (noncardiac surgery) [101]. Patient enrolement in another phase III study (cardiac surgery) was stopped in 2001 due to increased rate of neurological complications [99].

Presently, all clinical investigations on Oxygen™ are suspended. However, the manufacturers are seeking to perform additional multicentre-studies in Europe and the USA before filing for market approval.

Another second-generation PFC is OxyFluor™ (HemaGen Inc., St. Louis, MO), consisting of a 40% emulsion of perfluorodichlorooctane. This substance was designed to decrease the incidence of gas-embolism during extracorporeal circulation. Biocompatibility of this emulsion and its efficacy regarding oxygen transport and tissue oxygenation have up to date only been assessed in experimental studies [7, 83]. A clinical phase-I study in healthy volunteers is presently suspended (personal communication with the manufacturer).

The present state of clinical research on PFC is summarized in Table 19.3.

19.3.2 Hemoglobin Based Oxygen Carriers

In 1959 Perutz and Kendrew determined the molecular structure of hemoglobin (Hb) [78], for which the Nobel Prize in Chemistry was awarded in 1962. Basically, Hb is a tetramer consisting of 2α and 2β protein chains. Each chain contains heme as a prosthetic group, which is composed of a porphyrin ring containing ferrous iron (Fe^{2+}) in the center.

Hb is found in a saturated, O_2-carrying form (oxyhemoglobin) and in a desaturated form (desoxyhemoglobin). Contrasting PFC, the relationship between paO_2 and O_2-saturation (SaO_2) and thus O_2-content is sigmoidal. The O_2-affinity of Hb is reflected by the p50-value, i.e., the paO_2 corresponding to 50% saturation of Hb (physiologically, 26.8 mmHg, cf. Fig. 19.4).

In the erythrocyte, O_2-affinity is predominantly regulated by the allosteric factor 2,3 diphosphoglycerate (2,3 DPG). The conformation of the heme and protein moieties changes with binding and release of O_2: R-state ("relaxed," O_2-binding) and T-state ("tense," CO_2-binding). When O_2 is bound to Fe^{2+}, iron and the porphyrin ring are coplanar and iron is in a low reactive state. In the desoxygenated state, iron is moved out of the plane of the porphyrrin ring by a slight rotation of the monomers [77].

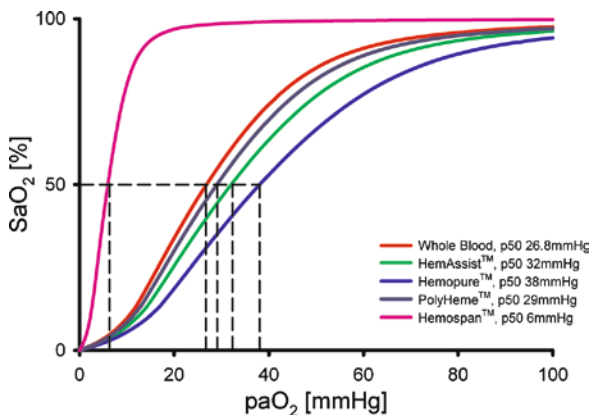


Fig. 19.4 Schematic synopsis of O_2-dissociation curves of whole blood and several HBOCs. O_2-affinity is reciprocally reflected by the specific p50 value

In this situation, Fe^{2+} is prone to oxydation to Fe^{3+} resulting in the formation of methemoglobin, superoxide anion, and hydrogen peroxide. These radicals may initiate deleterious cascades like lipoperoxidation, which is under physiological conditions (i.e., in the intra-erythrocytic milieu) inhibited by antioxidant molecules and specific enzymes (superoxide dismutase, catalase, GSH peroxidates, and methemoglobin reductase) [2, 8].

HBOC consist of isolated, chemically modified hemoglobin molecules. However, in these preparations, the abovementioned intra-erythrocytic regulatory mechanisms are absent.

Hemoglobin needed for the production of HBOC may originate from human (outdated RBC-concentrates) or animal (bovine or porcine) blood, or can be genetically engineered [99].

19.3.2.1 First-Generation HBOC

First experimental results of injection of isolated canine hemoglobin solutions in dogs were already reported by von Starck by the end of the nineteenth century [119]. The first human application of an HBOC with the intention to treat a severe hemorrhage was documented by Amberson in 1949. A 22-year-old woman suffered severe postpartum hemorrhage due to incomplete placental separation. After transfusion of all compatible blood products available in the blood bank, hemorrhagic shock was still persisting. In this situation, Amberson decided to infuse 2 L of a self-made hemoglobin solution. The results were impressive: already after infusion of 300 mL, shock-related hypotension reverted instantaneously, while the patient was breathing spontaneously. Despite a hematocrit of only 5.5%, hemodynamic stability and adequate vigilance were maintained, both indicating sufficient oxygenation of heart and brain. However, on day 9 post partum, the patient developed severe pyrexia and finally died from acute renal failure [3].

Despite the fatal outcome, this case report gives principal evidence about the potential of isolated hemoglobin to provide adequate O_2-transport and tissue-oxygenation – at least for a short term. In the following decades, research and development activities on HBOCs were intensified and the following unfavorable side-effects of isolated hemoglobin could be identified:

1. Contamination of the Hb-solution with erythrocyte-stroma and endotoxines.
2. Spontaneous dissociation of extracellular Hb-tetramers into αβ-dimers, α- or β-monomers. Consequences: Increase of plasma-osmolarity, accelerated glomerular filtration of monomers with reduction of their intravascular half-life.
3. Nephrotoxic effects related to erythrocyte stroma, endotoxines, and to the glomerular filtration of mono- and dimers with consecutive precipitation in the ascending part of the loop of Henle.
4. Markedly increased O_2-affinity (decrease of p50 to 10–15 mmHg with consecutive left-shift of O_2-dissociation curve.) due to the absence of 2, 3-DPG and the acidotic extracellular pH-milieu.
5. Increased formation of Met-Hb due to oxidation and missing erythrocytic enzymes (superoxide-dismutase, glutathionperoxidase, catalase, and Met-Hb-reductase).
6. Vasoconstriction of free Hb. Underlying mechanisms include scavenging of the endogenous vasodilator nitric oxide (NO-scavenging), augmented release of the endogenous vasopressor endothelin, stimulation of endothelin-receptors and adreno-receptors. The extravazation of Hb molecules is discussed as a prerequisite for these vasopressor effects [2].

19.3.2.2 Second-Generation HBOC

The development of second-generation HBOCs aimed at optimizing O_2-transport properties and attenuating undesirable effects [38, 49]. These efforts included the development of innovative methods of hemolysis, dialysis, crystallization, and filtration. Hereby, the contamination with erythrocyte debris and endotoxines could be reduced. Additionally, techniques for virus inactivation and elimination of prions – especially from bovine hemoglobin –were established. To avoid nephrotoxic effects related to the breakdown of the Hb-tetramer in mono- and dimers, a couple of chemical modifications of the Hb-molecule was developed (Fig. 19.5).

Crosslink of α-Subunits

The intramolecular crosslink of subunits increases molecular stability of extracellular Hb and reduces the rate of tetramer dissociation. Thereby, nephrotoxic effects are reduced, and intravascular half-live is increased.

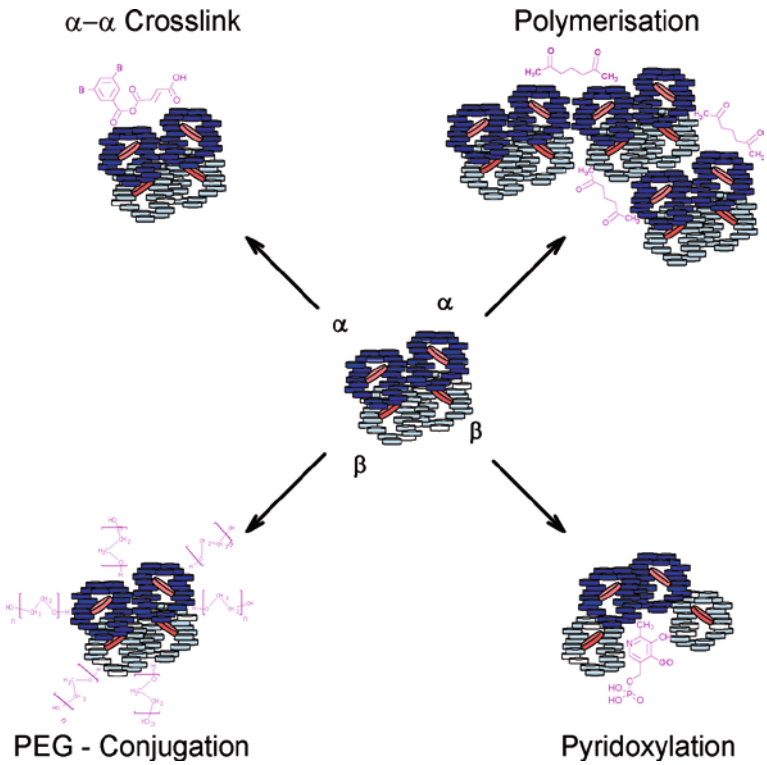


Fig. 19.5 Modifications of the isolated Hb-molecule (*center*) during the manufacture of HBOCs: intramolecular crosslink of α-subunits (α–α-crosslink), polymerization of Hb-molecules using glutaraldehyde or *O*-raffinose, insertion of pyridoxal-5′-phosphate acting as a 2,3-DGP-analog, conjugation of polyethylene-glycol to the surface of the Hb-molecule

This concept has been realized with the product diaspirin crosslinked hemoglobin (DCLHb, Hemassist™, Baxter Healthcare, Round Lake). Lysine residues (position 99) of both α-chains are cross-linked with 3,4-dibromosalicyl-fumarate. Aside from increased molecular stability, molecular conformation is modified, resulting in decreased O_2-affinity (p50 32 mmHg) and thus in a facilitated O_2-offloading to the tissues.

In many experimental and clinical studies, DCLHb provided adequate tissue oxygenation, in particular in situations with critically impaired O_2-supply [35, 58, 95]. However, DCLHb features strong vasoconstrictive properties, which have proven harmful for microvascular perfusion [70, 73]. Although, DCLHb was the main-favorite among the HBOCs for a long time [72], the process of clinical approval was abandoned in 1998 due to an unforeseeable trauma study performed in the USA (see below) [97].

Pyridoxylation

The reaction of the Hb-tetramer with pyridoxal-5-phosphate results in a β–β-crosslink at the site of terminal amino acids. The consequence is a decrease in O_2-affinity and facilitated O_2-delivery to the tissues (p50 30–34 mmHg).

Conjugation

The conjugation of macromolecules (polyethylene glycol (PEG), dextrane) to the molecular surface is another strategy to increase molecular stability and molecular size. This surface modification entails an increase of molecular diameter by adsorption of H_2O, which prevents glomerular filtration as well as extravazation through the endothelial barrier.

Genetically Engineered Hb

Using genetically engineered Hb (e.g., via bacteria and yeast-cultures) offers two incentives: (1) production could become independent of the scarce raw-material "outdated banked blood." (2) Selective exchange of particular amino acids modifies the tertiary and quaternary

structure of the Hb-molecule, thereby preventing the binding of NO [21] and vasoconstriction related to NO-scavenging [94].

In 1992, Looker and coworkers presented a modified recombinant human Hb designed as an oxygen carrying blood substitute. The modifications of the Hb-molecule included (1) a fusion of the two α-chains by insertion of glycine between the C-terminus of one and the N-terminus of the other chain and (2) the exchange of Asparagine residue (position 108) of the β-chain lysine [53].

The second-generation r-Hb (r-Hb 2.0) was a PEG-conjugated Hb-polymer, which featured 20–30-fold less NO-scavenging and thus less vasoactivity [38]. An experimental shock-model in pigs yielded promising results: regarding hemodynamic stability and 6-days survival, r-Hb 2.0 was as effective as whole blood and superior to conventional fluid resuscitation with crystalloid and colloids. In this study, fluid resuscitation with rHb2.0 resulted in similar pulmonary hypertension as following DCLHb. However, pulmonary hypertension following rHb2.0 lasted much shorter than after rHb1.1 [55]. Another experimental study indicated, that rHb2.0 restored pancreatic microcirculation after hemorrhagic shock in rats better than did rHb1.1 [118].

Since clinical application of r-Hb 1.1 yielded unfavorable effects including hypertension, hyperbilirubinemia, hyperamylasemia, and hyperlipasemia [39], the manufacturer stopped all research activities in this field . Results of a clinical phase-I study with r-Hb 2.0 have not been published to date.

Polymerized Human Hb

The Hb-molecule, free or already intramolecularly crosslinked, can be further stabilized by polymerization with glutaraldehyde or *O*-raffinose. This concept has been implemented with the products PolyHeme™ (Northfield Laboratories Inc., USA) and Hemolink™ (Hemosol Inc., Canada). Both products are based on Hb originating from outdated human blood. Polymerization has been realized with glutaraldehyde (PolyHeme™) or with *O*-raffinose (Hemolink™), respectively. The O_2-affinity of PolyHeme™ has been decreased to p50-values of 26–32 mmHg by additional pyridoxylation (see above) [32].

Polymerized Bovine Hb

The glutaraldehyde-Hb-polymer HBOC-201 (Hemopure™) has been developed by Biopure, Cambridge. Due to its large molecular size, this HBOC is void of nephrotoxic effects. Since prion-inactivation techniques have been implemented into the manufacturing process, the risk of bovine spongiform encephalopathy (BSE) seems to be extremely low, but is still a matter of concern [38]. Contrasting isolated human Hb, the O_2-affinity of extracellular bovine Hb is not regulated by 2,3-DPG, but by chloride anions. Indeed, the p50 of isolated bovine Hb is comparable with human intracellular Hb [9].

Due to the high incidence of infectious diseases among blood donors in South Africa, the bovine HBOC Hemopure™ (Biopure Inc., Cambridge) had been approved by the South African Ministry of Health on 6th April 2001. In 2002, Biopure filed for approval by the FDA, but the matter is still pending.

Maleimide-Activated PEG Modified Hb (MP4)

An innovative concept of tissue oxygenation at the site of microcirculation has been realized with the design of MP4 (maleimide-activated PEG modified hemoglobin Hemospan™, Sangart Corp.). MP4 is a low-dose HBOC (Hb content 4 g/dL) with high O_2-affinity (p50 5.9 mmHg) and high viscosity (2.5 cP) [113].

These counterintuitive characteristics of MP4 (high O_2-affinity) are based on the proposition that rapid O_2-unloading – as intended by conventional HBOCs with low O_2-affinity – induces tissue hyperoxia by arteriolar vasoconstriction, which impairs microvascular blood flow [110]. Contrasting this, the low O_2-affinity of MP4 entails that O_2-release is preferentially directed to hypoxic tissues, thereby avoiding hyperoxia in normoxic areas.

Due to the high viscosity of the MP4 formulation, local viscosity of the microcirculation is increased, thereby arousing endothelial shear stress with consecutive NO-release and vasodilation. As a consequence, functional capillary density and microvascular perfusion have been found increased in experimental models [121].

The low Hb-content allows for an effective exploitation of the raw material: one unit outdated RBC yields about four units MP4 (Table 19.4).

Table 19.4 Physico-chemical characteristics and actual state of clinical research on HBOCs

	Source of Hb	Concentration [g/dL]	MW [Da]	P50 [mmHg]	Indication	Phase of clinical testing
PHP™	Human	8	123,000	23.6	Hemodynamic instability in septic shock	II/III
HemAssist™	Human	10	65,000	32	Reduction of perioperative transfusion rate	Up to III, stopped
r-Hb 1.1™	Recombinant	5–10	64,000	31–32	Reduction of perioperative transfusion rate	I/II, stopped
r.Hb 2.0™	Recombinant	10	320,000	31–32	Reduction of perioperative transfusion rate	I/II, stopped
Hemopure™	Bovine	13	250,000	38	Reduction of perioperative transfusion rate	III, approved since 2001 in South Africa, FDA licence application filed in 2002
Polyheme™	Human	10	150,000	29	Reduction of perioperative transfusion rate	III, pivotal prehospital study completed in autumn 2006
Hemolink™	Human	10	120–180,000	39	Reduction of perioperative transfusion rate	III, stopped
Hemospan™	Human	4	95,000	6	Reduction of perioperative transfusion rate	III

PHP™ = pyridoxilated, polyethylene-glycol conjugated Hb (Curacyte Health Sciences, Munich, Germany). HemAssist™ = Diaspirin crosslinked Hb (DCLHb, Baxter Healthcare, Round Lake). r-Hb 1.1 = recombinant Hb, version 1.1 (Somatogen Inc., Boulder, later Baxter Healthcare). r-Hb 2.0 = recombinant Hb, version 2.0 (Baxter Healthcare). Hemopure™ = polymerized bovine Hb (HBOC 201, Biopure Corp., Cambridge). Polyheme™ = pyridoxylated, glutaraldehydepolymerized Hb (Northfiled Lab. Inc., Evanston). Hemolink™ = Hb raffimer (Hemosol Inc.). Hemospan™ = maleimide-activated polyethylene glycol-modified Hb (MP4, Sangart INC, San Diego)

19.3.3 Liposome Encapsulated Hb

First Hb-containing vesicles with synthetic membranes have been developed in 1957 by Chang and coworkers [11, 15, 16]. The encapsulation of Hb attenuates the vasoactive effects of stroma free Hb [20]. Moreover, erythrocytic enzymes (superoxide-dismutase, catalase, Met-Hb-reductase-systems, andDL homocystein) and allosteric regulators (e.g., 2,3 DPG analogs like hexainositolphosphate or pydridoxalphosphate) could be co-encapsulated to decrease the formation of Met-Hb and radical O_2 species [25, 65]. Due to their size(0.2–1 μm), hemoglobin vesicles are postulated not to pass the endothelial barrier, thereby avoiding NO-scavenging and vasoconstriction (Fig. 19.6).

However, the circulation time of these first "artificial red cells" (diameter: 1 μm) only amounted to very few hours, so that membrane surface properties were modified in order to increase plasma half life. These modifications included the use of different synthetic polymers, cross-linked protein membranes, alteration of surface charge, and the emplacement of sialic acid analogs [14]. Nevertheless, circulation time of these relatively large vesicles could only be marginally increased by these modifications [12].

In 1980, Djordjevich and Miller presented smaller Hb-vesicles (diameter 0.2 μm) based on the formation of lipid membranes [20]. The reduction of vesicle diameter substantially increased circulation time. This effect could be supplemented by conjugation of the

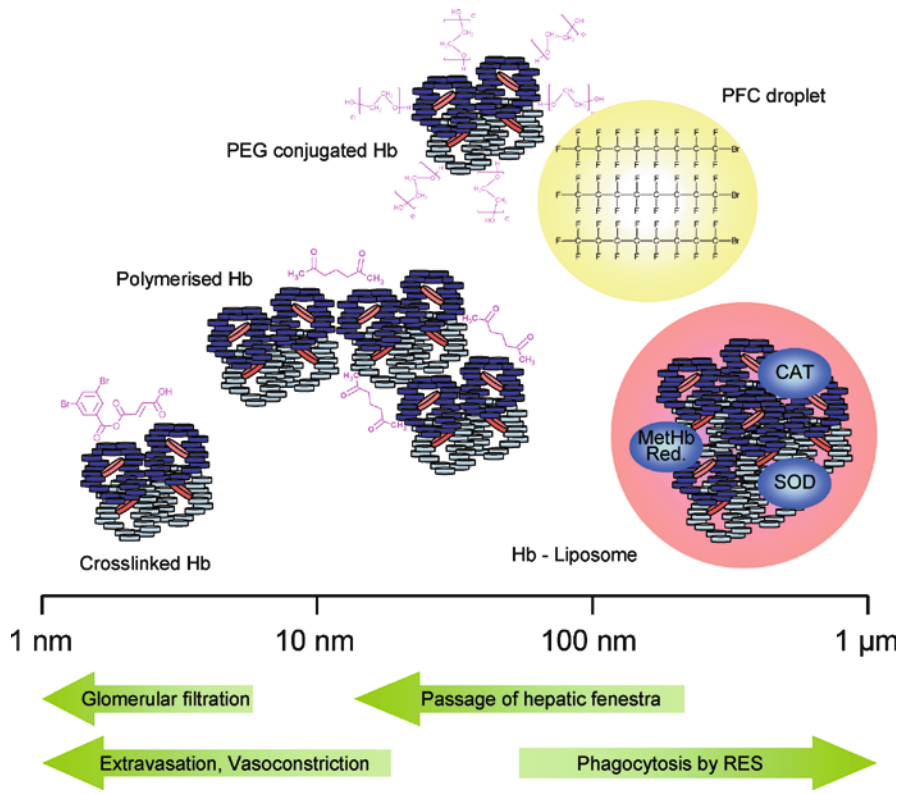


Fig. 19.6 Synopsis of molecular size of several hemoglobin based OCBS including pharmacokinetic and pharmacodynamic effects relative to molecular dimension. Modified stroma free hemoglobin formulations pass endothelial gaps thereby causing vasocontrictive effects. Due to their large molecular dimension, LEH formulations may not pass the endothelial barrier, thereby avoiding NO-scavenging. Like PFC emulsion droplets, LEH is subjected to phagocytosis by RES-macrophages. Modified according to Sakai et al. [85]

vesicle surface membrane with polyethylene glycol [56]. Moreover, PEG-conjugation seemed to enhance convective O_2-transport at the site of microcirculation [22] and prevent the aggregation of Hb-liposomes in the plasma [66, 87].

Another promising technique of encapsulation consists in the assembly of biodegradable nanocapsules (diameter less than 0.2 µm) made of polylactic acid. In vivo, this material can easily be degraded into water and carbon dioxide [13, 14]. The wall of these nanocapsules is stronger and more porous than the liposomal membrane, allowing incorporation of a higher volume of Hb-molecules and enzymatic systems. Moreover, these nanocapsules are permeable to glucose and small hydrophilic molecules, thereby permitting a "metabolic activity" and a physiologic function of co-encapsulated enzymatic systems.

Up to date, LEH is still in the preclinical stage [14, 105], while no data about the use of LEH in humans are available in the peer-reviewed literature. However, experimental data demonstrate the potential of LEH to provide adequate tissue oxygenation. In anesthetized hamsters, tissue oxygenation was found improved during hemodilution with LEH suspended in dextran 70 in hypoxic hamster flap tissue [18, 22]. Interestingly, the use of encapsulated Hb with a p50 of 15 mmHg was more efficacious than Hb with a p50 of 30 mmHg [18]. Moreover, Hb-vesicles suspended in dextran 70 were more efficacious than the one suspended in human serum albumin 8 g/dL [22].

In a hemorrhagic shock model in hamsters, resuscitation with a hemoglobin vesicle (p50 33 mmHg, Hb-concentrations of 3.8 and 7.6 g/dL, suspended in 8 g/dL human serum albumin) restored blood pressure quickly. In addition, functional capillary density and tissue oxygenation improved significantly [86]. Surprisingly, the efficacy of the hemoglobin vesicles with a hemoglobin concentration of only 3.8 g/dL was at least as good or better than the one with a hemoglobin concentration of 7.6 g/dL [86].

In an experimental model of acute normovolemic anemia in anesthetized dogs hemodiluted to their individual critical Hb-concentration, the infusion of 6% LEH formulation (p50 28 mmHg) improved tissue oxygenation for a short term and enabled a longer survival time when compared with placebo-treatment.

However, these effects could be maintained for a longer time in dogs transfused with autologous RBC-concentrates [71].

Comparably with PFC, LEH is eliminated by the RES. Indeed, the phagocytic activity of the RES was compromised after voluminous LEH-infusions in rats, partly resulting in immunosuppression [84].

Moreover, several authors reported proinflammatory effects of Hb-liposomes. A marked increase of IL-6 serum-levels was observed after LEH-infusion in mice [82]. In human blood cultures incubated with LPS, the presence of LEH potentiated the increase of TNF-α and IL-6 responses and the generation of superoxide [93].

Additionally, Szebeni and coworkers suggested a complement-mediated "pseudoallergic reaction," acutely following the infusion of LEH. Hereby, classical and alternative pathways of complement activation were evoked and variable degrees of inflammatory effects were documented in rats, pigs, and men [107, 108].

In humans, acute complement-mediated hypersensitivity reactions have been reported after application of liposomal formulations of cytostatic or antifungal drugs (i.e., doxorubicin, amphotericin,and daunorubicin). The severity of symptoms ranged from moderate (conjunctivitis, urticaria, nausea, flush, and pruritus) to severe (bronchospasm, hypoxemia, dyspnoea, acidosis,and shock) and the incidence varied between 3 and 45% [106]. The degree of complement-activation seems to correlate with size, surface charge, and cholesterol-content of the liposomes [106].

Among all artificial oxygen carriers, liposome encapsulated Hb or Hb-containing nanocapsules are the only blood substitutes mimicking the physiological milieu of the RBC. However, their design is complex and their large scale production is cost intensive. Moreover, important aspects of biocompatibility still need to be elucidated, before the investigation of these substances in humans can be initiated.

19.4 Clinical Application

Presently, only one HBOC and one PFC are approved for clinical routine use in patients, but these approvals are limited to South Africa, Russia, and Mexico. In 2001, the South African Ministry of Health approved the bovine HBOC Hemopure™ (Biopure Inc.).

Due to the high incidence of infectious diseases among blood donors in South Africa, the risk profile of any HBOC may be considered lower than that of allogeneic blood. The first generation PFC perfluoromethyl-cyclohexylpiperidin has been approved in Russia in 1996 (Perfortan™ 10%, Perftoran Corp.) and in Mexico in 2005 (PERFTEC™, KEM Lab.) [40, 124].

Although otherwise no OCBS have been approved for clinical routine to date, comprehensive data material about the application of OCBS is available. Second generation HBOCs and PFCs have been used in experimental models, as documented treatment attempts in singular patients (case reports) and in clinical phase I–III trials. Typically reported application scenarios include

1. Treatment of hemorrhagic shock
2. Treatment of acute intraoperative blood loss
3. Use as a transfusion alternative in hematological disorders
4. Potential transfusion alternative for Jehovah's Witnesses

19.4.1 Hemorrhagic Shock

19.4.1.1 PFC

Due to potential adverse effects related to the overdosage of PFC (compromised immunological function due to overload of the RES, see above), the manufacturers suggest strict dose limits. Therefore, PFC appears unsuitable as a primary resuscitation fluid for the treatment of hemorrhagic shock. However, the supplementation of conventional shock-therapy (i.e., crystalloids and colloids) with PFC may be advantageous. In splenectomized dogs, Kemming and coworkers demonstrated beneficial effects on survival and tissue oxygenation attributable to the combined use of fluid resuscitation with hetastarch, hyperoxic ventilation, and infusion of 4.5 mL/kg Oxygent™ 60% [47]. The same treatment modality was found beneficial in rats subjected to hemorrhagic shock: hepatic microcirculation, tissue oxygenation, and hepatocellular energy metabolism were restored most sufficiently in PFC-treated animals when compared with colloidal fluid resuscitation alone or with the transfusion of stored RBCs [75, 76].

Perftoran™ was used in the Afghanistan war in injured soldiers with suspected hemorrhagic shock. However, results of these application have never been published and the authors are not aware of any further documented attempts to use PFC in humans for supplementation of resuscitation from hemorrhagic shock.

19.4.1.2 HBOC

In contrast to PFC, HBOCs are hyperoncotic solutions that lend themselves as "oxygen transporting plasma expanders" for the initial treatment of hemorrhagic shock: hypovolemia can be treated effectively and decreased arterial O_2-content resulting from dilutional anemia is compensated by increased O_2-transport capacity.

Indeed, in experimental shock-studies, HBOCs were superior to conventional fluid resuscitation with crystalloids and colloids, when hemorrhagic shock was induced by withdrawal of >50% of circulating blood volume. The infusion of HBOC consistently effected a sustained stabilization of hemodynamics and tissue oxygenation and significantly decreased mortality [35, 64, 90, 103]. Moreover, the postischemic interaction between leukocytes and the endothelium could be attenuated by infusion of HBOCs based on human [42, 79] as well as bovine Hb [6].

HBOCs have been used in prehospital trials in severely injured trauma victims with hemorrhagic shock. In particular, the long-term favorite among the HBOCs, DCLHb™ (Baxter Healthcare, Round Lake) was tested in a multicenter trauma study performed in the USA. After enrolment of 112 patients, an interim-analysis yielded significantly higher 24- and 48 h mortality in patients treated with DCLHb [97]. There were severe deficiencies regarding design and performance of the study (under-resuscitation and over-proportional enrolment of desperate cases in the DCLHb-group),and the study was terminated prematurely and never restarted [96]. Additionally, in a multicenter study of 85 patients with acute ischemic stroke, the infusion of DCLHb was associated with serious adverse events (fatal brain and pulmonary edema, renal and pancreatic insufficiency); severe stroke and treatment with DCLHb were identified as independent predictors of worse outcome [88].

Thereupon, Baxter shifted research- and development activities towards genetically engineered HBOCs (r-Hb). While the first-generation r-Hb 1.1 was structurally an analog to DCLHb and had identical side-effects, the second-generation r-Hb (r-Hb 2.0) was a PEG-conjugated Hb-polymer, which featured 20–30-fold less NO-scavenging [38]. An experimental shock-model in pigs yielded promising results: regarding hemodynamic stability and 6-days survival, r-Hb 2.0 was as effective as whole blood and superior to conventional fluid resuscitation with crystalloids and colloids [55]. However, since the clinical application of r-Hb 1.1 yielded unfavorable effects comparable with the side effects of DCLHb (i.e., hypertension, hyperbilirubinemia, hyperamylasemia,and hyperlipasemia [39]), Baxter stopped all research activities in thisfield. Results of a clinical phase-I study with r-Hb 2.0 have not been published to date.

Contrasting this, PolyHeme™ proved to be an effective resuscitation fluid, when 171 patients suffering massive hemorrhage were treated with the HBOC. Compared with a historical control group, 30-day-mortality could be reduced significantly (64.5 vs. 25%) [31]. However, this report does not comment on potential side effects of PolyHeme™.

In July 2006, Northfiled completed another prehospital phase III study aiming at the enrolment of 720 patients at 16 US-trauma centers. Primary endpoint of this study was the 30 days mortality, secondary endpoints were the reduction of allogeneic blood transfusions and the incidence of multi organ failure [41, 63]. The results of this trial have not been published in the peer-reviewed literature data to date. However, a news release on the manufacturers provides a preliminary data presentation, suggesting that the results of this trial are difficult to interpret: in 124 out of the 714 enrolled patients, major protocol violations had been observed. The chairman and chief executive officer, Dr. S.A. Gould, reports that in the PolyHeme™-group, there were more patients with protocol violations, more protocol violations per patient, and most of these patients had predictors of poor outcomes present prior to treatment. Regarding the primary endpoint, no differences between the groups could be observed. However, an increased rate of myocardial infarction was detected in trauma victims resuscitated with PolyHeme™ (www.northfieldlabs.com, internet-research 17th October 2010).

19.4.1.3 Liposome Encapsulated Hemoglobin

For resuscitation from hemorrhagic shock, LEH has been used only in experimental models to date. In these studies, several LEH preparations improved systemic O_2-transport [112] and cerebral tissue oxygenation [111, 122] after fluid resuscitation. Of note, LEH do not have any oncotic effect, so the treatment modality of LEH in shock is similar to that of PFC, i.e., supplementation of colloidal fluid replacement with oxygen carrying liposomes.

19.4.2 Treatment of Acute Intraoperative Blood Loss

19.4.2.1 PFC

In elective surgery with anticipated severe blood loss, the application of PFC as a part of a multimodal treatment strategy provides significant blood sparing potential: prior to surgery, whole autologous blood is withdrawn and collected in blood-bags, while normovolemia is maintained by simultaneous infusion of crystalloid and colloidal solutions (acute normovolemic hemodilution, ANH). In the case of intraoperative blood loss with further decrease of Hb-content during acellular fluid replacement, adequate tissue oxygenation may be maintained by the combination of hyperoxic ventilation and repetitive coadministration of low-dose boluses of Oxygent™. In the best case, the retransfusion of autologous blood can be postponed until surgical bleeding is under control, thereby avoiding spilling out of transfused RBCs via ongoing surgical blood loss.

The concept of bridging extensive blood loss by extreme hemodilution under the protection of hyperoxic ventilation and application of PFC has been patented as "augemented hemodilution" (a-ANH™) by Alliance Pharmaceutical Corp [99].

In splenectomised dogs, Habler and coworkers demonstrated that this concept allowed the extension of acute normovolemic anemia from Hct 21% to Hct 8% without any sign of impaired tissue oxygenation or compromised myocardial contractility [33, 34].

In patients undergoing cardiac surgery, Frumento and coworkers found that the application of 2.7 g/kg Oxygent™ provided adequate gastrointestinal tissue oxygenation at Hb to 6.6 ± 0.4 g/dL [27]. In noncardiac surgical patients (orthopedic and general surgery), low-dose-bolus administration of 60% Oxygent™ (0.9, 1.8 or 2.7 g) allowed the postponement of the transfusion of allogeneic blood by 80 min [100]. In a recent multicentre phase-III-study, the number of pRBC-units transfused until postoperative day 3 was significantly lower in patients treated with PFC [101].

However, some methodological concerns arose about the latter study regarding the comparability of the groups (preoperative ANH and hyperoxic ventilation have exclusively been performed in the PFC-group) [109]. Indeed, hyperoxic ventilation alone has been demonstrated to significantly increase the tolerance of acute normovolemic anemia by providing sufficient oxygenation of vital organs [33, 57, 74, 120].

Nevertheless, due to the high incidence of adverse and serious adverse events in clinical phase-III trials, all clinical research activities on Oxygent™ are suspended at present.(see above).

Contrasting this, first-generation PFC Perftoran™ was approved in Russia in 1996 and in Mexico in 2005 under the name PERFTEC™. In Russia, about 4,500 patients have been treated with Perfotran™ 10% (4–30 mL/kg) until 2002, 1,823 of them within clinical studies. Most of these studies are published in Russian; these manuscripts predominantly report significant blood savings attributable to the infusion of Perftoran™ [54]. A recent study investigating the efficacy of PERFTEC™ in 30 patients undergoing cardiac surgery demonstrates reduction of allogeneic RBC-transfusions in the PFC-group and the authors report no serious adverse events. However, the difference in RBC-transfusion rate was not statistically significant, the study population was very small, and the authors used heterogeneous transfusion triggers in their study protocol [116].

19.4.2.2 HBOC

Aside from fluid resuscitation from hemorrhagic shock, HBOCs are also suitable for the treatment of intraoperative blood loss. During isovolemic replacement of lost blood, the O_2-transport properties of the HBOC allow for hemodilution to a lower hematocrit than do crystalloid and colloid solutions. In the best case, the transfusion of allogeneic blood can be postponed until surgical bleeding is under control.

HBOCs have been tested in several clinical phase III studies, including cardiac and noncardiac (general, vascular, trauma) surgery [28, 31, 44, 51, 89, 102]. Frequently observed side effects consisted in increased systemic and pulmonary arterial resistances, decrease of cardiac output, jaundice, increased activities of amylase, lipase and hepatic transaminases [44, 51, 89, 102]. Whether the increased enzyme activities must be judged as signs of pancreatitis or may be related to interference with photometric laboratory tests has not been fully elucidated [45] yet.

Contrasting this, neither elevation of liver enzymes nor adverse events were observed in clinical phase-I studies with Hemospan™. In a phase II study of 90 patients undergoing orthopedic surgery under spinal anesthesia, the infusion of up to 500 mL Hemospan reduced the frequency of hypotensive episodes to 45–48% (vs. 87% in the control-group). Three serious adverse events, including two deaths were reported, but none was considered related to study treatment [67, 68]. The effect of Hemospan™ on hypotension after induction of spinal anesthesia was also studied in a recently completed phase-III trial, which compared the volume expansion effect of Hemospan™ with a hydroxyethyl starch preparation (6% HES 130/0.4). Results of these trials are to be expected in the near future (personal communication). While the majority of presently available results regarding Hemospan™ address its safety profile, the blood sparing potential of this Hemospan still remains to be proven in randomized prospective trails.

Although HBOCs sufficiently increased O_2-delivery in most clinical studies and thereby substituted the transfusion of allogeneic RBCs in the acute perioperative phase, a sustained reduction of allogeneic blood transfusion (up to postoperative day 7) has only been reported by two authors [17, 89]. However, the blood-sparing potential was limited to only 260–600 mL pRBC and the clinical relevance of this finding has been critically discussed by the authors themselves. A reason for this may be the short intravascular half life of HBOCs. The short term application only postpones the time point of allogeneic blood transfusion. To achieve an effective reduction of RBC-transfusions, HBOCs must be infused over a longer term, theoretically until the erythropoiesis can provide sufficient quantity of autologous RBCs. Regarding the long-term use of HBOCs, only case reports are presently available [52, 61].

19.4.2.3 Liposome Encapsulated Hemoglobin

Since the use of LEH-formulations in humans has not been documented so far in the literature, no results about the use of LEH in the treatment of acute intraoperative blood loss are available at present. However, a couple of experimental models have simulated the scenario of acute normovolemic anemia requiring transfusion of allogeneic RBCs to maintain adequate tissue oxygenation. Overall, the results of these studies demonstrate the potential of several LEH-formulations to provide adequate tissue oxygenation in situations with critically impaired O_2-transport capacity [10, 18, 22, 71]. Whether these findings will translate to clinical benefit still remains to be elucidated.

19.4.3 Use as a Transfusion Alternative in Hematological Disorders

Several case reports have documented the use of OCBS (predominantly HBOCs) as a transfusion alternative in hematological disorders such as sickle cell anemia or autoimmune hemolytic anemia. Indeed, the individual benefit presented in these reports is imposing and the universal compatibility of OCBS may be particularly attractive in these hematological conditions (decreased probability of alloimmunization, absence of allogeneic antibodies). Nevertheless, data from randomized prospective trails are missing thus far to appraise the significance of OCBS in the treatment of hematological disorders appropriately.

Raff et al. reported the case of a 40-year-old woman with a sickle cell crisis after a hip operation. In lieu of compatible RBC-concentrates, the authors infused five doses of polymerized human Hb (PolyHeme™). This treatment in combination with erythropoetin, resulted in a sustained increase of Hb-concentration and finally in the patient's recovery [80].

Gonzalez et al. assessed the safety of Hemopure™ in 18 adult sickle cell patients in a randomized phase I/II study. Patients were not in crisis at the time of study, but the authors concluded from their safety and efficacy results, that Hemopure™ might be effective in the management of vaso-occlusive episodes [29].

Lanzkron et al. reported a case of Jehovah's Witness with sickle cell anemia complicated by acute chest syndrome. Comorbidity of this patient included

thrombocytopenia, bilateral pulmonary infiltrates, staphylococcal sepsis, and pulmonary embolism. The patient received 12 units of PolyHeme™ over the course of 13 days, recovered rapidly and could finally be discharged home with no allogeneic blood transfused [52].

Another patient population that might benefit from OCBS are patients with hematological disorders autoimmune genesis. Mullon et al. reported the case of a 21-year-old woman presenting with severe autoimmune hemolytic anemia. The patient received 11 units of Hemopure™ over 7 days. Although hematocrit had decreased to 4.4% in the meantime, the patient survived without immediate or long-term evidence of ischemic end organ injury.

19.4.4 Potential Transfusion Alternative for Jehovah's Witnesses

For religious reasons, Jehovah's Witnesses decline the transfusion of allogeneic and even stored autologous blood. Contrasting this, the application of fractions derived from blood (including clotting factors, albumin, globulins, and particularly hemoglobin) is not prohibited by the religion. The decision is finally left to the individual as a "matter of conscience" to determine whether the use of the product is in violation of the doctrine on blood.

Indeed, a couple of case reports of the successful use of OCBS in Jehovah's Witnesses are documented in the literature. For example Cothren et al. report about a 39-old Witness suffering placental abrpution complicated by disseminated intravascular coagulation (DIC). The patient was admitted with an Hb of 2.9 g/dL. The infusion of 18 units PolyHeme™ in the course allowed the abstaining from any RBC-transfusion during her entire hospital stay, while the patient completely recovered and finally survived surgery.

The same authors also report a 44-year old Witness who sustained multiple trauma after a motor vehicle collision and was infused with five units PolyHeme™ along with erythropoietin [19].

Moreover, Agrawal et al. presented the case of a Jehovah's Witness receiving chemotherapy for acute myeloid leukemia. Due to continuously decreasing RBC-counts, the patient received a total dose of 1,230 g of Hemopure™. Although sufficient cerebral oxygenation could be established in the course of treatment, the patient experienced pulmonary edema, renal failure, and accumulation of hemosiderin-laden macrophages in the bone marrow and finally succumbed to the disease after 18 days of treatment. Whether these complications were related to the HBOC-therapy, remains unclear [1].

19.5 Expert Opinion

In a recent survey comparing the acceptability of donor blood and blood substitutes among general public and blood donors in the UK and in Holland, blood substitutes were perceived riskier than banked blood despite the knowledge of risks inherent in the transfusion of allogeneic blood [26].

This finding reflects very well that several safety issues of OCBS are still unresolved. While adverse effects of PFC on immunological function are predominantly dose-dependent, the toxicity of HBOCs appears to be more complex. Nephrotoxicity of first-generation HBOCs could be significantly reduced by crosslinking Hb subunits or by polymerizing Hb tetramers. However, vasopressor effects resulting in compromised microvascular perfusion and autooxidation of extracellular Hb resulting in the formation of Met-Hb and reactive oxygen species hallmark the toxicity of second-generation HBOCs.

Besides the unresolved issues regarding the safety profile of OCBS, evidence concerning their efficacy is still incomplete, as the assessment of efficacy in clinical trials is problematic for several reasons. First, the clinical response (parameters of central hemodynamics, global O_2-transport, and tissue oxygenation) to the infusion of HBOCs has been highly heterogenous in clinical and experimental studies. Second, the transfusion practice and the choice of transfusion triggers show significant variability among clinicians and also among study protocols involving the administration of OCBS. Thus , it is difficult to establish whether a transfusion has actually been avoided and to draw conclusions on the blood sparing potential of the OCBS. Third, we do not have sufficiently validated tools for monitoring tissue oxygenation in the clinical setting, in particular when oxygen delivery must be estimated independently from actual hematocrit values.

Moreover, our basic understanding of physiologic regulation of oxygen delivery and utilization may still

be incomplete [104]. Indeed, essential paradigms regarding oxygen delivery (e.g., impact of low oxygen affinity and low viscosity) have recently been challenged by the innovative design of Hemospan™, indicating that a better understanding of oxygen delivery at the site of microcirculation and oxygenation of tissues, cellular, and subcellular structures may be required for an appropriate efficacy-assessment of OCBS. To this end, most target parameters may predominantly be assessed in experimental models, and these experiments should be predictive of response in humans, incorporate stress conditions, and be used to systematically evaluate the effect of variation of Hb structure, biochemistry, and physical chemical properties [23].

Concerns about blood supply (imbalance between demand and availability of blood products, transfusion related risks) underline the high socio-economic relevance of OCBS. However, a prerequisite for their approval and introduction into clinical routine use is the proof of their safety and efficacy. Although we are still lacking evidence in this regard, new insights have been gained into the physiology of oxygen-delivery to the tissues and some aspects of the toxicity of HBOCs have been identified in greater detail. This knowledge may be the basis for further research and developing activities aiming at the identification and manufacture of ideal OCBS.

The manufacture of OCBS is presently highly expensive (cf. limitation/critical view), so that in economical terms, the use of OCBS would not be an alternative to the transfusion of allogeneic at present. In the view of still unresolved safety-problems, unproven efficacy and enormous costs, the implementation of OCBS into the clinical routine appears not to be foreseeable in the very near future. Instead, some more basic research activities will be mandatory to find the ideal OCBS with optimal oxygen delivery to the tissues, a safety profile at least comparable with that of blood, and a manufacturing technique that allows economically viable large scale production.

19.6 Five-Year Perspective

Till date, only two OCBS have obtained nationally limited approvals for clinical routine use in humans: the bovine HBOC Hemopure™ (South Africa) and the PFC Perftoran™/PERFTEC™. Whether these substances will also be approved in European or US-American countries within the next 5 years cannot be anticipated at present.

However, the manufacturer of Hemopure™ had filed an FDA-approval in 2002. While this procedure is still pending, several clinical phase II trials in hemorrhagic shock and nonsurgical indications are under preparation [104]. The HBOCs Hemospan™ and PolyHeme™ are in an advanced stage of clinical testing. Whether the manufacturers of these substances will also apply for FDA-approval will predominantly depend on the results of the corresponding clinical trials.

Regarding Hemospan™, comprehensive data from clinical phase III studies will be available in the near future. Aside from the proof of safety, particular evidence of the blood sparing potential of this innovatively designed HBOC has to be expected from clinical results. Likewise, more insight into safety and efficacy of PolyHeme™ will have to be obtained in the next 5 years. To this end, the multicenter trauma trial completed in 2006 was announced as a pivotal study, but the publication of these results in the peer-reviewed literature is still awaited with great expectation.

Maybe, additional clinical and prehospital trails of Hemopure™, PolyHeme™, and Hemospan™ are required, before an application for FDA approval appears reasonable. Whether these trials will actually be performed and if so, whether they will provide desired evidence regarding safety and efficacy of these substances, remains to be seen.

Alternative innovative technologies applicable for the manufacture of OCBS include Hb-aquasomes made of ovine Hb-molecules adsorbed to spherical hydroxapatite cores and PEG-conjugated hyperpolymers of porcine Hb (Hb-concentration 3 g/dL, MW 800 kDa, glutaraldehyde-polymerized). A 14% suspension of Hb-aquasomes was reported to possess almost physiological O_2-transport properties (p50 28.4 mmHg), to be void of vasopressor effects and effective in tissue oxygenation in an experimental study [50]. Biocompatibility of porcine Hb-hyperpolymers has been tested in volunteers [4], though further clinical data are presently not yet available.

At present, no OCBS meets the requirements of safety, efficacy, and availability at reasonable costs, which presently appears to impede progress in the field. Therefore, a workshop sponsored by the US-American National Heart, Lung, and Blood Institute was convened

in March 2006 to identify potential goals of basic science research to improve progress in the development of HBOCs [23]. Aside from a list of several toxicologic issues requiring clarification (cf. "Limitations/Critical view"), the research recommendations elaborated by this workshop also addressed metabolism and phamacokinetic properties of HBOCs, further investigation of physiological principles of oxygen transport and delivery, and establishment of valid clinical monitoring techniques targeting tissue oxygenation. The authors conclude that "There is substantial amount of work to be done and abundant opportunity for research of direct relevance and great interest to the transfusion medicine community." [23]

Paralleled by enhanced basic science research activities, comprehensive data about HBOCs currently under clinical investigation (i.e., Hemopure™, PolyHeme™ and Hemospan™) should be expected in the next 5 years. Whether and when new clinical research activities with first- or second-generation PFC formulations will be reinitiated, is presently not foreseeable.

19.7 Limitations/Critical View

Up to now, none of the currently known products has been able to entirely meet the high quality requirements postulated by the FDA for introduction into clinical routine. In particular, it was not possible to establish whether the safety profile of OCBS is comparable or even superior to stored RBCs. Indeed, many HBOCs having entered clinical trials foundered on the shoals of unexpected adverse events [104].

In a recent meta-analysis of data pooled from clinical trials with several HBCOs, Natanson and colleagues found a 30% increase in the risk of death and nearly a threefold increase of myocardial infarction in patients infused with HBOCs [62]. However, this meta-analysis included trials with different HBOCs and not all HBOCs behave in the same way. The different chemical modifications (crosslink, polymerization, and surface modifications) entail allosteric conformations with variable degrees of NO-scavenging and vasoactivity, and a significant variation of basic characteristics such as O_2-affinity, oncotic pressures ,or viscosity. It is therefore impossible to extrapolate effects and side effects of one HBOC to another. Nevertheless, the safety-profile of most HBOCs still requires clarification, before these substances can be approved for routine clinical use. Moreover, most HBOCs and PFC-emulsions have hardly been investigated till now in critically ill patients. Therefore potential interactions between concurrent stress, particularly local or systemic inflammation, and OCBS-related side effects cannot be precisely estimated at present.

Aside from cardiovascular morbidity and mortality, serious adverse events related to the infusion of OCBS include gastrointestinal distress and hepato-pancreatic toxicity. Whether these effects are exclusively attributable to the vasoconstrictive effects of HBOCs, is unclear. However, the toxicity of extra-erythrocytic Hb is also increased by autooxidation in lieu of intra-erythrocytic protective enzymes, finally resulting in the formation of Met-Hb and reactive oxygen species with increased oxidative stress to end-organ tissues.

Till date, only little is known about the catabolism of HBOC, their interaction with haptoglobin ,and the question of possible toxicity of acutely occurring amounts of bilirubin. Moreover, the impact of HBOC formulation excipients on product toxicity and stability have not yet been conclusively evaluated. It may be speculated that these factors contribute to gastrointestinal distress and other adverse effects observed after HBOC-administration.

Moreover, potential immunogenicity of HBOCs (chemically modified Hb-molecules might be recognized as exogenous substance thereby triggering an immune response in humans) has not been completely elucidated to date. Aside from the fear of prion contamination, bovine Hb might even have a greater chance of causing immunogenicity in humans due to its xenogeneic origin.

Biocompatibility of liposomal Hb-formulations and their potential to induce immune responses in humans has not been appraised till now. Central questions regarding biocompatibility in humans have not been resolved yet and the clinical relevance of TNF-α production by mononuclear macrophages as well as potential immunomodulating effects of LEH is not fully understood yet.

Apart from unresolved safety issues, convincing evidence about the efficacy of OCBS regarding the definitive replacement of allogeneic RBC-transfusions is still lacking. The use of OCBS within a multimodal blood-sparing strategy including cell salvage may not be reasonable, since extracellular Hb as well as

PFC-molecules may be eliminated by the washing-progress of the cell-saving-device. One of the most critical shortcomings of all prevalent OCBS is their short in vivo half live, which makes them rather suitable for short-term gaps (e.g., acute blood loss, ischemia, bridge until compatible red cells become available) than for sustained replacement of allogeneic blood transfusions. Indeed, several clinical studies demonstrated a blood sparing effect of OCBS in the early perioperative phase, while only two studies demonstrated a reduction of allogeneic RBCs sustained beyond postoperative day 7 [17, 89]. However, the cumulative blood sparing potential amounted to 260–600 mL RBC-concentrated and the clinical relevance of this finding was critically discussed by the authors themselves.

To entirely avoid the transfusion of allogeneic RBCs, OCBS should theoretically have to be administered until the patient's erythropoiesis has provided sufficient autologous RBCs. This long-term application of OCBS has neither been implemented in study protocols, nor does it appear rational for economic reasons. It should be borne in mind that OCBS are very expensive at present, since manufacturers attempt to recoup their developmental costs. Actually, the price of HBOCs even exceeds the cost of banked blood. For example two units of Hemopure™ (cost: $700–$1,000) provide the same Hb-content as one unit of donated blood (cost: $200) [5].

These figures indicate that large scale production at reasonable prices cannot be expected in the near future, but the production of OCBS in a larger scale may also be a prerequisite to support widespread clinical and preclinical research activities in the field.

The manufacture of most HBOCs is dependent on the availability of outdated human RBCs. Whether the production of HBOCs can be enhanced by increasing blood collection rates or even by inclusion of currently deferred donors to harvest RBCs for pathogen-inactivated Hb is deemed a controversial issue among experts.

The blood sparing potential of PFC-emulsions is in the first place narrowed by the dose-limitations recommended to avoid an RES-overload with consecutive immunosuppression (see above). The only currently approved PFC-formulation (Perftoran™ and PERFTEC™, respectively) is a 10%-PFC emulsion and the O_2-transport capacity of higher-concentrated preparations such as Oxygent (60%) may be considerably higher. However, clinical research activities on Oxygent™ are presently suspended due to unfavorable side effects in several clinical trials (see above). Hence, an optimal PFC formulation appears not to be presently available.

Although some of the presently available OCBS have partially demonstrated promising results, the philosophers' stone – i.e., a product meeting the requirements of safety, efficacy, and cost-effectiveness – is not yet found and we are still several years away from the introduction of OCBS in clinical routine.

19.8 Conclusion/Summary

The growing imbalance between increasing demand and decreasing availability of allogeneic blood underlines the socio-economic significance of safe and effective artificial O_2-carriers as an alternative to transfusion of allogeneic RBC. The blood-sparing potential of both types of artificial O_2-carriers currently under investigation (HBOCs and PFC) has been proven in experimental as well as in clinical studies. As a part of a multimodal treatment strategy including normovolemic hemodilution and hyperoxia, the low-dose-administration of PFC effectively increases intraoperative anemia tolerance. Although the bovine HBOC Hemopure™ was approved in South Africa for treatment of acutely anemic surgical patients in 2001, the approval of a particular HBOC by the FDA is presently not foreseeable. Nevertheless, further development of safe and effective synthetic O_2 carriers remains an issue of substantial interest. At present, the implementation of further basic science research activities appears necessary to achieve this goal.

Suggested Readings

Review Articles

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Tissue Engineering of Blood Vessels: How to Make a Graft

20

Andrew A. Gassman and Howard P. Greisler

20.1 Introduction

Folkman and Hochberg [46] noted in 1973 that, unlike persistent two-dimensional (2D) tumor cell growth, three-dimensional (3D) growth is self-limiting. Observations from that cell culture environment demonstrated an initial phase of exponential population growth that was followed by central spheroid cell necrosis. Despite continually replenishing the nutrient supply that bathed the gelatin-impregnated cell aggregates, all cells greater than 150–200 μm away from the spheroid surface died. Eventually a homeostasis was met when cells proliferating on the surface replaced those cells beyond this distance. Cell viability was a function of nutrient and oxygen diffusion, and beyond the diffusion limit of each nutrient resulted in an environment incompatible with cell survival. Additionally, this work implicated poor removal of metabolic waste and accumulation of inhibitory byproducts as another mechanism contributing to regulation of cellular growth. Although some cells (i.e., endothelial cells and fibroblasts) within tissue exposed to relative hypoxia demonstrate increased cellular proliferation [48, 175], tissue that is exposed to severe hypoxia or anoxic conditions beyond nutrient diffusion distance cannot survive.

Later work by Folkman and Haudenschild [45] described how endothelial cells could be grown in culture so as to differentiate in capillary tubules. This seminal work opened up the possibility of artificially developing vascular tissue in culture. This description of angiogenesis would subsequently open up entire fields of study that seek to describe how blood vessels are formed, the chemical signals that contribute to their growth, inhibitors of their development, and a myriad of truths that would subsequently unmask the key biological parameters that define physiologic and pathologic vascular behavior. The information gained has subsequently provided scientists with both the tools to control vascular growth and a target by which they compare their efforts for vascular development.

Tissue engineering is the pursuit of seamlessly replacing tissue damaged by natural or pathological processes with a functional alternative. Langer and Vacanti [93] defined Tissue Engineering in 1993 as an interdisciplinary field that attempts to provide solutions to tissue creation and repair. Their work highlighted the critical importance of nutrient exchange and the limitation diffusion distance plays in 3D cell culture. They proposed to overcome this limitation by concurrent vascularization during a tissue construct's development and maturation. Since then, there have been many attempts and strategies to vascularize tissue. The development of the microcirculation is critical for the development of 3D tissue replacements.

The properties of a tissue engineered construct arise from the cellular and extracellular components and how those cells interact with their microenvironment [8]. Thus, environment cues and matrix compositions are critical to the recapitulation of tissue function. Without microvascular network formation the microenvironment will be inhospitable to tissue formation. In this discussion we hope to explain the key concepts related to vascular biology that the tissue engineer should understand when setting out to design both

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their tissue of interest and the microvascular network that will nourish it. A clearer understanding of the parameters of how the vascular tree develops and how their implanted construct will remodel after implantation is critical to the success of both the tissue's formation and its long-term survival.

When engineering a microvasculature, there are some key philosophical distinctions that must be made to organize the myriad of construction methodologies. For example, there are two temporal options when attempting to vascularize a tissue engineered construct. One approach develops a microvasculature prior to implantation into the tissue's final position. Using this methodology, the capillary structures develop under either in vitro culture conditions or in vivo at an ectopic site or in another organism completely. These techniques develop the capillary networks initially and then require that they anastamose with more proximal vasculature once transplanted into its final position. This methodology is limited by its requirement for multiple interventions, potential donor site associated morbidities, and lengthy time of development. Another approach involves the postimplantation development of a capillary system within the tissue's ultimate desired location. Until recently this approach required the insertion of material that would foster a continuous vascular ingrowth from proximal vessels to the microcirculation. This method limits the tissue engineer's resolution in construct design. Unlike the prior approach, this method does not allow for direct placement of cells in a predetermined pattern, because those patterned cells beyond the diffusion limit at the time of insertion do not survive. Instead, the construct must be patterned in a way to promote specific cellular recruitment and attachment in a 3D pattern that will resemble or foster the eventual development of the tissue of interest and its supporting microvascular network. Tissue constructs can be patterned or designed by two general methodologic approaches. The tissue engineer can either use techniques termed cellular patterning to directly position cellular components within the construct, or localize growth factors or other biomolecules within the device that foster specific cellular recruitment and directed vascular ingrowth from host tissue [3]. As we shall see these two methods can be used in conjunction with each other to strike a balance between the specificity of cellular patterning and the recruitment of the growth factor derived methods.

20.2 Expert Opinion

The field of Tissue Engineering is expanding rapidly. Every year more approaches are being developed to recreate an ever-expanding variety of tissues. We believe that the major, still unresolved challenges to successful clinical utilization of engineered tissue constructs are, in no particular order:

1. Immune reactivity and cell sourcing
2. Vascularization of engineered 3D constructs
3. Control of postimplantation remodeling
4. Short and long term regulation of cellular phenotype
5. The need for individualization to optimize performance among patients with varying comorbidities and demographic characteristics

Nutrient and gas exchange remains a critical requirement and a critical challenge. This should be among the first design questions addressed in developing tissue engineering strategies. As discussed above, general approaches to vascularization include either the engineering of a microvasculature in vitro, or the generation of a microvasculature early in the postimplantation period. Our understanding of the regulation of angiogenic processes is steadily improving, but remains far from complete. We have learned a great deal about initiating angiogenesis within in vitro model systems using a variety of angiogenic peptides, biomechanical stimuli, and microarchitectural patterns. However, we understand less about the regulation of neovessel stabilization vs. regression and about the control of spatial organization of engineered microvascular networks, particular in response to in vivo remodeling processes.

The induction of angiogenic mechanisms is greatly influenced by a host of variables relevant to tissue engineering. By way of example, the scaffold material, in part selected for its utility in the replacement of a specific tissue type, impacts neovascularization by virtue of its biochemical, its biomechanical, and its topographical characteristics. The incorporation into the tissue construct of other cell types, e.g.: smooth muscle cells (SMCs), hepatocytes, cardiomyocytes, etc., similarly impacts the induction, the distribution, and the durability of the neovasculature. Consequently, no single strategy for vascularization of engineered tissues is likely to be optimal for tissues in general.

A great deal of progress has been made in promoting microvascularization of engineered tissue constructs in vitro, and recent studies have shown promising results in selected short term in vivo models. However, it is imperative that we establish a more integrated knowledge of the effects of the large number of variables of construct design, intended implant location, and patient-related variables (age, diabetes, nutrition, steroid use, etc.) on the development and durability of microvascular networks either engineered in vitro or induced in vivo following construct implantation.

20.3 Biology of Neovascularization

Neovascularization can occur via two mechanisms, vasculogenesis or angiogenesis. Vasculogenesis is the de novo formation of vascular structures from the assembly and differentiation of precursor or progenitor cells (i.e., angioblasts). This typically embryonic developmental process, until recently, was difficult to emulate in either in vivo or in vitro tissue engineering applications. Angiogenesis is the process whereby new vascular structures are derived from preexisting blood vessels [145]. Angiogenesis is the mechanism of vascular development most tissue engineers have utilized when attempting to neovascularize tissue engineered constructs. Angiogenesis typically proceeds after an external stimulus destabilizes quiescent vascular structures. Subsequently, endothelial cells can differentiate into tissue/substrate-invading processes termed angiogenic sprouts, form lumens, and finally stabilize these newly formed structures. Although vascular supporting cells or pericytes (i.e., SMCs and fibroblasts) play an important role in stabilizing vessels, endothelial cells are critical to tissue vessel invasion and lumen formation. Therefore, endothelial cells shall be the main focus of our discussion and any descriptions of other cell types shall be in terms of how those cells influence neovascularization.

20.3.1 Mechanism of Neovascularization

Angiogenesis in vivo commences with the degradation of the basement membrane, retraction and removal of pericytes, and remodeling of perivascular matrix proteins. This process is termed vessel destabilization. After endothelial cells and pericytes experience angiogenic stimuli such as hypoxia or vascular endothelial growth factor (VEGF), they upregulate the production of angiopoietins. Angiopoietins (Ang) are a family of cytokines that bind the extracellular endothelial cell-specific Tie receptors. When Ang-1 binds its Tie-2 receptor it causes transphosphorylation and subsequent downstream activation of several intracellular protein kinase pathways to promote endothelial cell survival and migration [30]. Ang-2 acts as a competitive antagonist of Ang-1, by both destabilizing the cell-cell contacts and preventing Tie-2 receptor phosphorylation and signaling [140, 158]. Additionally, proliferating endothelial cells have a tendency to disrupt their cell-cell contacts contributing to further destabilization [5]. Coordinated Ang stimulation is also important for the survival of the destabilized vessel. Without other stimulation it can induce endothelial cell apoptosis and vessel regression, but in the presence of VEGF it promotes endothelial cell survival and further vessel sprouting [107].

The endothelial cell's angiogenic invasion across the basement membrane, which serves partially to stabilize mature vessel morphology, and through the surrounding matrix is required for microvascular development. Matrix and basement membrane proteins are sensitive to degradation by serine proteases (i.e., plasmin and matrix metalloproteases or MMPs). Activated endothelial cells increase expression of surface urokinase receptors that bind the inactive proenzyme and convert it to the active form. Active urokinase then promotes the conversion of plasminogen to plasmin [118]. Plasmin then degrades fibrin, fibronectin, and laminin while activating MMPs, a large diverse group of proteases capable of degrading a variety of matrix proteins [116, 125].

Angiogenic sprouting occurs after pericytes recede, exposing underlying endothelial cells. Endothelial cells form filamentous processes by migrating, proliferating, and degrading matrix proteins in an organized fashion toward the source of the angiogenic signals. If there are growth factors trapped within the scaffolding matrix, then scaffold degradation can also locally control sprout direction. The process of invasion and matrix degradation in turn serves as a feedback loop propagating angiogenic invasion. The exact direction an angiogenic sprout travels is determined by three main processes. These include chemotaxis, haptotaxis, and mechanotaxis. These are defined as cellular movement directed by soluble chemokines, scaffold-bound attractants, and

mechanical stresses, respectively. The sprout's direction and cellular movement are ultimately determined by the summation of these signals' intracellular signaling pathways. There is an interconnection between integrin receptors and growth factor receptors via the intracellular proteins such as GTPases (guanine nucleotides). Originally believed to be just involved in cytoskeletal formation and organization, these proteins play an important role in cellular activation and movement [206]. Integrin receptors are involved in a number of growth factor associated pathways, via the stimulation of chaperone proteins. Their activation leads to the phosphorylation of focal adhesion kinases (FAKs) altering integrin protein structure, thus increasing cellular attachments to extracellular matrix (ECM).

Cellular movement toward an angiogenic stimulus is effected by intracellular signaling pathways contributing to the formation of actin cytoskeletal elements around focal adhesions. For example, VEGF, after binding its receptor, transduces a signal for chemotaxis intracellularly via a series of GTP binding proteins termed the Rho GTPases (e.g., Rho, RhoA, Rac, and Cdc42) and their associated kinases (e.g., ROCK). RhoA, Rac1, and Cdc42 regulate cell shape changes through effects on the cytoskeleton and cell adhesion [206]. This family of proteins is critical conformational changes within the actin cytoskeleton. Rho regulates the formation of actin stress fibers and cellular contractility. Rac is generally required at the leading edge for cellular extension and new adhesion formation, and Cdc42 controls the formation of cellular projections [143].

Sprouting endothelial cells move from a vessel's luminal surface, through degraded basement membrane, and into the surrounding matrix via the formation of filipodia and exert their own mechanical force through focal adhesions. Filipodia are cytoplasmic actin-filled membrane projections that form when a cell senses gradients of growth factors [49]. Focal adhesions are matrix-specific convergences of cytosolic structural elements (i.e., actin filaments) that occur along the cell membrane. Actin stress fibers connect to the substrate via a focal adhesion at one end and assembled through actin polymerization at the other end. After the growth factor binds its receptor, Rho GTPases such as Cdc42 are activated. Cdc42 stimulates cell polarity and the production of these actin projections along its axis of stimulation. The actin filaments lengthen by polymerization secondary to the activation of other intracellular proteins including phosphatidylinositol 3-kinase (PI3K) and Rac proteins. Polymerization then causes cytoplasmic protrusions, termed lamellipodia, at the cell's leading edge. Transmembrane integrins at focal adhesions are capable of sensing ECM then selectively stimulating either pathway, via Cdc42 or Rac activation. Focal adhesions are increased in fillipodia or lamellipodia and act as the point of traction for forward propulsion via the development of branching or filamentous actin fibers, respectively [143]. Stress fibers are actin filaments arranged in reverse and linked by myosin chains. They are elongated by the Cdc42/Rac activation and contracted with RhoA/ROCK. This arrangement allows the cells leading edge to maintain propagation while the trailing edge is able to pull itself from one focal adhesion to another. Mechanical stress, particularly fluid shear, can stimulate endothelial cell proliferation, movement, and orientation. The glycocalyx lining the cell's apical surfaces transduces external fluid shear via transmembrane integrin receptors. These receptors and mechanical stimulation will both be discussed at greater length below.

Lumenogenesis is the formation of endothelial cell lined chains of interconnecting compartments that permit blood flow from the preexisting vasculature into the neovasculature. Folkman and Haudenschild provided evidence that in the presence of angiogenic stimulation, endothelial cells can form capillary like structures with lumens [45]. They later suggested that this process occurs via intracellular vacuole formation and coalescence. These coalescing vacuoles form by $\alpha_2\beta_1$ integrin and Rho GTPase-dependent pinocytic mechanisms by which the plasma membrane and extracellular contents are internalized leading to vacuole formation [32, 34, 35, 38]. Concurrently, projections from endothelial cells sense and form junctional contacts with neighboring endothelial cells to form more complex multicellular and patent capillary structures.

Each step of neovascularization does not occur in the absence of another. For example lumen formation may occur concurrently with sprout formation and endothelial cell matrix invasion. Specifically, vacuoles may be visible in endothelial cells which are actively sprouting [33]. The exact mechanisms explaining how these steps in angiogenesis relate is still quite unclear. Although these processes likely occur as a continuum and not as discrete steps, we have described them as such to clearly point out their unique importance for vascular ingrowth and microvascular network formation.

Vascular structures must retain a shape that permits blood flow, possess vasoreactivity, and possess a selective degree of permeability. For example, VEGF can induce excessive neovascularization with chaotic and excessive vessel fusion [37]. Excess fusion can result in a phenomenon termed "vascular lakes" or structures that no longer retain cylindrical vascular shape and instead form large cavernous sinuses that do not promote laminar blood flow. To regulate intravascular pressure precapillary arterioles have vasoreactive or contractile properties. These features originate from SMCs within the vessel's media layer. Although there is some evidence that some small diameter (<4 mm) tissue engineered blood vessels display a degree of vasoreactivity [216], there has been minimal work evaluating the contractile properties of engineered microvasculature. Additionally, the appropriate display of surface proteins such as selectins (e.g., ICAM and VCAM) is necessary for cell-specific extravasation. To the best of our knowledge no work has yet analyzed the endothelial cell surface adhesion proteins and their cell specificity within a tissue engineered microvasculature. Furthermore, a vascular structure that has excess permeability may neither be able to adequately perfuse tissues nor be able to select an appropriate cell to move into surrounding tissue. VEGF has been studied extensively in the construction of microvascular structures. However, it has the potential to cause vascular structures to take on chaotic and leaky conformations. In fact, when it was originally discovered it was termed vascular permeability factor (VPF) [43]. A specific discussion of the microvascular functionality will be saved for a later discussion.

20.3.2 Angiogenic Growth Factors

The most commonly described angiogenic cytokines include VEGF [84], fibroblast growth factor (FGF) [197], tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β) [214], and the angiopoietins [183]. In vivo, these growth factors may be derived from an inflammatory state such as that which occurs after synthetic material is implanted. Understanding how the inflammatory mediators and the locally secreted cytokines foster wound healing, foreign body reactions and neovascularization is paramount to the development of functional implanted tissue engineered constructs. Although a thorough examination of how all these principles interrelate is beyond the scope of this chapter we will attempt to point out their broader intersections throughout our discussion.

VEGF participates in the regulation of many aspects of angiogenic invasion. It is an endothelial cell-specific cytokine capable of inducing migration, proliferation, and microvascular differentiation with sprouting and tube formation [84]. VEGF increases both vascular permeability and fenestration. Although posttranslational modification alters the cytokines availability and activity, all six known isoforms (membrane bound and secreted) of this heparin-binding cytokine are derived from a single gene's alternative splicing. VEGF promotes von Willebrand release, integrin expression, interstitial collagenase expression, and plasminogen activator (PA) and plasminogen activator receptor (PA-R) expression. Directly blocking VEGF with antibody significantly decreases microvessel growth, cellular proliferation, and capillary tube formation [18, 212]. Microvascular endothelial cells exposed to insulin release VEGF in a sustained autocrine fashion that can result in excessive neovascularization [106]. Vessels created by sustained growth factor delivery (i.e., VEGF) have a tendency to regress in the absence of physiologic demand or when tonic exposure ceases [41, 47].

VEGF is a potent angiogen that induces endothelial cells to take on an invasive phenotype in both fibrin and collagen hydrogels [65, 173]. VEGFs clinical applicability has been greatly improved by a greater understanding of its absorbance and release kinetics within these extracellular matrices [153]. Attaching soluble VEGF isoforms to fibrin matrix proteins promotes both sustained and controlled release. Subsequently, microvascular growth results in more organized, linear vascular networks with substantially less vessel branching and tortuosity [39] (Fig. 20.1). Ruhrberg et al. [149] found that matrix-bound VEGF (via heparin binding) provides spatially restricted stimulatory cues that polarize endothelial cells and thereby guide angiogenic sprouting and vascular branch formation. To accomplish this, they engineered mouse embryos that did not express VEGF in their matrix or those whose VEGF was solely matrix-bound. Animals that lacked matrix-bound VEGF demonstrated fewer but larger blood vessels. Those animals that overexpressed the matrix-bound VEGF demonstrated thin highly branching vascular structures that were present even in ectopic sites. VEGF localization in the extracellular

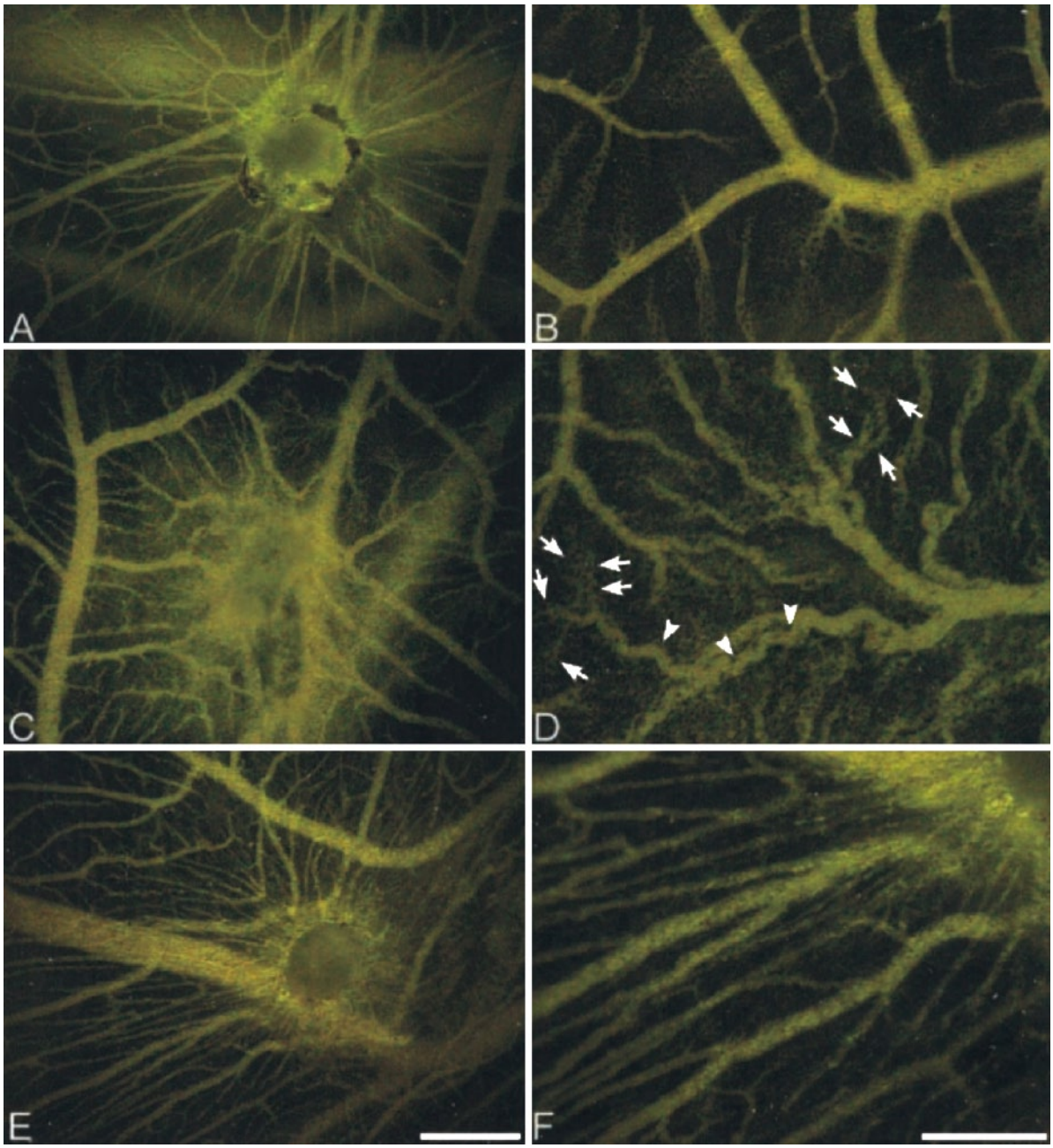

Fig. 20.1 In vivo video fluorescence microscopy of $VEGF_{121}$-induced vascular networks. Fibrin gel matrices formulated with 5 μg α_2PI_{1-8}-$VEGF_{121}$ or 2 μg native $VEGF_{121}$ were grafted atop embryonic day 9 chicken CAMs. Representative still video images show CAM vasculature perfused with FITC-dextran 48 h after grafting. (**a**, **b**) Responses to control grafts made of fibrin alone. (**c**, **d**) Fibrin formulated with free-diffusible native $VEGF_{121}$. (**e**, **f**) Fibrin formulated with matrix bound α_2PI_{1-8}-$VEGF_{121}$. Release of both $VEGF_{121}$ forms profoundly increased densities of arterial/venous and capillary vessels. However vessel hierarchy as well as vessel morphology of CAM microvasculature induced by formulations of α_2PI_{1-8}-$VEGF_{121}$ in fibrin appeared much more normal compared with those induced by freely diffusible $VEGF_{121}$ release. Bars = 1 mm (**a**, **c**, and **e**); 0.5 mm (**b**, **d**, and **f**) (*White arrows*) demonstrate small tortuous capillaries. Reprinted with permission from Ehrbar et al. [39]. © 2004 Lippincott Williams & Wilkins, Inc.

space provides a control point for regulating vascular branching pattern. Although VEGF typically binds to matrix proteins with heparin linking molecules, we shall later describe engineered variants that can also binds fibrin directly.

FGF was originally described as an isolate from bovine central nervous tissue (i.e., pituitary for type 1 and hypothalamus for type 2) and received its name for its profound stimulation of fibroblasts in culture [52]. It is a family of potent pro-angiogenic growth factors directly involved in endothelial cell migration, proliferation, differentiation, and sprouting [71, 80, 97, 123]. FGF has 22 recognized isoforms. However the two most commonly described are FGF-1 (i.e., acidic fibroblast growth factor or aFGF) and FGF-2 (i.e., basic fibroblast growth factor or bFGF) [90]. Both have been described in angiogenic invasion and construct neovascularization [41], but highlighting their specific differences is beyond the scope of this chapter. There is a known synergistic effect on angiogenic potency when VEGF is combined in vitro or in vivo with these FGF polypeptides [99, 208] (Fig. 20.2).

As with VEGF, manipulating the absorbance and release kinetics of FGF has augmented its applicability. FGF is produced by a variety of cells including endothelial cells, macrophages, SMCs, and fibroblasts. Once secreted, the cytokine may serve to stimulate its secreting cell in an autocrine fashion or bind to ECM in close proximity to vasculature [196]. Heparin sulfate is also typically found co-localizing in peri-capillary tissue with FGF. However, unlike VEGF, FGF binds with a relatively high degree of affinity to matrix compounds such as fibrin with saturation without cross-linking molecules [154]. Heparin and heparan sulfate proteoglycan both promote FGFs effects on cell proliferation. Additionally both molecules serve a protective function by preventing proteolytic degradation (i.e., trypsin, thrombin, plasmin), of the cytokine [104, 148]. FGF is important for angiogenic development and has been suggested as a critical component for the long-term survival of vascular endothelial cells within a 3D culture system [157]. As we shall see later, unique modifications in FGF allow for resistance to degradation and thus prolonged availability for the development of durable capillary formation.

TGF-β at some concentrations is a pro-angiogenic growth factor that has been shown to stimulate endothelial cell differentiation and tubule formation [156]. It is one of the many substances released from α-granules during platelet degranulation within the fibrin coagulum in wounds and around implants. It is a potent recruiter of inflammatory cells, such as macrophages, which subsequently orchestrate the local inflammatory response and foreign body reaction [135]. It should be noted that although in vitro TGF-β is inhibitory to angiogenic sprout formation at high concentrations, at

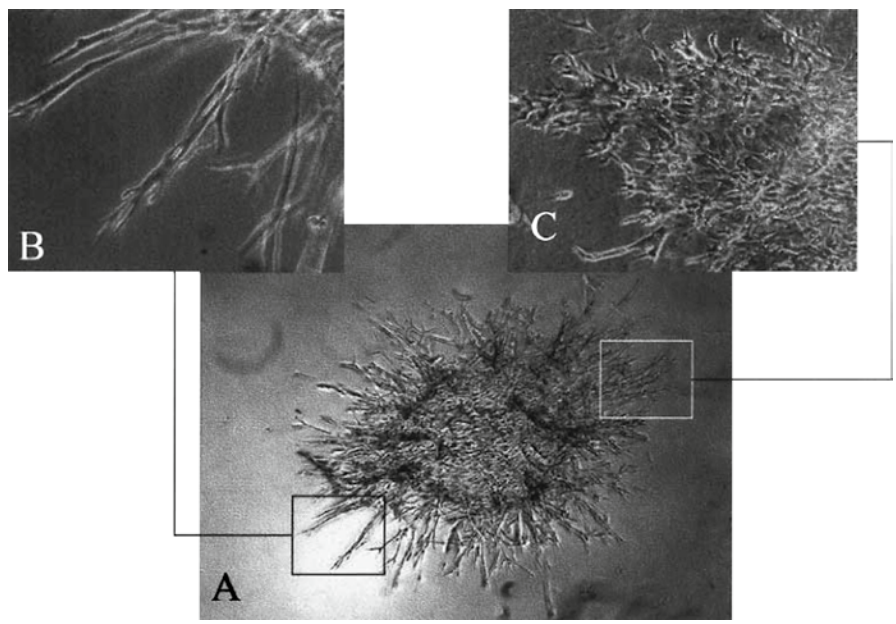

Fig. 20.2 Angiogenesis under inverted microscope. (**a**) Assay disk was exposed to 100 ng/mL FGF-1 plus 100 ng/mL VEGF. Pictures were taken at day 4 (original magnification 4×). (**b**) Endothelial cell sprouting (original magnification 4×). (**c**) Capillary network formation (original magnification 20×). Reprinted with permission from Xue and Greisler [208]. © 2002 Elsevier

low concentrations in vitro and in vivo it is pro-angiogenic. This dichotomy is owed in part to the low concentration-dependant recruitment of leukocytes that possess their own cascade of proangiogenic growth factors [100]. When platelets become entrenched within a fibrin clot they secrete TGF-β into the surrounding matrix. As such, it is slowly released in a degradation demanded fashion. Specifically, when plasminogen is converted to plasmin to remodel or degrade an established clot, TGF-β is released. The liberated cytokine is then free to both promote local cellular differentiation and inhibit cell migration [144]. TGF-β also stimulates cells to produce PA inhibitor, an inhibitor of the enzymatic activation of plasmin. This step serves as a method to regulate growth factor release and fibrin degradation. TGF-β is one of the most important cytokines regulators of fibrin degradation and endothelial cell proliferation, migration and capillary tube endothelial cell in vivo [100]. Although TGF-β has five isoforms (i.e., 1–5), TGF-β_1 has been the most extensively described in vitro and in vivo. TGF-β is an important counterpoint to the other proliferative cytokines. In order to produce not only angiogenic sprouts but also functional capillary-like tubules, endothelial cells must decrease proliferation and increase differentiation. TGF-β at low concentrations fosters differentiation. At higher concentrations cellular differentiation ceases. As such a critical balance must be struck between proliferation and differentiation.

TNF-α, or cachexin, is a protein secreted by leukocytes and macrophages during inflammation such as that which occurs secondary to the implantation of graft material. It has a broad array of functions ranging from stimulation of granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin-1 (IL-1) synthesis to endothelial cell stimulation in vivo. In vitro, it inhibits cell proliferation while promoting tube formation [85]. Perhaps TNF-α's greatest role in angiogenesis is its ability to increase endothelial cells' gene transcription. It induces human endothelial cells to increase the transcription of both proteolytic enzymes and cell surface adhesion sequences. In vitro, TNF-α increases the secretion of urokinase type plasminogen activator (uPA), a potent fibrinolytic enzyme critical for vascular invasion of fibrin matrices, and several MMPs (i.e., MMP-1, 3, and 9) [60, 194]. In fact, when human dermal microvascular endothelial cells (HDMECs) are cultured on the surface of fibrin hydrogels and subsequently induced to invade into the hydrogel with FGF and VEGF, no capillary tube differentiation occurs until the sprout structures are exposed to TNF-α [87].

20.3.3 Cell/Matrix Interactions in Neovascularization

ECM is a general term that refers to the collection of biological chemicals and proteins that intertwine to form a solid but porous environment in which cells reside. The ECM provides structural support to tissues, transmits mechanical stimulation, and stores cytokines and other mediators of cell signaling. Cells that reside within a particular matrix interact with it via contacts termed integrins. These receptors convey information to a cell pertaining to the biochemical identity of the matrix and the mechanical forces exerted on the matrix. Each receptor type in turn is capable of initiating a specific intracellular signaling cascade that is individual to the type of signal received by its receptor. These signaling pathways induce the cell to change its behavior (i.e., proliferation, synthesis, migration, etc.). Specifically, capillary morphogenesis is dependent on the composition of the ECM [33]. Ultimately microvasculature development is secondary to the endothelial cell's interaction of forces and biomolecules within its own environment [195].

When integrins bind a protein they recognize a particular portion of the peptide sequence termed the binding motif. For example, the vitronectin receptor on endothelial cells is termed $\alpha_v\beta_3$. This integrin receptor has been well described on invasive endothelial cells, and has been demonstrated in healing wounds and granulation tissue [9, 17]. $\alpha_v\beta_3$ is nonspecific to endothelial cells and is also present on other cells including fibroblasts. Although the vitronectin glycoprotein receptor, $\alpha_v\beta_3$ recognizes a variety of proteins, it most notably recognizes the protein motif arginine-glycine-aspartic acid (RGD) present in vitronectin, fibronectin, and several others as well. This unique attribute of the $\alpha_v\beta_3$ receptor has been exploited to design cell-binding peptide sequences within synthetic scaffolds [102, 111]. As will be discussed below, these adhesion peptides have been employed to pattern the microvasculature within tissue engineered constructs. Furthermore, additional peptide motifs confer greater

specificity to endothelial pattering (i.e., plasma fibronectin variant motif REDV (Arg-Glu-Asp-Val)) [112].

20.4 Engineering Microvascular Networks

20.4.1 Cellular Patterning

Cellular patterning is the process that aims to specifically position cells within a scaffold in the hope of ultimately determining their relative position within a final tissue's ECM. To pattern microvasculature one must have the ability to specifically select endothelial cells and adhere them to a construct with a relatively high degree of spatial fidelity. This task is quite daunting considering the intricacy of capillary networks. Cell surface peptide sequences have proven to be a useful means of cellular identification. These proteins can be covalently linked to matrix proteins or scaffolding materials in the hope of localizing cells to the embedded adhesion sequences.

Chen et al. [22] demonstrated that an endothelial cell's contact with ECM greatly determines its growth, proliferation, and overall survival. Cells cultured in a nonadhesive environment with individual fibronectin coated islands demonstrated matrix binding and integrin signaling regardless of matrix island size. However, a reduction in binding area resulted in cellular apoptosis. Cells unable to spread demonstrate cell cycle restriction and remain in S phase. Additionally, linear patterning of fibronectin reduced cellular spreading and fostered the formation of quiescent, luminal capillary tube like structures, restricted to areas of patterned ECM [36]. Furthermore, the patterned ECM also determined the cellular response to angiogenic and chemotactic factors such as platelet derived growth factor (PDGF) and FGF [134]. Thus, the tissue engineer gains another level of patterning fidelity. There is however a theoretical likelihood that the addition of pro-angiogenic factors will result in tissue development and proliferation in un-patterned locations when a relatively nonadhesive substrate is used to pattern a microvascular network.

Cells bind the ECM via integrin receptor binding of unique peptide sequences. RGD recognizes the integrin receptor $\alpha_v\beta_3$ [112, 150]. This peptide motif is the minimal sequence required for cellular adhesion, and in vitro it is capable of adhering cells to a substrate and promoting cellular spreading [61, 137]. The RGD sequence was originally described in fibronectin. However the sequence occurs in other ECM compounds (i.e., vitronectin, collagen, VWF, and fibrinogen). Implanted engineered constructs are exposed to all blood borne cellular and molecular components including bone marrow derived circulating endothelial progenitor cells. The RGD sequence immobilized onto the construct (formulated into a cyclic RGD), has been reported to attract endothelial progenitor cells from circulation and bind them in a shear resistant manner [4].

Amino acid substitutions in the RGD sequence and changes in adjacent amino acids alter the specificity of peptide binding. Typically, a fourth amino acid including serine (S), threonine (T), or valine (V) enhances cellular adhesion [63]. Hubbell et al. [67] designed an endothelial cell specific attachment factor Arg-Glu-Asp-Val (REDV). The fibronectin derived REDV peptide was shown to bind endothelial cells but not fibroblasts, vascular SMCs, or platelets. The REDV peptide binds the endothelial cell integrin $\alpha_4\beta_1$ [112]. This specificity is derived from the α_4 integrin recognizing REDV while α_v recognizes RGD exclusively. This matrix modification gives the tissue engineered added specificity when culturing endothelial cells into a tissue construct.

Mann et al. [110] noted a key limitation to cellular adhesion motifs. When endothelial cells and vascular SMCs bind a peptide adhesion motif, they often produce less ECM. It was proposed that matrix production was mediated by an adhesion event that the binding sequence abrogates. Without the production of matrix, proteins constructs engineered by this method could lack the physical characteristics that matrix proteins confer to tissue and be in jeopardy of structural failure. The same group also found that these effects could be ameliorated by the addition of pro-synthetic growth factors such as TGF-β and subsequently they were able to hold these cytokines in conjunction with peptide adhesion sequences [109].

There is a great deal of interaction between growth factor signaling and integrin receptor expression. Besides sharing several intracellular signaling pathways, growth factors such as TGF-β increase integrin expression [27, 51]. For example, FGF induces

endothelial cell migration by up regulating integrin $\alpha_v\beta_3$ and thus promoting additional ECM interaction [165]. VEGF up regulates integrins $\alpha_v\beta_3$, $\alpha_1\beta_1$, $\alpha_2\beta_1$, and specifically blocks $\alpha_1\beta_1$, reducing VEGF signaling [163, 164]. These interactions are critical because the addition of growth factors stimulate cellular migration, proliferation, and differentiation both directly as mentioned previously and indirectly by fostering matrix binding and thus activating many of the same intracellular activation pathways (i.e., IP3, cyclic adenosine monophosphate (cAMP), GTPase, etc.).

20.4.1.1 Effects of Scaffold Materials on Neovascularization

The scaffold material serves as the physical template for both cellular material and matrix proteins. As will be discussed in greater detail below, the body typically uses an inflammatory response to both degrade and replace or to isolate construct material after implantation. The tissue engineer can specifically utilize these responses to induce in the host both matrix production and neovascularization following implantation of the construct. Although dependent on intermediary steps, synthetic materials offer variety of options (i.e., degradation characteristics), which may be used to foster a constructs microvasculature formation.

Scaffolds can be of either biologic or synthetic material. The decision regarding which to use depends on specific attributes both present within the material and required by the tissue construct. Biologic scaffolds typically comprise collagen, fibrin, elastin, and other ECM compounds (i.e., hyaluronic acids, fibronectin, and glycosaminoglycans) that serve an accessory role in structural modification. Typically these materials are organized as 3D hydrogels. Hydrogels are linked water-soluble biologic polymers that have strength characteristics that are similar to soft tissues. Their high fluid content and porosity makes them efficient at transporting nutrients, wastes, and for peptide delivery. Synthetic materials may also be formed into hydrogels, and in that application they do have some notable advantages over their biologic counterparts. Where biologic materials may have inconsistencies from lot to lot, synthetic materials can be reliably manufactured. Synthetic materials including polyesters can be produced in diameters equivalent to naturally occurring fibers via processes termed electrospinning or leaching (described below). Additionally, synthetic hydrogels, with comparable porosity to biologic hydrogels, can be patterned to foster vascular network formation and chemically altered to prefer endothelial cell ingrowth to other cell types.

Collagen has been used as a biologic scaffold for tissue engineered constructs and wound repair. Collagen is well suited for tissue engineering applications because it readily incorporates into host tissues, is biodegradable, and is relatively weakly antigenic [94]. It can be formed into a number of configurations depending on the tissue application. For example, type-I (fibrillar) collagen has been formed into either sponges or hydrogels. Sponge formation from lyophilized collagen requires ultraviolet irradiation crosslinking for this otherwise amorphous hydrogel to promote cellular ingrowth [121]. Collagen sheets derived from decellularized cadaveric skin have also been described in wound repair and organ scaffolding [215]. The addition of specific cellular components (i.e., endothelial cells, keratinocytes, osteocytes, fibroblasts, and SMCs) and accessory ECM molecules (i.e., glycosaminoglycans and elastin) has also been described. However, the collagen biopolymer has also been criticized for many reasons. For example, there is a great deal of variation between production batches and its arginine side chains interact with platelets. Collagen initiates platelet adhesion and aggregation promoting thrombus formation [120]. Thus, it is both potentially hemostatic and potentially thrombogenic. Collagen is found extensively throughout the body, including vascular structures, as a structural protein. It has been used biomimetically to form implantable hydrogels that in vivo subsequently displays both capillary self assembly and microvascular invasion [201]. Fibrin is another biopolymer used extensively in tissue engineering applications and used in both wound healing and as a surgical sealant. It is an attractive material for use in tissue engineering because it is biocompatible and stimulates both endothelial cells and pericytes. It also increases pericyte proliferation as demonstrated by bromodeoxyuridine (BrdU) incorporation into DNA synthesis, cell count, and cAMP production [176]. Fibrin readily adheres to cells and can be derived from a patient's own blood supply thus removing the risk of a foreign body reaction to the engineered substrate [77]. Fibrin hydrogels induce cellular synthesis of cytokines that are secreted locally. For example, fibrin-based dermal

replacements comprising fibroblasts and keratinocytes secrete more VEGF into their culture supernatant solution than their collagen-based counterparts [65].

Fibronectin is a high molecular weight glycoprotein commonly found in both plasma and a variety of extracellular cellular matrices. It is also the prototypical matrix protein studied in relation to cellular integrin receptors. Integrins are transmembrane heterodimeric (α and β subunits) protein chains that transduce environmental signals, including ECM composition, to intracellular signaling pathways. Capillary formation is augmented when cells are cultured on malleable surfaces such as matrix compounds that bind integrin receptors. Specifically, cells seeded on plates of increasing fibronectin density demonstrate poor cell viability and rounding with low density and spreading and growth with high fibronectin density. Although it is not a load bearing protein, when cells bind fibronectin they may display some cellular retraction and differentiation into branching tubular networks [71]. It should also be noted that similar effects were present when varying the amounts of matrix proteins such as gelatin or type IV collagen. The variety of biological compounds that gives rise to neovascularization offers some insight into the complexity of native artery formation (Fig. 20.3).

Synthetic alternatives to the aforementioned biological proteins have been used to develop microvascular networks. Poly-α-hydroxyacids are a group of widely studied polyester compounds that have been used for several decades in the form of absorbable suture and graft materials. The degradation of these polyesters results in the generation of acidic species, which may result in local inflammation. Factors that determine the rate at which these substances are resorbed include molecular weight, the presence of co-polymers, site of implantation, and local stress [203]. Although polyglycolic acid (PGA) is rapidly degraded within the body (<3 months), resulting in relatively rapid biomechanical weakening, it can be formulated with other copolymers to foster structural characteristics that make it more suitable for specific tissue requirements. Polylactic acid (PLA) undergoes hydrolysis at a lower rate, and polylactide-co-glycolide copolymer (PLGA) embodies characteristics of both polymers [44]. Sponge made from these synthetic materials used for microvessel ingrowth can be manipulated to alter both porosity and degradation characteristics [66, 218]. Although these polyesters do not directly bind cellular components, after some hydrolysis the surface binds serum proteins and cellular material. Additionally RGD peptide adhesion sequences may be added to the surface directly or through the inclusion of other polymers to promote further endothelial cell adhesion [209]. These adhesion sequences may serve one of two functions. They either retain the endothelial cells within the construct allowing them to locally proliferate and form vascular structures after the addition of growth factor, or they serve as a pattern for microvascular invasion.

Polyethylene glycol (PEG) is the prototypical cell patterning substrate. It is a synthetic biopolymer hydrogel that is extremely nonadhesive to both proteins and cells. PEG structures are produced by polymerization via photoiodination (i.e., photopolymerization). The hydrogel features created via photolithographic photopolymerization of PEG diacrylate demonstrate great fidelity for larger features >200 μm. However features <80 μm have also been described. Besides structural feature formation, cellular encapsulation is also possible by this same method. It is also important to note that the radiation required to assemble PEG hydrogels by these methods does not reach cytotoxic levels. Besides its exquisite patterning resolution, PEG is well suited for the formation of microvasculature because of its binding specificity. It is a typically nonreactive substrate that does not readily bind most cell types. As such, it is a "blank slate" that when patterned with endothelial cell adhesion sequences will effectively only bind endothelial cells. When combined with growth factor stimulation PEG can confer a high degree of fidelity when patterning the microvasculature with adhesion sequences.

Photopolymerization also provides the tissue engineer with several key advantages when designing microvascular networks with PEG hydrogels. This process allows the substrate to be patterned into 3D structures directly onto tissue surfaces, and allows the incorporation of adhesion peptides [59, 61], specific degradation sequences [162], and growth factors [109]. Once incorporated, these peptides are then released in a local cell-demanded fashion. These design features can be included individually or concurrently. If each feature is connected to a photolinking molecule, or photoinitiator, that is activated with a discrete energy intensity corresponding to a particular wavelength of light, then multiple feature types can be attached concomitantly [128, 151]. Thus, PEG can retain a high

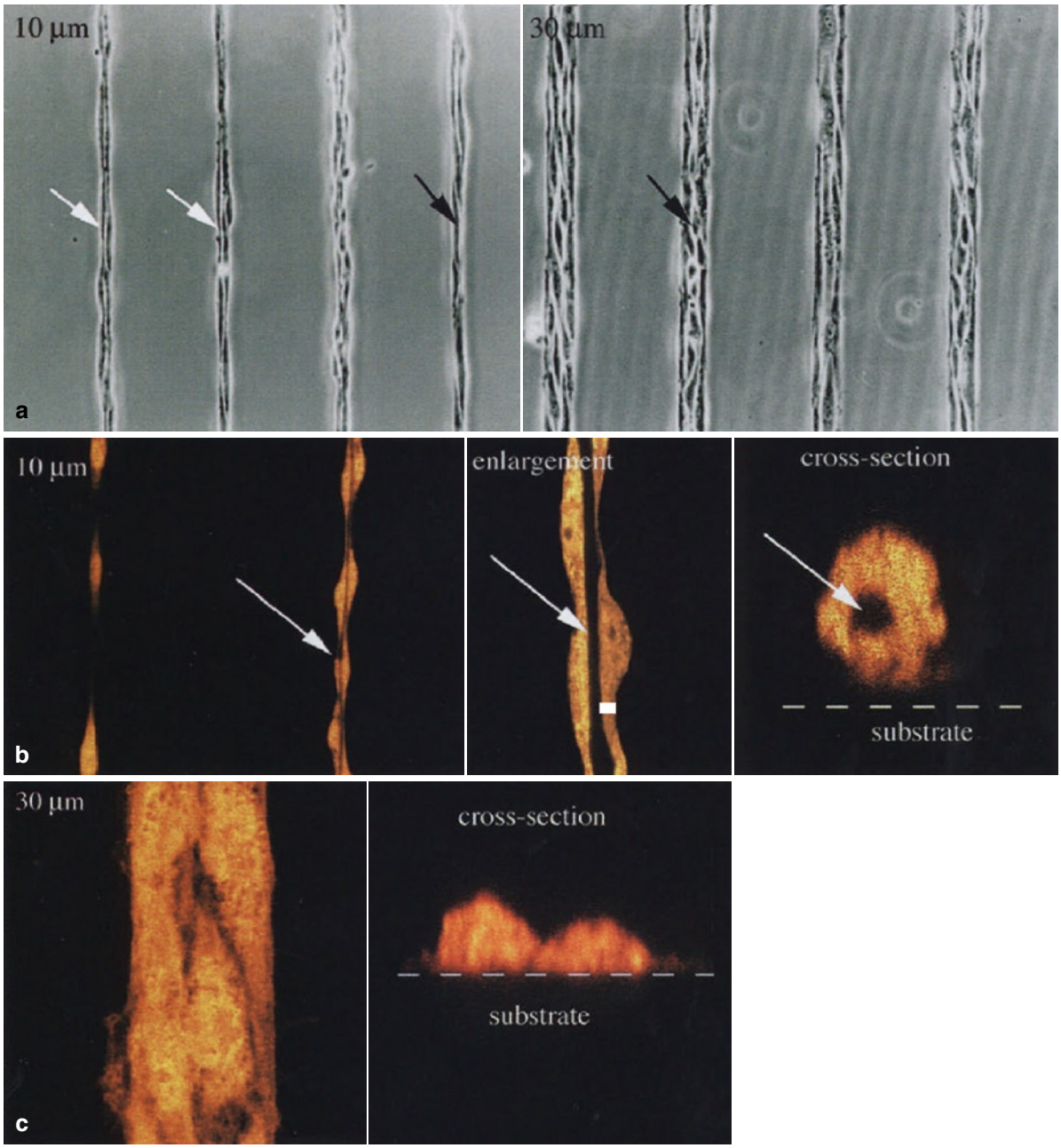

Fig. 20.3 Capillary tube formation on linear islands (**a**, **b**) and directional extension of motile processes on square islands (**c**). (**a**) Phase-contrast micrograph of capillary endothelial cells cultured for 72 h on an unpatterned region (*left*) or 10 µm lines (*right*) coated with the same density of fibronectin (X200). Note the capillary tube formation is limited to the linear islands. (**b**) Confocal microscopic images of fluorescein-stained cells cultured on 10 µm line showing a central cavity extending along several cell lengths when viewed in a horizontal (*left*: 1,000×) or vertical cross-section (*right*: 3,000×) (*White arrows*) demonstrate capillary structure with lumen formation. (*Black arrows*) arrows demonstrate capillary structure without lumen formation Reprinted with permission from Dike et al. [36]. © 1999 Springer

degree of fidelity for multiple cellular adhesion and growth factor patterns beyond the resolution gained by photolithography (described below).

Polyglycerolic sebacate (PGS) is a tough degradable biopolymer that, like PEG, does not result in a significant inflammatory response [200]. This material

allows the attachment of adhesion motifs such as RGD. This type of elastomer lends itself well to microfabrication processes that rely on artificially casting microvascular patterns. Although the material could potentially degrade over a prolonged period of time once implanted, initial perfusion of the construct is derived from the manufacturing of microchannels and not microvascular ingrowth from surrounding tissue. The patterned network then relies on the nonadhesive properties of the material or endothelial attachment from its adhesion motifs to prevent network thrombosis [44].

20.4.1.2 Microfabrication Techniques for Engineering Microvascular Networks

Endothelial cell culture in three dimensions differs from 2D culture due in part to cellular arrangement, mechanical forces, additional cell-matrix contacts, and proximity for cell signaling. These differences have been demonstrated by disparity of cellular activities between 2D and 3D culture systems in response to biochemical stimuli [13]. To accommodate for these differences, mathematical modeling has been employed to characterize the mechanical behavior (i.e., flow, pressure, distension, and hematocrit and rheologic characteristics) of the microcirculation to help scientists predict the optimal design of cellular patterning techniques. To get endothelial cells to form capillary-like structures in three dimensions is a complex process that has required increasingly complex methods of cellular patterning.

The process of fabricating biologic or synthetic materials into structures capable of capturing and fostering cellular growth in reproducible patterns is termed microfabrication. For the purposes of our discussion, this terminology refers specifically to the patterning of microvascular networks prior to implantation. Ideally, this process entails positioning endothelial cells and their supporting pericytes in an orientation that fosters the formation of a stable microvascular network that is capable of completely perfusing its target tissue. To accomplish this goal the tissue engineer may directly position cellular components in the construct or place cues that attract and adhere the cellular components after implantation.

Micromolding or microcontact printing is a type of microfabrication in which microelectromechanical systems (MEMS) technology, derived from microchip fabrication, is used to create complex patterns from molded patterned reliefs [12]. To create such small and intricate patterns, photolithography is typically employed. Briefly, lithography involves the etching of branching trench patterns onto a silicon or Pyrex substrate via chemical erosion or radiation exposure. To maintain resolution, a protective layer termed the resist is applied to the substrate prior to either the dry or wet etching methods. A positive photoresist is one in which ultraviolet light in the pattern of interest causes the exposed material to become soluble. After the removal of soluble components, the photoresist leaves an accurately deigned area of substrate exposed underneath and ready for etching. A negative resist follows all the same principles; however the resist substance becomes insoluble on exposure to ultraviolet radiation and remains soluble without it. Photolithography uses electromagnetic radiation shown through a patterned filter or resist onto the inert substrates (usually a silicon wafer, creating detailed and complex structures (channel resolution ~100 nm). Then either biologically active or inert materials are poured over these substrates to form a micromold relief. The molded relief then serves as the capillary-like channels for subsequent microvascular patterning. These channels are then covered with another layer of substrate to generate an enclosed capillary-like bed. Stacking micromolds has been suggested as one method to create 3D microvascular networks seeded with endothelial cells and other parenchymal cells [7]. Kaihara et al. [79] demonstrated that endothelial cells and hepatocytes could be successfully co-cultured on polymer substrates under constant flow. The resultant monolayer both retained its synthetic functionality and was capable of being folded into more complex structures.

Papavasiliou et al. [133] demonstrated an adaptation of noncontact photolithography to stack microchannels with high degree of resolution. Through a patterned photomask, an eosin photoinitiator and the dependant amine groups on the 2D PEG hydrogels were functionalized into a complex covalently bound 3D hydrogel. As such, a detailed three-dimensionally printed gel is built by sequentially stacking patterned hydrogels. 3D printing is the process whereby a series of patterned materials formed in the x and y plane are applied together in the z-axis to form a 3D structure. Each successive layer of the 3D structures is produced in the same manner as the previously mentioned

negative resist methodology. The summation of the layers results in a three-dimensionally printed network of microchannels suitable for microvascular ingrowth.

Laser lithography is very similar to photolithography, but has greater resolution in the *z*-axis. Where photolithography instantaneously emits electromagnetic radiation across an entire microvascular pattern, laser lithography typically uses confocal lasers to draw out the pattern with a single beam of radiation. The confocal laser can more accurately control the depth of radiation penetration. Similar to photolithography, the laser's radiation can be used to pattern a photoresist, used to create 2D and 3D surface patterns in hydrogels, or cell-adhesive monolayers [59]. More recently, two photon confocal lasers have been used to accurately spatially control the photoreactive process. Specifically, Lee et al. [96] demonstrated that cells, photopolymerized into collagenase-sensitive PEG hydrogels, will migrate out of the hydrogel along a designed pattern when a two photon laser is used to crosslink soluble cell-binding peptide motifs to that hydrogel. Most notably, the two-photon methodology retains the resolution of confocal photomasking with even depth penetration in the *z*-axis of the already formed hydrogel (from 80 to 430 μm for single and two photon, respectively). In essence, this technology is another improvement of cellular patterning through the use of adhesion sequences. However this improvement is significant for two reasons. First, the spatial precision of the laser crosslinking process allows for increasingly complex structure formation by attaching multiple sequences in different locations with a high degree of fidelity. This advantage can allow for the development of capillary structures alongside parenchymal cells in complex tissues where architecture is critical for ultimate function. Secondly, the deep laser penetration can pattern endothelial cell specific adhesion sequences within already assembled thick hydrogels to provide a "road map" for the invasion of microvascular networks deep into a hydrogel well beyond the 200 μm diffusion limit.

Laser-based micropatterning has also been developed to directly position individual or groups of cells without the use of adhesion motifs. Specifically, laser-guided direct writing, described by Nahmias et al. [127] involves positioning cells confined within a laser beam onto 2D surfaces or within biologically active hydrogels. Particles and cells, according to their refractive index, are pushed via the optical force exerted by a weakly focused beam of radiation into their final position. Importantly, this weak radiation does not have a statistically significant effect on cell viability. Of note, endothelial cells have a smaller refractive index than other cell types such as neurons. As such, they are more difficult to trap within a laser beam and experience less pushing forces when acquired. To overcome this limitation, endothelial cells may be cultured on VEGF-linked polystyrene beads. These cell-coated beads can be readily held in suspension over matrix proteins and directly pushed into place by a laser. Despite demonstrating that these cells can be cultured to form lumens, there are some limitations with this method. Laser-guided direct writing requires a great deal of time and effort to place an individual cell. Once the cell has been placed it is susceptible to migration while additional cells are being placed. Nonetheless, this work represents an initial step in high fidelity cell-specific microvasculature network formation.

Dip pen nanolithography (DPN) is another microfabrication technique borne out of nanotechnology and microelectronic manufacturing. DPN general has very high resolution and involves the use of an atomic force microscope (AFM) or scanning force microscope. These instruments achieve resolution at fractions of a nanometer. These microscopes work by dragging or tapping a cantilevered, extremely fine tip across the surface of interest. As the cantilever assembly moves in response to the surface, a laser-diode detection system senses the assembly's movement. A high-resolution map of the surface topography can then be generated. When AFM is used for DPN, the fine tip of the cantilever assembly applies a chemical to a surface much like a quill pen or dip pen applies ink to a writing surface. The number, orientation, and spacing of the cantilevered tips may be designed to specification. DPN can generate surface features with a resolution of 12 nm [190]. Although the degree of resolution may go beyond what the tissue engineer would require for individual cellular placement, DPN represents a high-resolution method for the direct application of biochemical substrates. DPN can be used to create microchannels similar to those mentioned above. However, this method may also be used to apply and detect proteins such as those that contain the peptide sequences RGD or REDV to the surface of biologic hydrogels. Thus DPN represents a potential method of patterning the microvasculature with an unprecedented level of resolution.

Magnetic culture techniques involve the addition of ferromagnetic particles to culture conditions. Once integrated into matrix material or cell structures, those particles may be influenced by external magnetic forces to form microvascular structures. Alsberg et al. [2] suggested that aligned fibrin fibrils could direct angiogenic invasion of hydrogels and microvascular patterning. They demonstrated fibrin fibrils aligned radially around magnetic microbeads and that microvascular cells then cultured on this patterned matrix demonstrated alignment along those discrete fibrils. Lin et al. [101] demonstrated that endothelial cells (umbilical vein and microvascular) bound to magnetic microbeads via an RGD binding sequence may be patterned using magnetic probes. The probe was capable of conferring specific shapes when these structures were grown in culture, and they demonstrated angiogenesis in vitro within both collagen and Matrigel. Ito et al. [74] demonstrated that endothelial cells and pericytes laden with internalized magnetic cationic liposomes could be patterned by a magnetic field. This technology has been used to grow individual cells, cellular sheets, and cord-like structures with precision shaping on low adhesion surfaces. Furthermore, once the magnetic field is released, the cellular structures are freely mobile to be re-positioned with or without magnetic assistance [72].

20.4.2 Recruitment of Microvascular Networks by Application of Angiogenic Biomolecules

20.4.2.1 Controlled Growth Factor Administration

Rather than directly placing cells within a construct, the tissue engineer can use growth factors and other biomolecular signals in a myriad of configurations to pattern and foster the development of a microvasculature within tissue. To develop these capillary networks, endothelial cell chemoattractants and mitogens can be used to induce endothelial cell invasion and then both proliferation and differentiation into intact capillary structures within the tissue of interest. The tissue engineer must decide if the growth factors are best administered systemically or locally. Systemic administration is much easier, but it typically results in more side effects than local administration. Additionally, local administration confers greater control in the actual amount of cytokine the tissue of interest receives. Next, one must decide if the growth factor will be released once en mass or administered at a lower sustained concentration from a storage source. The immediate delivery is termed a burst. Like systemic administration, higher concentrations of angiogens may be associated with aberrant arterial architecture. Finally, one must decide if the growth factor should be free to diffuse or be attached to a substrate. When attached to a substrate, its release or bioavailability is then related to the degradation of the substrate or the linkages that connect it to the substrate. Typically substrate adhesion results in even lower concentrations of growth factor delivery because the cytokines are administered in a cell demanded fashion. However, the tissue engineer must also ensure that the process of binding does not change the cytokine's activity. Once bound, some growth factors may undergo 3D conformational changes that alter the exposure of their cellular binding ligand.

The systemic administration of growth factors can augment neovascularization. However, there are a myriad of considerations and unwanted side effects common to systemic administration that make this an unfavorable form of angiogenic induction. For example, systemic administration of VEGF has been associated with hypertension, atherosclerotic plaque formation, and excessive leaky blood vessel formation. FGF systemic administration has been associated with vasodilatation, intimal hyperplasia, and hypertension [105]. In vitro the "systemic" administration of growth factor typically entails ubiquitous distribution that extends throughout the entire culture environment. Hudon et al. [68] demonstrated in vitro that within a cultured skin substitute, the addition of either bFGF or VEGF increases the number of capillary tube structures per cross-sectional area. Davies et al. [31] demonstrated porous polyurethane scaffold neovascularization and microvessel ingrowth with VEGF (15, 150, 1,500 ng/mL). However, over the 42 day experimental course, copious growth factor was required to generate this angiogenic response. Furthermore, most angiogenic growth factors do not remain active in vivo for prolonged periods. They are subject to rapid degradation by enzymes within the circulation. Thus to achieve a specific effect, higher doses of the cytokine must be used in vivo to overcome its destruction. The scaffold itself can be used as a reservoir for growth

factor administration. For example, VEGF has been incorporated into 3D leached porous scaffolds comprising PLGA copolymer with and without mineralization ($CaCl_2$/alginate). Mineralization was performed to analyze the suitability of this type of construct for bone tissue vascularization. These matrices were shown to slowly elute VEGF over 15 days with 70–80% of the VEGF eluted out of the material by 10 days [126]. The eluted VEGF stimulated human microvascular endothelial cell proliferation, and subsequent work demonstrated that when these constructs were subcutaneously implanted into immunodeficient mice there was 260% increase in mouse microvascular construct ingrowth [136]. Later microspheres of similar composition (i.e., 85:15 lactide-to-glycolide in PLGA) were used to elute VEGF, and they demonstrated similar results [172].

Microspheres provide additional options by allowing slow growth factor elution into a variety of tissues and scaffolds. The PLGA co-polymer microsphere has been employed within both ischemic and irradiated tissue. In both circumstances microspheres designed to slowly release VEGF demonstrated significantly more neovascularization and capillary formation than control scaffolds [78, 179]. More recently, this polymer was reformulated into a hydrogel with similar results in microvascular formation in ischemic tissue [171]. Besides PLGA, VEGF has been loaded within a variety of substances including calcium alginate microspheres, alginate beads, and chitosan albumin, and the exact concentration of VEGF to be administered varies with delivery method [105]. These benefits are not unique to VEGF. FGF incorporation into PLGA microbeads induced four times the microvascular ingrowth into rat peritoneal mesentery when compared to beads without FGF. Although there was no comparison between VEGF and FGF in angiogenic potential, this work demonstrates that there exists a benefit from the slow elution of angiogens directly into the tissue target of neovascularization. It is also important to note that vessels formed by stimulation with VEGF typically results in both abundant and unstable immature vascular capillaries. As we shall discuss in depth later, without the support of perivascular cells or sustained cytokine delivery from scaffold depots, immature capillaries are vulnerable to regression.

Biologic scaffolds can also be used as a supply for angiogenic growth factor elution. In nature, the ECM serves as a supply for growth factors. Cytokines secreted by cells that reside within tissue may bind the surrounding proteins either directly or indirectly. These compounds may also slowly diffuse through the material. Thermodynamic, electrostatic, and fluid movement through the matrix interstitices determines the rate at which these chemicals move through the intertwined matrix proteins. Once an angiogen elutes through a tissue it can signal cellular activation or angiogenic induction of any cellular material with which it comes into contact along the way. For example, FGF-1 incorporated into either fibrin or collagen hydrogels augmented both the epithelialization and angiogenic invasion of in rabbit ear wound model [132]. Additionally, FGF-1 has been incorporated into fibrin glue lining the surface of aortoilliac or throacoabdominal aortic interposition grafts [54]. Once in place, the fibrin slowly eluted the cytokine resulting in endothelial cell recruitment and adhesion to the graft surface.

Biologic materials may also serve as suitable microparticles for eluting angiogenic growth factors that promote tissue microvascularization. Gelatin beads containing FGF induce increased microvascular development when implanted into the subcutaneous tissue of mice when compared to empty bead controls [83]. Additionally, gelatin microbeads loaded with FGF and subsequently administered intramuscularly to rabbit gracilis muscle flaps generate an augmentation of capillary network density and perfusion [217]. This study noted that the local and controlled release of growth factor not only demonstrated more robust microvascular formation but also greater tissue viability. These results taken together suggest that either synthetic or biologic based substrates are capable of adequately administering growth factors in a sustained fashion to elicit robust microvasculature development.

Growth factor combinations represent another possible solution for the development of microvascular networks. Many of the same interactions that have been well documented in vitro exist when cytokines are concurrently administered in a controlled release fashion in vivo. Like in in vitro angiogenesis assays, VEGF and FGF act synergistically in vivo. Specifically, when they are administered within murine subcutaneous Matrigel implants they promote augmented microvascular ingrowth [99]. It should be noted that this advantage was greatest at 2 weeks (i.e., largest vascular volume and number of new blood vessels) of culture. However, this particular advantage was no

longer evident by 6 weeks as control groups caught up to treatment groups in both categories. Rophael et al. [147] demonstrated synergism in direct microvascular outgrowth from existing blood vessels for the combination of angiogenic growth factors VEGF, PDGF, and FGF. Besides augmenting initial microvascular recruitment, additionally growth factors may confer greater vessel stability. Peirce et al. [135] demonstrated that the combination of VEGF and Ang-1 was superior to VEGF administration alone. In this work, one or both cytokines were released from alginate microbeads within the dorsal skin fold of adult rats. When VEGF was administered alone there was increased microvessel number and length. However, this benefit was lost after 14 days when vessel regression began to occur, returning these values to control levels. The addition of Ang-1 maintained the increased vessel density through the end of experimentation (21 days). Similarly work examining the release of growth factors from porous bi-layered (PLGA) scaffolds demonstrated that VEGF alone yielded many chaotic immature vessels in an ischemic mouse limb. Once again, the addition of another cytokine, in this case PDGF within a separate PLGA layer, yielded more mature vessels that lasted 6 weeks in vivo [23].

Attaching a growth factor to a substrate or scaffolding material confers several advantages to the development of microvasculature. The cells recruited to the surface of the substrate may be selected by the specificity of the attached cytokine. Additionally, as the scaffold material degrades, more growth factor is released. Thus, as a cell invades or degrades the substrate more growth factor is released. In essence, the gradual degradation of the scaffold elicits a self-titrating release of cytokine. Furthermore, only locally released lower concentrations of cytokines are present within the recipient, essentially limiting the possibility of aberrant or excessive vascular growth at distant sites. For example, when growth factors are directly attached to implantable scaffolds, they demonstrate greater microvascular organization and direction within in vivo assays such as the chorioallantoic assays and hydrogels implanted into rat subcutaneous tissue [221]. VEGF and TGF-β are prime examples of growth factors that can be directly attached to hydrogels (i.e., PEG) surfaces and used to pattern 3D features [128, 202]. When attaching VEGF to PEG surfaces, the tissue engineer can control not only cell-demanded release of the cytokine but also the type of bond linking the growth factor to the substrate. Seliktar et al. [162] developed and demonstrated that a PEG–VEGF linkage could be formulated for cytokine release that is dependent on MMP-2 cleavage. Therefore, not only can the cell demanded-growth factor liberated be cell specific (i.e., VEGF) but the release of the growth factor can also be specific to cells that are locally secreting the protease required for its liberation. This method could potentially provide a very intricate means by which to pattern microvascular networks within biologically active hydrogels.

Greisler et al. [56] demonstrated that the addition of FGF-1 to the surface of synthetic materials, via a fibronectin-heparin linkage, elicits greater endothelial cell adherence and growth than substrates that lack the mitogen. Additionally, 1 week after applying FGF-1 to nondegradable graft material (i.e., Dacron) and degradable material (i.e., polydioxanone or PDS), the cytokine retention was 44% and 23%, respectively. Later work comparing fibrin glue to the fibronectin linkage system demonstrated that fibrin containing heparin and FGF-1 induced greater endothelial cell recruitment and retention than the fibronectin delivery system [57]. Additionally, when implanted in vivo, the fibrin delivery of FGF-1 and heparin induced significantly greater endothelial cell recruitment and microvascular invasion of the synthetic graft [54]. Additional work subsequently demonstrated that endothelial cells could also be selectively recruited to the graft material over other cell types (i.e., pericyte SMCs) by altering the heparin concentration [82, 167]. FGF bound to heparin and subsequently immobilized within cross-linked collagen matrices has also been used to promote endothelial recruitment, seeding, and neovascularization of engineered blood vessels [205].

Shireman and Greisler examined the rate at which growth factors are released from fibrin hydrogels and their mitogenic effect on endothelial cells. It was found that unbound FGF and VEGF exhibit initial burst of growth factor elution from the hydrogel over the first 24 h of culture. Specifically, 70% of FGF-1 and 56% of VEGF is eluted from fibrin glue within 24 h [167]. Subsequently, 66% of the remaining FGF-1 and 58% of the remaining VEGF were released by 96 h. Although this initial burst is significant in a closed in vitro culture system it may not have much benefit in vivo. In vivo the initial burst may be lost to the circulation and may not contribute to local endothelial cell adhesion and angiogenic invasion. After the initial burst release,

the rate of growth factor release was found to be heparin dependant. For example, at 96 h, hydrogels containing 5 units/mL of heparin released 58% percent of the VEGF that was loaded during fabrication, while hydrogels with 500 units/mL released 76%. Within this model, the quantity of VEGF loaded and the cell type overlying the hydrogel did not greatly alter the rate of release. Wissink et al. [204] examined the rate of release of FGF from cross-linked collagen hydrogels with and without heparin. The most notable differences between these studies included the use of collagen hydrogels and their subsequent crosslinking with *N*-(3-dimethylaminopropyl)-*N′*-ethylcarbodiimide and *N*-hydroxysuccinimide. In the first 6 h 30% of the growth factor was eluted from nonheparinized matrices and 2% from the heparinized matrices. At 28 days, the nonheparinized and heparinized gels had released 100% and 65% of their FGF, respectively.

Angiogenic growth factors can be covalently linked to biologic hydrogels. During coagulation thrombin not only cleaves fibrinogen forming fibrin monomers but it also activates factor XIII. The active factor XIIIa, a transglutaminase, cross-links the clot by creating an amide bond between glutamine and lysine residues on adjacent fibrin molecules. Schense and Hubbell [161] demonstrated that a growth factor could be attached to a fibrin hydrogel during this phase of coagulation. Specifically, growth factor peptides attached via a heparin-binding domain to a factor XIIIa substrate (NQEQVSP) within a bi-domain peptide can be enzymatically linked to the fibrin hydrogel in place of an amide crosslink. The heparin-binding domain, synthesized from antithrombin III, serves as a linking molecule connecting fibrin to heparin binding growth factors such as FGF, VEGF, and TGF-β [155]. These growth factors are then released in a cell-demanded fashion in which release is mediated by heparinases and plasmin activity. The type of linking molecule and its sensitivity to enzymatic degradation then determines the rate of release from the hydrogel [40]. This sustained controlled release of growth factors such as VEGF results in more organized microvascular network formation [41]. In essence, linking growth factors directly to fibrin matrices allows for slow, cell-demanded cytokine liberation similar to the PEG hydrogels previously discussed but in a biologic hydrogel.

Mutant growth factors are cytokines that have amino acid substitutions within their peptide sequence. This change can confer several advantages to the protein including alterations in activity and additional resistance to degradation. Growth factors can be engineered to take on specific properties that they do not exhibit in nature. For example, extensive work within the author's laboratory has demonstrated that engineered mutant varieties of FGF-1 can display heparin independence [210], resistance to thrombin degradation [168], increased mitogenicity for both endothelial cells and SMCs [210], and specificity for endothelial cells [211]. One mutant in particular represents a great opportunity to foster endothelial growth and development. R136K is a mutant of FGF-1 in which the amino acid arginine has been replaced by a lysine. This mutation confers relative endothelial cell specificity, heparin independence, and resistance to proteolytic degradation [42]. Each of these benefits represents a significant advantage when attempting to embed an angiogenic growth factor within a tissue engineered construct that will promote sustained growth factor functionality and mature microvascular network formation.

A promising strategy for localized and prolonged delivery of bioactive peptides to tissue engineering scaffolds links cytokines to collagen binding peptide motifs. Ishikawa et al. [73] described a fibronectin/collagen binding motif termed collagen binding domain (CBD) that was derived from a naturally occurring binding domain in Clostridium histolyticum. This peptide sequence coupled to epidermal growth factor (EGF) and placed in collagen gels or fibrillar collagen sponges displays high affinity to a variety of collagen subtypes (types I–IV), prolonged growth factor availability, and promotes faster epidermal wound healing when placed in wounds. The author's laboratory has examined if CBD would confer these same advantages and augment angiogenic sprout formation when ligated to mutant FGF-based growth factors. Combining both proteins allows for specific and cell-demanded administration of R136K. When compared to wild type cytokine, R136K-CBD had significantly superior binding to and retention on collagen. The addition of the CBD binding motif did not adversely affect the cytokine's effect on endothelial cell mitogenicity, chemotaxis, or angiogenesis as measured by thymidine incorporation, modified Boyden chamber assays, and pelleted endothelial sprout formation in fibrin disks, respectively [16]. Taken together, this compound could be patterned within a scaffold to specifically induce microvascular ingrowth while minimizing the need for growth factor or heparin replenishment. Thus, this chimeric mutant protein represents a

powerful cell-demanded tool for angiogenic patterning that the tissue engineer may use to construct a microvascular network.

20.4.2.2 Utilization of Mesenchymal Mural Cells to Enhance Microvascular Network Formation

Supporting cells are other means of providing growth factors and augmenting microvascular viability. Local growth factor secretion has a profound influence on endothelial cells and vascular structures that are immediately adjacent to the secreting cell. This paracrine stimulation can be used to augment capillary ingrowth and the sustained release of growth factor from the supporting cells also represents a means of supporting the developing microvascular structure. Fortunately, for the tissue engineer there are a variety of cells that locally secrete growth factor, and those that do not secrete levels high enough to be considered proangiogenic can be induced to do so.

Adipose tissue implanted subcutaneously often undergoes continuous volumetric loss post implantation. It has been proposed that the volume deficit is attributable to adipocyte death from both mechanical manipulation and ischemia secondary to slow revascularization of transplanted tissue [15]. Masuda et al. [113] demonstrated that adipose cells are intrinsically pro-angiogenic and produce high levels of VEGF. The subcutaneous transplantation of fragmented omentum with intact adipocytes and microvascular endothelial cells augments capillary development in vivo. It should also be noted that throughout the entire experiment (i.e., 12 weeks) the capillary density was greater than in the surrounding tissue, most notably on day 4 (233% greater).

When pancreatic islet cells are induced to secrete angiogens, the area around that tissue demonstrates microvascular invasion. During pancreatic cell transplantation typically less than 30% of transplanted cells are capable of engrafting and surviving [152]. This is due in part to limited neovascularization [219]. Islet cells are completely dependent on neovascularization for perfusion post transplantation. Islet cells, unlike adipocytes, do not naturally secrete high levels of angiogenic growth factors. However, when transfected with adenovirus either expressing angiopoietin-1 (Ang-1) or VEGF the cells locally secrete those gene product cytokines. After implantation into diabetic mice, the transfected islet cells induce greater microvascular density and subsequently better functional response to hyperglycemia and insulin administration when compared to controls [177, 219]. The addition of Ang-1 to microvascular networks formed in response to VEGF may play a pivotal role in the microvasculature's ultimate functionality, as Ang-1 has been shown to stabilize the permeability of leaky vessels formed in response to VEGF administration [191].

Reconstruction of burn wounds with skin substitutes has been limited by relatively poor graft survival. These failures were due primarily to the poor vascular supply to the dermal replacements. In an attempt to develop a microvasculature in vitro prior to implantation, Black et al. [10] cultured human umbilical vein endothelial cells, keratinocytes, and fibroblasts within collagen sponges in vitro. Successful reconstruction of dermal anatomy was due in part to the local secretion of VEGF by embedded keratinocytes. These microvascular constructs were later successfully transferred from in vitro culture to in vivo grafts. In vivo they were able to inoculate with the host microvasculature and provide perfusion [141]. Supp et al. [181] demonstrated that the keratinocytes used in skin substitutes could be cultured to overexpress VEGF via retroviral transfection. Subsequently, those skin substitutes induced a greater number of invading dermal blood vessels to enter the construct and subsequently decreased the time until neovascularization [182]. At 14 weeks they noted that the grafted tissue's vascular network did not exhibit pathologic transformation common to malignant tissues capable of secreting angiogenic growth factors. Later work demonstrated that the real benefit to the VEGF overexpression was augmentation of microvascular ingrowth from the immunodeficient mouse host wound bed and not the proliferation of engrafted human endothelial cells [180].

Overexpression of the anti-apoptotic gene, Bcl-2, is another strategy that has been employed to develop a microvasculature within engineered constructs. Overexpression of this gene confers cell viability by preventing involution of already existing vasculature in even poor culture environments (i.e., hypoxic or poor nutrient exchange). Bcl-2 transcription occurs in response to cellular binding of pro-angiogenic growth factors (i.e., VEGF, etc.) or may be generated by Bcl-2 gene transfection [129]. To promote the neovascularization of a skin substitute, Bcl-2 was overexpressed

in human umbilical vein endothelial cells that were seeded in acellularized dermis. The skin substitute had blood flow just below the epidermis 4 days after transplantation and after 2 weeks 75% of Bcl-2 expressing samples had both an epithelialized surface and a perfusing microvasculature [159]. Bcl-2 has been used to promote dermal microvascular endothelial cell incorporation into PLLA sponges. After Matrigel containing these cells filled the interstices of the PLLA sponges, the constructs were implanted into the subcutaneous tissues of SCID mice. These constructs exhibited a sustained fivefold increase in the number of microvessels and a fourfold decrease in the number of apoptotic cells when examined 7 and 14 days later [130]. Besides augmenting the ability of endothelial cells to neovascularize acellular dermis and abdominal wall implants, an overexpression of Bcl-2 is able to foster neovascularization of implants within ischemic hind limbs of immunodeficient mice [160].

Mature, stable capillaries typically comprise endothelial cells and pericytes. Pericytes are the cells that surround endothelial cells and support them by producing matrix proteins and secreting local mediators that contribute to both endothelial viability and proliferation. Pericyte recruitment and incorporation is generally considered necessary for the maturation and stabilization of capillary networks formed either in vitro or in vivo.

Pericytes assist endothelial cells in the formation of microvascular networks. For example, endothelial cells cultured in vitro together with vascular SMC-derived pericytes can be made to differentiate and form capillary sprouts with longer average sprout length and sprout durability. Co-culture angiogenic induction of endothelial cells by either SMCs or fibroblasts is at least partially due to the local secretion of soluble factors such as VEGF by these cells which, in a paracrine fashion, induces endothelial plasminogen expression, cordlike structure formation, and mitogenesis [91, 122]. Additionally, the local secretion of growth factors and ensuing augmentation of angiogenic activity is more pronounced in 3D culture. For example, fibroblasts grown in 3D culture on PLGA co-polymer scaffolds demonstrate a 22-fold increase in the expression of VEGF mRNA than those that are grown in monolayer culture, and the conditioned media from the 3D culture stimulate endothelial cell proliferation, integrin $\alpha_v\beta_3$ expression, and vessel formation in chorioallantoic membrane assays [220]. In co-culture, both SMC-derived pericytes and the endothelial cells alter their genetic expression and synthesis of growth factors. For example, co-cultured SMCs increase their gene expression of VEGF, PDGF, and TGF-β, and co-cultured endothelial cells significantly increase their expression of VEGF [62]. These in vitro findings have been supported by in vivo observation. Specifically, mitotically active pericytes within a tumor microvasculature extend to greater lengths than their trailing endothelial cell counterparts. These findings suggest that pericytes play a role in both angiogenic induction and guidance of endothelial invasion [131]. Additionally, Levenberg et al. [98] demonstrated that mural cell components improved in vitro vascularization of cardiac myocytes seeded on synthetic scaffolds and promoted their in vivo microvascular survival after subsequent implantation into immunodeficient mice. This study demonstrated that co-culture (i.e., endothelial cells and myocytes) and, to an even greater extent, tri-culture (i.e., endothelial cells, myocytes, and fibroblasts) promoted endothelial cell adhesion, capillary lumen development, and lumen expansion within the tissue over solitary endothelial cell culture.

Pericytes also promote the formation of microvascular networks through the stabilization of existing networks. For example, capillary-like structures formed in vitro from endothelial cell/SMC co-culture are less responsive to destabilizing and pro-angiogenic cytokines such as VEGF and Ang-2 [88]. Pericyte stabilization may be mediated in part by the production of Ang-1 and Tie-2 receptor phosphorylation [1, 142]. Pericytes can also produce tissue inhibitors of metalloproteases (TIMPs), which serve to regulate the activity of a number of MMPs. Regulation of MMPs in turn decreases several angiogenic processes including matrix invasion [92]. Koike et al. [86] demonstrated that human umbilical vein endothelial cells grown in co-culture with 10T1/2 murine mesenchymal precursor cells in collagen gels produce longer lasting functional microvessels. Similar microvascular results were achieved using adult dermal fibroblasts along with endothelial cells during the fabrication of endothelialized dermal tissue replacements [10]. Additionally, these microvessels can both remain patent and demonstrate functional vasoreactivity in vivo for over 1 year [3]. Work examining tumor microvasculature has demonstrated that microvessels that have not recruited pericytes are more vulnerable to endothelial apoptosis on cessation of tonic VEGF stimulation [6].

The importance of microvessel pericytes has been demonstrated in several attempts to prevascularize adipose tissue. Inosculation and perfusion of endothelial networks represent a means to supply the metabolic needs of adipose tissue. As such, much work has been undertaken to improve adipose tissue transfer volume and subsequently rapidly establish a microvascular supply. To do so endothelial cells must form a continuous lumen and connect to the host's arterial and venous vasculature. One approach used the in vivo co-culture of HDMECs and preadipocytes within fibrin hydrogels. Subsequently, the engineered adipose tissue developed a microvasculature that reached perfusion by day 7 within a chorioallantoic membrane model [14]. Another approach involves the application of adipose-derived whole microvessel fragments in immunodeficient hosts. When microvessel fragments are seeded within collagen gels and then placed in the subcutaneous tissue of immunodeficient mice, they inosculate with the host vasculature as early as day 1. These capillary structures have resultant blood flow at day 14 and a mature vascular network at day 28 [166]. This work demonstrates that pericytes' role in sprout augmentation and stabilization also extends to inosculation and vessel integration. Additionally, both microvascular cells and microvascular fragments can be directed via a micropatterned scaffold to orient developing microvasculature [20].

20.4.2.3 Dynamic Biomechanical Stimulation

The local environment around a cell greatly determines its angiogenic potential. Not only do local growth factors contribute to cellular induction but also the mechanical forces transmitted through the local environment. Forces external to a cell are transmitted intracellularly by matrix-integrin interactions [69]. Besides providing a means to connect cytoskeletal elements with external structures, the integrins are points of mechanotransduction including ion channels and membrane bound tyrosine kinases. Blocking these integrin receptors subsequently reduces intracellular signaling associated with mechanical stress [75]. Key to the process of mechanotransduction is the focal adhesions linking cytoskeletal elements and integrin receptors. Besides serving as points of contact where cells exert their force for locomotion, focal adhesions translate external mechanical stimulation or cellular distortion onto the cytoskeletal structure where cytoskeletal protein complexes integrate multiple signaling cascades.

When a force is applied to a cell through integrin-bound matrix proteins, the stress causes the alignment of intracellular actin filaments and myosin chains and their associated intracellular proteins. When cells are subjected to intralumenal shear stress, they experience forces on their apical surface or glycocalyx. The resultant deformation of the cell from flow then is transmitted to their basilar membrane focal adhesion attachments. The intracellular tension between the apical and basilar surface integrins then proceeds to cellular activation as discussed above. Fibers originating from focal adhesions orient key cellular machinery including tyrosine kinases, ion channels, inositol lipid kinases, and growth factor receptors [76]. Additionally, when mechanical movement and tension are transduced through a cell, two proteins necessary for intracellular signaling can be brought into close apposition, fostering signal transduction. Thus, extracellular stimulation can be rapidly transduced directly to individual molecules along cytoskeletal elements without the need for soluble chemical signals.

Endothelial cells exposed to shear stress have a signaling pattern that promotes locomotion. Shear stress promotes Rac activation resulting in actin polymerization and inhibition of RhoA. Thus, cells will develop lamellipodia in the direction of flow and exhibit contracture along their trailing edge. Shear stress activates two signal-transduction pathways within endothelial cells. The first, based on protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activity, is Ca^{2+} independent, while the other, phospholipase C activation, is Ca^{2+} dependent [192]. PKC is both ubiquitous and an intracellular messenger that is necessary for endothelial cell proliferation, endothelial cell migration, and capillary tube formation. Down regulation and blocking of these compounds lead to decreased migration and decreased response to cytokine induced migration, proliferation, and endothelial tube formation [213].

Cytoskeletal re-organization in response to the direct application of force to the cell represents a complex intracellular signaling sequence. Focal adhesions are organized subsequent to the activation of the GTPase Rho and its critical downstream activation molecules, Rho associated kinase (ROCK) and mDia [115]. ROCK stimulates cellular contraction by promoting

the phosphorylation of myosin light chains, which increases actin-myosin interactions and subsequent contraction. mDia promotes actin polymerization and regulates alignment of stress fibers with microtubules. Both ROCK and mDia control cytoskeletal rearrangements to provide scaffold stabilization. Additionally, mechano-sensitive ion channels are opened secondary to force applied to the cell membrane and integrin receptors. When activated, the channels open permitting calcium influx. The intracellular cytoplasm can then contribute to contractility and cytoskeletal reorganization [108].

When basement membranes are thinned, vascular structures are more susceptible to mechanical deformation. This mechanical sensitivity allows for increased transmission of local tensional forces to endothelial cells' intracellular actin cytoskeletons [70]. The cells are then able to proliferate and sprout in the direction of tension formation. Blocking cellular cytoskeletal tension decreases sprout formation whereas fostering it with Rho activation promotes sprout branching behavior [124]. Recently it has been suggested that aberrant sensation of mechanical stimulation and GTPase activity (i.e., Rho and ROCK) play a role in angiogenic stimulation of tumor capillary beds [50]. Tissue engineering has been applying mechanotransduction to in vitro culture environments for over a decade. Bioreactor systems have been employed to exert in vivo-like conditions to engineered constructs that are created in vitro. The dynamic culture environment preconditions or exposes the tissue to the physical forces (i.e., strain and shear stresses) that it will experience after implantation [64]. The mechanical stimulation fosters matrix production [185], cellular organization [220], MMP production, growth factor synthesis [146], cellular proliferation, and mitogenesis.

Mechanical stress either acts directly on endothelial cells or indirectly by increasing the local concentration of growth factor. Lee et al. [95] demonstrated that mechanical stimulation is capable of releasing growth factors trapped within hydrogels. Alginate hydrogels laden with VEGF were implanted into the subcutaneous tissue of immunodeficient mice. The hydrogels fostered microvascular tissue ingrowth, and this effect was more pronounced with intermittent mechanical stimulation. It should be noted that the mechanical stimulation also caused pulsatile release of growth factor from within the scaffold. Cheng et al. [24] noted that mechanical strain induces the release of FGF from vascular SMCs, and the quantity of release was proportional to the percent strain exerted on the cells. Pericytes also foster angiogenic invasion and stabilization under mechanical stimulation. Finally, TNF-α is capable of inducing both cellular proliferation and apoptosis, and endothelial cell exposure to shear stress significantly inhibits TNF-α's effect and even upregulates some anti-apoptotic genes [25].

Mechanotransduction of dynamic shear stress has been used to foster endothelial cell invasion into tissue engineered constructs. Takei et al. [186] developed a method to create microvascular tubes (diameter 550 μm) using collagen hydrogels seeded in vitro with endothelial cells. These tubules were subsequently exposed to luminal shear and/or VEGF. They found that endothelial cells invade their surrounding hydrogel when exposed to luminal shear (4.1 dynes/cm^2). Subsequently adding VEGF to the culture environment augmented the distance of invasion (from 250 to 800 μm) and the formation of capillary-like tubes structures within the hydrogels. Kang et al. [81] demonstrated that human umbilical endothelial cells cultured within a parallel-plate flow chamber on type-I collagen hydrogels could be induced to invade the biological substrate by exposing the cells to fluid shear stress. The shear stress also enhanced Akt and MMP-2 activation. Within this model, the invasion distance was proportional to the magnitude of shear, and the maximal shear stress-induced invasion was noted at approximately 5 dynes/cm^2.

Cyclic strain is also a potentially powerful stimulus for microvascular network formation. Cyclic mechanical strain has been shown to inhibit endothelial cell apoptosis [103] and increase cellular proliferation [178, 193]. Von Offenberg Sweeney et al. [198] noted that bovine aortic endothelial cells grown in a 5% strain at 1 Hz culture environment for 24 h demonstrated greater migration and capillary tube formation when seeded on type-I collagen gels as compared to those grown in static culture. This work highlights the need for exploration into the role strain plays in neovascularization. Work by Matsumoto et al. [114] also noted that uniaxial cyclic strain enhances endothelial cell proliferation, orients angiogenic sprout perpendicular to the direction of force application, and reduces sprout branching patterns. Finally work by Pietramaggiori et al. [138] (Fig. 20.4) demonstrated that within a rabbit ear wound model, cyclic mechanical strain is superior to tonic strain in the development of dermal microvasculature.

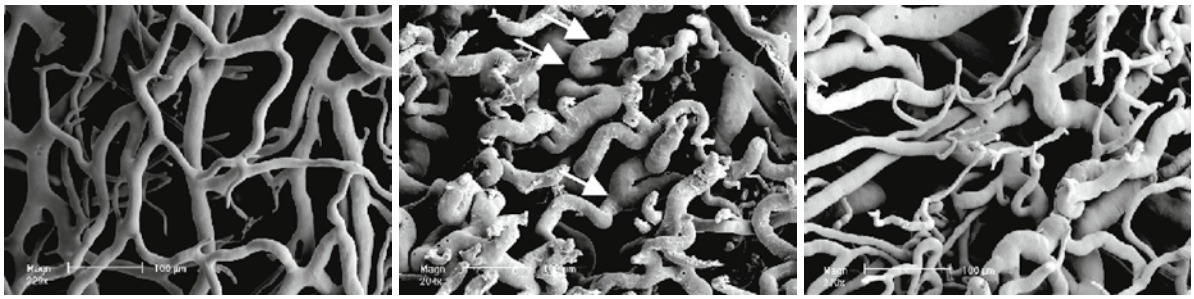

Fig. 20.4 Effects of mechanical force on vasculature of perfused tissues. Corrosion casting of the vasculature in a sham ear and postcontinuous and cyclic stretch. It is possible to notice how vessels change diameter size along their course (*single arrow*) and draw less rectilinear trajectories (*double arrow*). Reprinted with permission from Pietramaggiori et al. [138]. © 2007 Lippincott Williams & Wilkins, Inc.

Specifically, comparable levels of microvascular development and wound healing were seen at 4 days and 8 h for tonic stimulation and cyclic strain, respectively. Taken together this work would suggest that dynamic culture could be a robust angiogenic stimulus for microvascular network formation. Efforts to specifically address this topic via in vitro bioreactor systems are under way in the author's laboratory.

20.4.2.4 Postimplantation Remodeling and In Vivo Recruitment of Microvascular Networks

It is important to note that much of what we know of angiogenesis stems from in vitro work. Although in vitro models of angiogenesis are invaluable in attempting to understand the basic cellular and molecular mechanisms related to angiogenesis, translating these findings to an in vivo setting is challenging. Many of the same principles may not hold up within a physiologic environment. For example, as previously mentioned the angiogenic induction of the growth factor TGF-β varies between in vitro to in vivo. Additionally, Matrigel, an in vitro scaffold matrix derived from murine basement membranes, supports fibroblast capillary-like tube formation when these cells typically lack the intrinsic capacity to do so in vivo. Furthermore, in vitro microvascular networks typically form when endothelial cells are embedded within a scaffold material and exposed to one or two known pro-angiogenic signals (mechanical or biochemical stimulation). However, in vivo networks exist within complex, dynamic environments that are exposed to a myriad cytokines and mechanical stimuli. Thus it is difficult to determine the relative effect, if any, of a single growth factor within this dynamic environment until it is tested in vivo.

Implanted tissue constructs are subject to postimplantation remodeling secondary to local inflammation. The human inflammatory response is a complex system of cellular signaling sequences that protect the body from further insult after injury. These efforts typically involve walling off and/or degrading foreign substances within the body [19]. As neither of these actions would augment the functionality of implanted tissue replacements, great efforts have been undertaken to develop materials that elicit minimal inflammatory responses (i.e., PEG and PGS). However, no substance is truly inert [53]. Engineered substances such as PEG derive their relative inert qualities from poor protein and platelet adhesion. Although this represents a great reduction in coagulum and inflammatory mediator production, it may not stop the generation of some elastase and complement activation [174, 184]. Resorbable biologic materials may represent a superior alternative in microvascular substrate selection because of their ability to be hydrolyzed and replaced by native scaffolds. The constant turnover allows for the maintenance of structural integrity. Several attempts have been made to generate synthetic alternatives that perform in a similar fashion. However, a material that transitions seamlessly has not yet been reported.

After graft material is implanted, proteins from the recipient's serum typically coat it immediately. The types of proteins adsorbed along the construct's surface changes over time as some of the initial proteins are

eventually replaced in a process termed the Vroman effect [199]. Proteins such as fibrinogen lead to the formation of a coagulum composed of fibrin clot and platelets. While the fibrin clot remodels, other cell types are attracted to and invade this temporary matrix. These include endothelial cells, SMCs, and fibroblasts. The coagulum can continue to compact and remodel over days to 18 months [209]. However depending on the local inflammatory cytokine production or other signal sources the coagulum may serve as the basis for the formation of a dense, fibrous, and vascularized tissue aptly named fibrovascular tissue. Inflammatory mediators (i.e., IL-1 complement, thrombin, and fibrin fragments), platelets, and early arriving leukocytes such as neutrophils stimulate the chemotaxis and localization of monocytes/macrophages to graft materials. At the site of implanted material, macrophages then regulate inflammation, recruit fibroblasts that acutely and often chaotically deposit collagen and other matrix proteins, and orchestrate the subsequent tissue's neovascularization.

One of the oldest implanted synthetic materials, polyethylene terephthalate (PET) or more commonly known by its trade name Dacron, significantly induces macrophage recruitment and a large inflammatory response leading to fibrovascular invasion [57, 209]. To reduce the risk of construct thrombosis after implantation extensive efforts have been undertaken to promote continuous endothelial cell coverage of the luminal surface of these grafts.

In 1986, Clowes et al. [26] demonstrated in a baboon model that another porous synthetic material, expanded polytetrafluoroethylene (ePTFE), demonstrates neovascular ingrowth following implantation when expanded to a 60 μm internodal distance, unlike the lack of ingrowth into 30 μm ePTFE. Vascular casting of the constructs demonstrated clear microvascular structures penetrating the graft material. The microvasculature that invaded the local fibrovascular tissue subsequently seeded the lumenal surfaces of these grafts with endothelial cells (Fig. 20.5). The local inflammatory reaction secondary to construct implantation can be used to induce neovascularization. The tissue engineer can control the rate and duration of both scaffold degradation and the consequent inflammatory recruitment. Activated macrophages produce cytokines such as FGF and TGF-β, which may lead to endothelial cell recruitment and scaffold neovascularization. The relative amounts of these locally produced cytokines are determined in part by the material and more specifically by its rate of degradation [119]. The addition of the more stable lactide component confers stability because it is not hydrolyzed as readily. PDS grafts are less susceptible to hydrolysis and degrade over 6 months. Work by Greisler et al. [58] demonstrated that PLGA, PDS, and PET all stimulate a local inflammatory response, which modulates cell proliferation, fibrovascular invasion, and

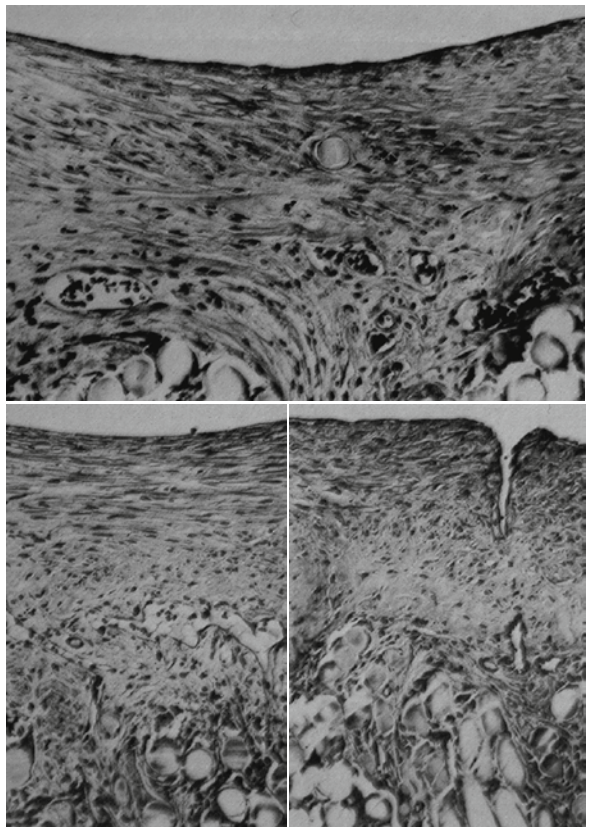

Fig. 20.5 (*Top*) Mid-portion of PDS Specimen at 2 months showing extensive capillary infiltration of inner capsule. Also seen are apparent transinterstitial myofibroblasts migration into the inner capsule and layering out of these cells into longitudinal and circumferential orientations and smooth luminal surface (hematoxylin-eosin, X250). (*Bottom left*) Midportion of PDS specimen at 2 months showing transinterstitial capillary invasion into the inner capsule perpendicular to luminal surface of specimen and its subsequent orientation parallel to luminal surface of specimen (hematoxylin-eosin, X180). (*Bottom right*) Midportion of PDS specimen at 2 months showing transinterstitial capillary invading the inner capsule and communicating with the luminal surface. Endothelial-like cellular luminal surface of the specimen appears to be continuous with capillary wall. Remainder of tissue resembles that seen in *top* and *bottom left* portions of this illustration (hematoxylin-eosin, X180). Reprinted with permission from Greisler et al. Arch Surg. 1987;122(6):715–21. © 1987 American Medical Association

capsule formation. The faster resorbing PLGA had a maximum local mitotic index after 3 weeks that was 4 times higher than PDS at 4 weeks and 26 times higher than the PET. At 12 weeks the PLGA construct had been completely replaced by fibrovascular tissue and all luminal surfaces were endothelialized. This result was unlike that of the PET construct, which did not degrade or exhibit endothelialization.

Faster resorption is not always superior in ultimate construct design. For example, when compared to PDS or PLGA/PDS mixtures, the faster resorbing PGA vascular grafts demonstrate greater mechanical deformation in high pressure perfusion environments (i.e., aneurysmal dilatation) [55]. This result was attributed to scaffold resorption occurring prior to ingrowth of sufficient fibrovascular tissue. Therefore, the tissue engineer must select scaffolding material that not only accommodates microvascular network formation but also resorbs at a rate compatible with the kinetics of tissue regeneration prior to the point of structural failure. One approach to this problem is to use composites of several materials. For example, to protect the structural integrity of PGA, in environments like the aorta where the physical demands make early degradation a serious risk, it can be combined with other more slowly resorbed or nonresorbable polyester compounds such as PDS or polyhydroxyalkanoate (PHA) [169]. PHA has high tensile strength and long-term durability. The combination of the two scaffold materials allows for neovascular ingrowth and structural durability.

PEGs inert characteristics and ability to bind specific cellular adhesion motifs make it a reasonable choice for a synthetic scaffold material when attempting to promote post implant formation of a microvasculature in vivo. However, for this substrate to fully incorporate tissue the initial material must degrade and resorb over time. PEG diacrylate can be made biodegradable by incorporation of the crosslinking peptide sequences Leu-Gly-Pro-Ala (LGPA) or Val-Arg-Asn (VRN) to form hydrogels that are susceptible to collagenase or plasmin degradation respectively [202]. Additionally, these degradation sites are specific to their particular enzyme's cleavage. PEG may also be made susceptible to MMP-2 degradation by formulating the cysteine rich PEG with a dicysteine protease sensitive oligopeptide (GPQGIWGQ) [162]. These types of cleavage sites are stable until the expression of their specific serine protease. The substrate remains intact, providing structural support until there is enough cellular invasion to initiate degradation. Cell demanded hydrolysis allows these hydrogels to be stable, biocompatible, and biodegradable. It is presumed that once enough cells have localized to degrade the scaffold there should also be enough cellular and matrix material present to structurally support the new tissue and microvasculature. More recent work has examined biologically based synthetic scaffold materials. Peptide sequences of argi nine-alanine-aspartate-alanine (RADA) have been used to formulate self-assembling 3D scaffolds. These fibers allow the tissue engineer to formulate matrices with nano-scale fibrous and porous topographies that mimic the configuration of natural matrix proteins. Networks of these compounds may be assembled with MMP-2 specific degradation sites also allowing for their gradual degradation and replacement [21].

Another more recent approach to developing microvasculature and fibrovascular tissue involves direct vessel exposure. Specifically, an in vivo technique described by Tanaka et al. [189] exposes vasculature and then surrounds the vessels with a noncollapsible chamber. The chamber both creates potential space and protects it from collapse. This space is then either filled with a specific angiogenic matrix (e.g., PLGA or Matrigel) [28] or subsequently filled with fibrin after the initial extravasation. Fibrovascular tissue then replaces the coagulum and the exact extent of the microvascular development is determined by the local administration of growth factor [188]. This chambered vascular pedicle demonstrated the greatest lumen microvascular outgrowth into fibrovascular tissue and implanted tissues (i.e., acellular dermis) when constructed in a flow-through configuration as compared to both loop graft or end vessel [187]. Biologic hydrogels form fibrovascular tissue, and laminin rich hydrogels favor adipogenesis [29]. In all tissue types there has been well documented microvessel formation at 2 weeks that extends from the vascular pedicle and supports tissue growth throughout the chamber volume of 12 mm diameter by 6 mm height [139].

20.4.2.5 Progenitor Cells

Vasculogenesis is a relatively new method of tissue engineered construct neovascularization. As previously mentioned vasculogenesis relies on the development of endothelial progenitor cells to differentiate

into mature vascular structures. Some progenitor cells located either within the bone marrow or in circulation, display surface markers such as CD31 like their mature endothelial cell counterparts. However, they often possess receptors more common to their hematopoietic lineage including CD133 and CD34. Progenitor cells can then be cultured either within in vivo or in vitro culture environments. One study evaluating the growth kinetics of endothelial progenitor cells indicated that within 40 days of in vitro culture a progenitor population could be grown to 10^{14} cells as compared to 10^{8} for differentiated microvascular endothelial cells [117]. For reference, the total number of eukaryotic cells in an adult human has been estimated at 10^{14}. Although this significant growth rate attenuated with increasing passage, the rapid increase in cell volume would be advantageous in replacing tissue in a timely fashion. The endothelial cells derived from progenitor cells have great potential for neovascularization. Mature endothelial cells derived from progenitor cells are capable of invading PLGA copolymer scaffolds in vitro when seeded with vascular SMCs [207]. Taken together, endothelial progenitor cells represent a promising strategy for neovascularization.

Progenitor cells have also shown great promise in neovascularizing tissue engineered substrates in vivo. For example, Kung et al. [89] demonstrated that acellularized dermis could be successfully vascularized by endothelial progenitor cells derived from peripheral blood. These cells were seeded with keratinocytes onto the dermis 3 h prior to transplantation onto the backs of SCID mice. There was microvascular growth and perfusion half way through the dermal tissue at 2 weeks that lasted the duration of the experiment. Of note, acellular controls only had minor tissue invasion at the graft edges by 4 weeks. Melero-Martin et al. [117] demonstrated that the injection of Matrigel into the subcutaneous tissue of immunodeficient mice revealed that endothelial progenitor cells could also produce a functional subcutaneous microvasculature within 7–10 days.

Melero-Martin compared human endothelial progenitor cells' ability to form microvascular networks in vivo to differentiated human umbilical vein endothelial cells and found no difference. The progenitor cells were, however, more likely to form microvascular networks than differentiated dermal microvascular endothelial cells. Sieminski et al. [170] reported different results in another comparison in vitro between endothelial progenitor cells, human umbilical vein endothelial cells, adult dermal microvascular endothelial cells, and neonatal dermal microvascular endothelial cells. Within collagen hydrogels, progenitor cells were superior to all other cell types in average length of angiogenic sprouts and percent lumen formation when stimulated by angiogenic stimuli such as VEGF, FGF, and phorbol myristate acetate (PMA). This work also noted that blocking integrin $\alpha_2\beta_1$ attenuated progenitor microvascular network formation while $\alpha_v\beta_3$ blockade did not have significant effect.

Progenitor cells also demonstrate greater sprout formation and cellular proliferation when they are grown in co-culture. Wang et al. [201] placed human endothelial progenitor cells within collagen/fibronectin hydrogels and implanted them within the cranial windows of SCID mice to demonstrate microvascular network formation. When grown in isolation, the progenitor cells quickly developed into vessel-like structures. However these did not inosculate with the host vasculature and subsequently regressed by day 7. When 10T1/2 were added with the endothelial progenitor cells to the hydrogel, perfused microvascular networks were seen after 2 days in culture (Fig. 20.6). After ligating the left anterior descending coronary artery, Bonaros et al. [11] injected human endothelial progenitor cells and skeletal myoblasts into the infarcted myocardium of athymic mice. They found that the presence of each cell type promoted the engraftment of the other. These findings suggested two-way paracrine stimulation by the local increase of VEGF and TGF-β between the progenitor cell types. Additionally, these cytokines were expressed at higher levels in the ischemic peri-infarction tissue.

Endothelial progenitor cells could potentially represent a tool that allows the tissue engineer to generate microvascularized tissue completely within the in vivo environment. This would remove some of the obstacles inherent in organogenesis including implant remodeling, degradation, and disintegration. There would be no foreign body reaction if all proteins and cells were derived from the host. However, work with progenitor cells is still relatively new. The tissue constructs we have discussed typically need angiogenic signals such as exogenous cytokines, co-culture, or local hypoxia to differentiate into a microvascular network. Further studies will be needed to assess the long-term viability of the microvasculature developed using this approach. Most importantly, while immunodeficient animals are

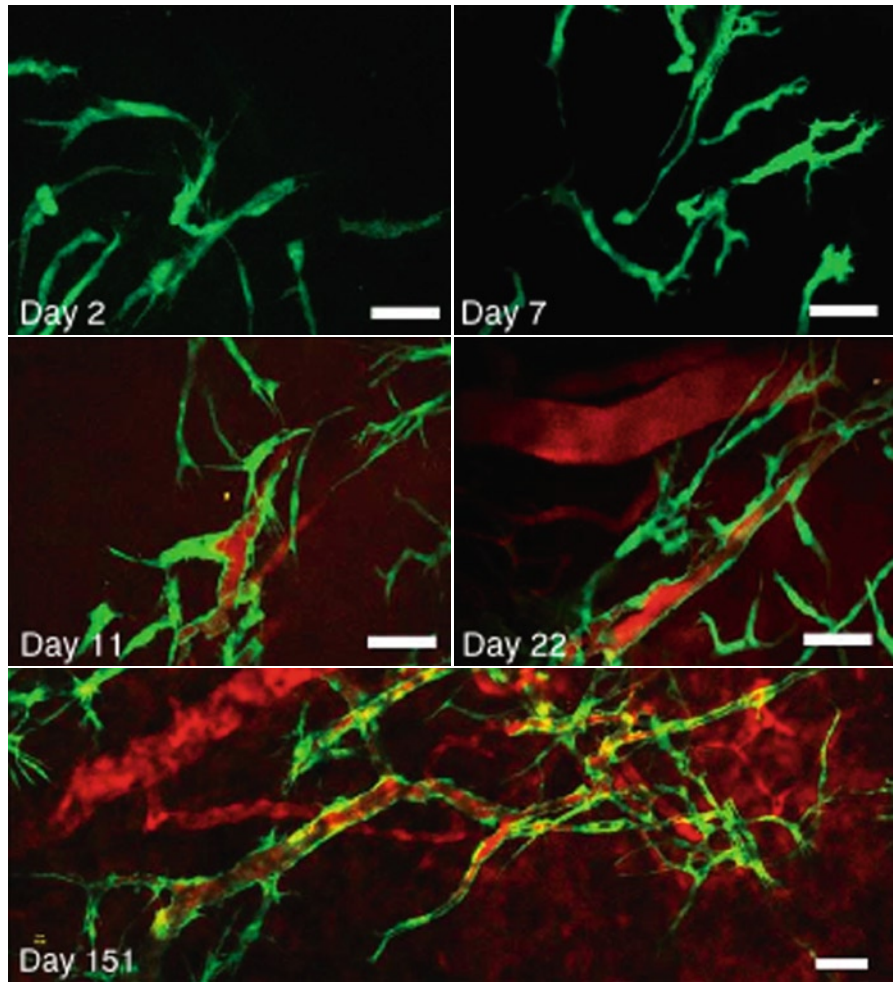

Fig. 20.6 Human embryonic stem (hES) cell-derived endothelial cells form functional vessels in vivo. hES cell-derived endothelial cells (GFP^+) were mixed with 10T1/2 in a collagen gel, and implanted into cranial windows in SCID mice. Images were taken at day 2, 7, 11, 22, 151 after implantation. After 11 days, rhodamine-dextran was injected into the tail vein to highlight perfused vessels. Engineered vessels were stable and functional for more than 150 days in vivo. Green, hES cell expressing enhanced green fluorescent protein (EGFP); *red*, functional blood vessels with contrast enhanced by rhodamine-dextran. Scale bar 50 μm. Reprinted with permission from Wang et al. [201]. © 2007 Nature Publishing Group

important models to study the in vivo angiogenic behavior of cells that would otherwise be subjected to immunologic rejection shortly after implantation, they do not demonstrate how an engineered microvasculature will behave in human subjects with an intact immune system and inflammatory remodeling pathways. Although studies thus far have been very encouraging, more investigation is needed to determine the ultimate utility of this new tool.

20.5 Five Year Perspective

Over the next 5 years, environments that foster development and multidisciplinary approaches to tissue engineering are likely to produce some very exciting results. Several techniques mentioned in this chapter and probably others not yet published should be employed in tandem to develop both a functional and long-lasting microvasculature.

Recent developments in our understanding of angiogenic growth factors and our ability to generate desired mutant and chimeric mutant angiogenic growth factors, and a growing number of potentially advantageous delivery strategies for them, should greatly enhance our ability to engineer the development of durable microvascular networks. As we have discussed, cytokines may be engineered to have matrix-binding components and mutations created to limit enzymatic degradation, enhance angiogenic potency, and increase endothelial cell specificity. These elements confer both patterning capabilities and allow delivery over longer periods of times at lower concentrations.

Co-culture models have also suggested useful strategies to be employed for microvascular development. As we have discussed, the addition of SMCs and fibroblasts, vessel fragments, and adipocytes have all shown to foster the development and long-term stability of angiogenic networks. Although we do not yet fully understand all the cell-cell interactions within these environments, the field of tissue engineering is gaining greater facility with intricate models, and increasing the complexity within a culture environment will likely offer more options potentially useful in fostering microvascular development.

Similarly, recent studies addressing the effects of biomechanical forces including shear stress and cyclic strain on the induction of angiogenic activity are of fundamental importance to the field. Angiogenesis is an extraordinarily complex process or, more appropriately, a group of interrelated processes as described above and it is quite probable that optimization of these processes will depend on recapitulating the in vivo environment with the use of biomechanically dynamic co-culture environments.

Another exciting recent development in tissue engineering is the use of progenitor cells in the development of microvascular networks. This group of cells, either derived from circulating blood, bone marrow, or adipocytes, represents a major step forward in the development of patient/self derived capillary networks. As endothelial cells are relatively highly immunogenic, this approach to cell sourcing could obviate issues of immune response. In both in vitro and in vivo settings these cells can expand rapidly and, in the presence of the right growth factor milieu and/or supporting cell types, differentiate into stable microvasculature. Endothelial progenitor cells can be readily isolated and expanded for use in vitro. However, also of potential utility would be the development of technologies that allow the homing of progenitors to an area of interest, followed by strategies designed to direct their differentiation and angiogenic development - a significant advancement for the field possible over the next several years.

20.6 Limitations/Critical Views

Vascularization of any 3D tissue construct is critical to the construct's viability, to the phenotypic characteristics of the viable cells, and to the structural and biomechanical integrity of the construct's matrix. All cells in vivo reside within 200 μm of a capillary network, but the density of that network, as well as the density of other cell types in the tissue, varies among tissues. Tissues with lower metabolic requirements, e.g.: cartilage, have lower density microvascular networks. With this information the tissue engineer may determine the relative size and density of the microvascular network that is required for their application, and then design a vascular pattern that will accommodate an appropriate level of nutrient supply and waste removal. The tissue's complexity requires the development of equally intricate microvascular structures. While our ability to initiate angiogenic mechanisms by which to generate a microvascular network has dramatically improved in recent years, we are just beginning to successfully control the 3D architecture of these networks and still have relatively little understanding as to how to control their long term durability and function in the postimplant remodeling environment and in the absence of chronic delivery of angiogenic peptides. Unfortunately, the tissue engineer has a finite level on control of the ultimate pattern of the microvasculature.

Successful incorporation of an engineered tissue construct occurs when a connection of microvasculature from host vessels to the region of tissue that requires perfusion is established. This can be achieved either by inosculation with the constructs' microvasculature engineered prior to implantation, or by postimplantation recruitment of invading capillaries. The latter process occurs naturally in areas of inflammation, but the newly formed microvessels quickly regress following the elimination of the inflammatory stimulus. As we have discussed above, approaches to engineer microvascular networks include cellular patterning and biomolecule patterning. In essence, these two approaches represent two different philosophical approaches to the same challenges. The first, cellular patterning represents the tissue engineer's control over individual cell types and occurs ideally when cells are aligned by adhesion peptides, microchannels, lasers, magnets, etc. into a configuration that supports the formation of capillary structures that are suitable for blood flow. These approaches are typically employed within an in vitro setting and allow the tissue engineer more direct control in the initial fabrication of the microvasculature. The other schema, biomolecule patterning, is an approach used in both in vitro culture and in vivo

environments. This technique has less control on individual cellular components but instead patterns the microvasculature on a larger scale. Growth factors that are directly added or liberated from matrix/microparticles, or released by adjacent cells recruit the cells of interest and then foster invasion into these constructs. Although this approach does not guarantee the formation of microvasculature within a specific configuration, Folkman and many scientists since have demonstrated the angiogenesis occurs in similar fashions under reproducible conditions. As discussed, recent developments in scaffold micro-architecture engineering allow a precise localization of selected attachment factors, growth factors, etc., by which to gain control over the 3D positioning of a recruited neovascular network. When growth factor patterning occurs within the in vivo setting the microvasculature is both recruited and remodeled concurrently. Conversely, the architecture of the precisely designed cellular-patterned structures undergoes remodeling after implantation. Thus, patterning the microvasculature, in its current state, entails a certain degree of randomness. Either vascular structures are defined initially and subject to remodeling or the structures are guided into place by the relatively less precise spatial control into the site where they will be required. Lacking total control in microvascular patterning may not adversely affect the ultimate application of a tissue-engineered construct. However, the tissue engineer should appreciate that the approach taken determines the order in which cellular components should be added to developing tissue, and currently there are limits on the resolution of microvascular network formation after implantation remodeling.

Tissue engineering the microvasculature is the key to an engineered construct's survival. Tissue necrosis has relegated the application of many "off the shelf" tissue reconstruction options to acellular scaffolds. To establish a pattern of microvasculature that is capable of supporting a variety of metabolically active tissues, biologic approaches appear to be the most readily accessible options at this time point. As discussed, the density and spatial organization of capillaries varies among tissues and should be recapitulated in the engineered tissue. Scaffold selection, based on requirements for different tissues, greatly impact vascularization potential, as do the presence of other required mesenchymal cells, the intended site of implantation, and critical variables intrinsic to the host into whom the construct is to be implanted. The multitude of complex hemodynamic and biomechanical forces, biochemical interactions, and cellular response under both physiologic and pathologic situations need to be considered in engineering the microvascular network and in predicting the long term viability and functionality of the engineered microvasculature.

20.7 Summary

Microvascular networks are critical for the development and preservation of engineered tissue. Survival is predicated on the requirement that any metabolic tissue must be within diffusion from its nutrient supply. Over the past 30 years many approaches have been undertaken to create continuous, patterned microvascular networks. Globally these approaches either rely or directly positioncells in a configuration that allows them form capillary structures or recruit the cells that will eventually comprise the microvascular structure to the site where they will be required. Recent work with modified growth factors, progenitor cells, dynamic stimulation, co-culture, and complex adhesion patterning have all demonstrated promising results for the development of designed, mature capillary networks. It is possible that a combination of techniques may be necessary to both develop the microvascular network and maintain its patency after implantation into the tissue recipients.

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Bone Tissue Engineering

21

J.C. Reichert and D.W. Hutmacher

21.1 Introduction

Bone is a complex, living, constantly changing tissue. Bone consists of cancellous and cortical bone. This architecture allows the skeleton to perform its essential mechanical functions. Cortical bone forms the outer layer of bone. This tissue gives bones their smooth, white, and solid appearance, and accounts for 80% of the total bone mass of an adult skeleton. The stiff cortical bone responds slowly to changes in loads and its main function is to support the body, protect organs, provide levers for movement, and (shared with cancellous bone) store minerals. Filling the interior of the organ is the trabecular or cancellous bone tissue, which is composed of a network of rod- and plate-like elements that make the overall organ lighter and allows room for blood vessels and marrow. Cancellous bone has a much larger surface area per unit volume and a greater rate of metabolic activity. The external surface of bone is covered by periosteum [42].

Periosteum and bone are connected by collagenous fibers referred to as Sharpey fibers. These fibers penetrate the entire cortex at sites exposed to high tensile forces directing fiber alignment. Microscopically, the periosteum consists of an outer, fibrous, firm layer (collagen and reticular fibers) and an inner, proliferative layer (cambium) which lies adjacent to bone and contains osteoblasts and osteoprogenitor cells. The outer fibrous layer provides elasticity and flexibility and facilitates the insertion of tendons, ligaments, and muscles. The cambium is capable of forming normal lamellar bone apposition on cortical bone that grows in width, and of forming primary, woven bone after a fracture.

The gross structure of a long bone can be divided into several regions (Fig. 21.1). The epiphysis defines the region between the growth plate or growth plate scar and the expanded end of bone, covered by articular cartilage. An epiphysis in a skeletally mature person consists of abundant trabecular bone and a thin shell of cortical bone. The epiphysis is the location of secondary ossification centers during development. The metaphysis is the junctional region between the growth plate and the diaphysis. The metaphysis contains abundant trabecular bone, but the cortical bone thins here relative to the diaphysis. This region is a common site for many primary bone tumors and similar lesions. The diaphysis is the shaft of long bones and is located in the region between metaphyses, composed mainly of compact cortical bone. The medullary canal contains marrow and a small amount of trabecular bone. The physis or epiphyseal plate is the region that separates the epiphysis from the metaphysis. It is the zone of endochondral ossification in an actively growing bone or the epiphyseal scar in a fully grown bone.

Bone has a rich vascular supply receiving 10–20% of the cardiac output. In long bones, one or two principal diaphyseal nutrient arteries represent the most important supply of arterial blood. These arteries pass obliquely through the cortical bone and divide into ascending and descending branches to supply the inner two-thirds of the cortex and medullary cavity. In case of dual blood supply, loss of one source of circulation can occur without adversely affecting the viability of the tissue.

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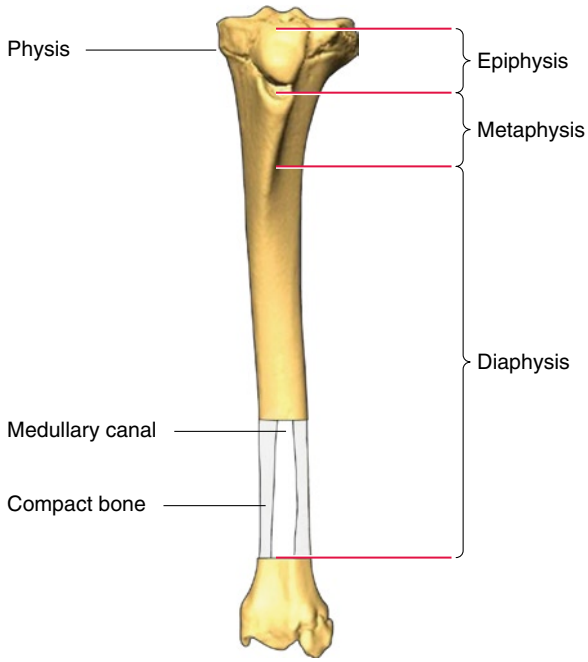


Fig. 21.1 3D reconstruction of an ovine tibia from CT data demonstrating the bone macrostructure

Numerous arteries supply metaphysis and epiphysis. These blood vessels mainly arise from the arteries that supply the adjacent joint, anastomose with the diaphyseal capillaries, and terminate in bone marrow, cortical bone, trabecular bone, and articular cartilage. In growing bones, these arteries are separated by the epiphyseal cartilaginous plates. Periosteal arterioles supply the outer layers of cortical bone and the periosteum.

The majority of bone mass is made of extracellular bone matrix. It consists of an organic component, primarily composed of type I collagen, which provides tensile strength and an inorganic component, primarily hydroxyapatite, providing compressive stiffness. Specialized populations of bone cells form, maintain, and remodel this matrix. Four types of bone cells are distinguished on the basis of their location, morphology, and function: osteoprogenitor cells, osteoblasts, osteocytes, and osteoclasts. Osteoblasts develop from undifferentiated cells while osteocytes form from osteoblasts. Osteoclasts arise from hematopoetic stem cells and develop from blood-borne monocytes. Monocytes are attracted to the bone matrix by chemotaxis triggered by a range of stimuli such as cytokines released by resident cells. These cytokines then stimulate monocyte differentiation into osteoclasts. The processes of bone modeling and remodeling require osteoclastic resorption of bone matrix and deposition of a new matrix by osteoblasts. Modeling shapes and reshapes bones during growth and stops at skeletal maturity. Physiologic remodeling does not change bone shape and consists of bone resorption and subsequent bone deposition in approximately the same location. As it continues throughout life, it appears to be important for the maintenance of the skeleton. Its exact function however remains unclear. Adaptive remodeling is the bone's response to altered mechanical conditions and may result in changes of strength, density, and shape. In recent years, the understanding of the processes associated with the control of bone cell function has increased significantly.

Trauma, tumors, infection, skeletal disorders, and bone disease often require surgical intervention and can result in large bone defects that need to be reconstructed (Fig. 21.2). Even though refinements in surgical techniques, implant design, and perioperative management have significantly improved the treatment of complex fractures and other skeletal defects, their reconstruction remains highly challenging [39, 67].

Over the years, bone grafts have advanced as the "gold standard" treatment to augment or accelerate bone regeneration [8] (Fig. 21.3). Traditionally, the following sources are used for bone augmentation:

1. Autologous nonvascularized bone graft: Cancellous or cortical bone taken from the iliac crest, distal femur, or proximal tibia. With their osteoconductive, and -inductive properties, autografts unite all characteristics positively influencing bone growth. However, autografts are limited in supply and associated with donor site morbidity and risk of infection [62].
2. Autologous bone marrow: Sterile aspiration of marrow from the posterior iliac crest. Marrow derived progenitor cells can then be isolated and expanded in vitro.
3. Autologous vascularized bone graft: Since the 1970s, vascularized bone grafts have gained a significant role in the reconstruction of complex skeletal lesions. Because of its anatomic characteristics, the fibula allows harvest of long vascularized diaphyseal of met-epiphyseal segments for transplantation and has therefore been applied the most. Vascularized bone grafts participate in the process of callus formation and are capable of actively reacting to mechanical stresses and remodeling. However,

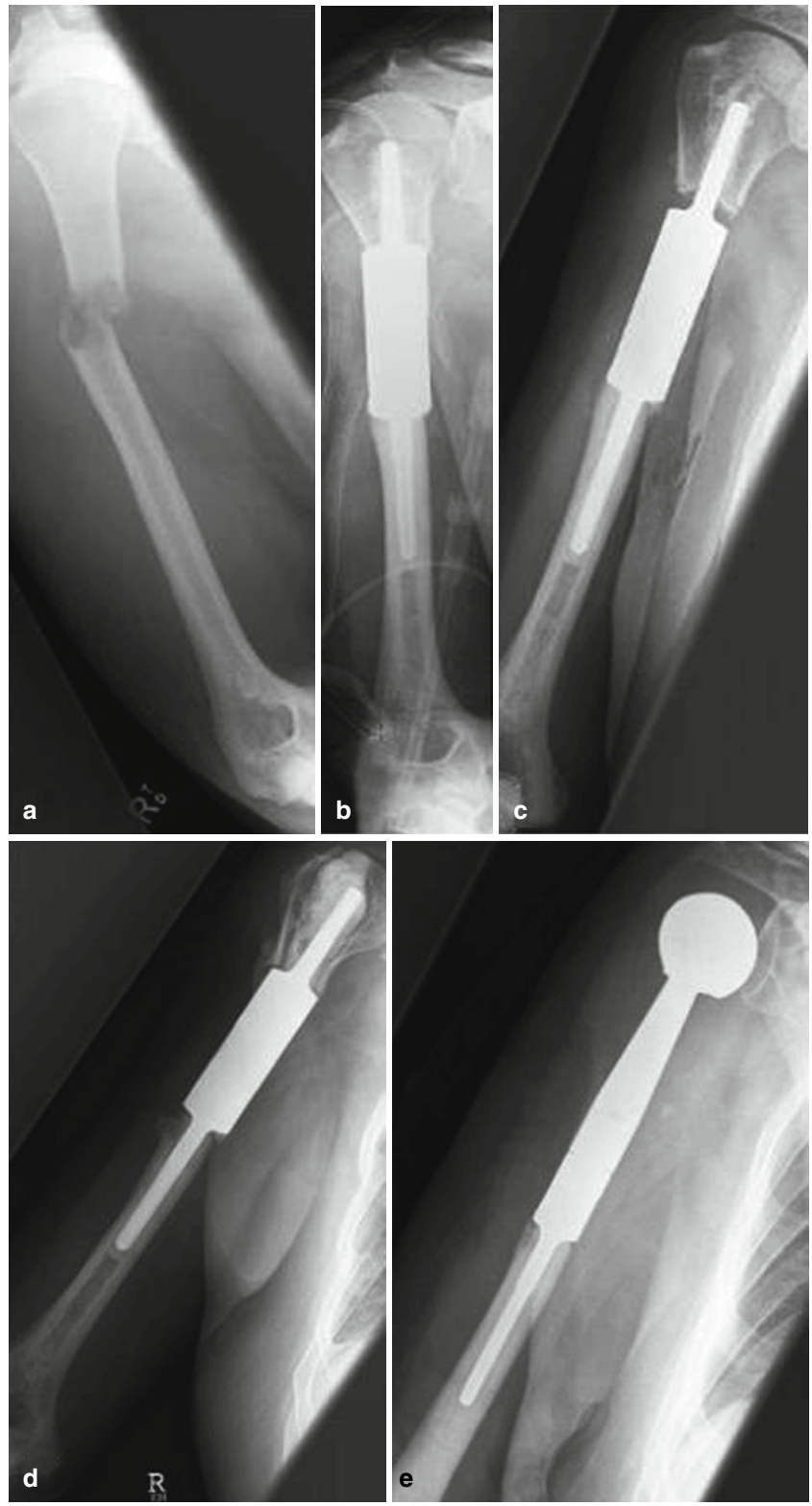


Fig. 21.2 The x-ray images show a pathological fracture resulting from solitary metastasis of a patient suffering from a renal carcinoma (**a**). Treatment of choice is by wide surgical excision which leaves a segmental bony defect. The defect was reconstructed with an intercalary prosthesis (**b**). This unfortunately failed (**c**) and was revised. After revision, failure reoccured (**d**) and further surgery was undertaken to convert the prosthesis to a proximal humeral replacement (**e**). With this new situation, the patient has less shoulder function than if the shoulder was able to be saved. If a suitable biological intercalary defect filler could be found, the patient's function may have been considerably better and he may have needed less surgery

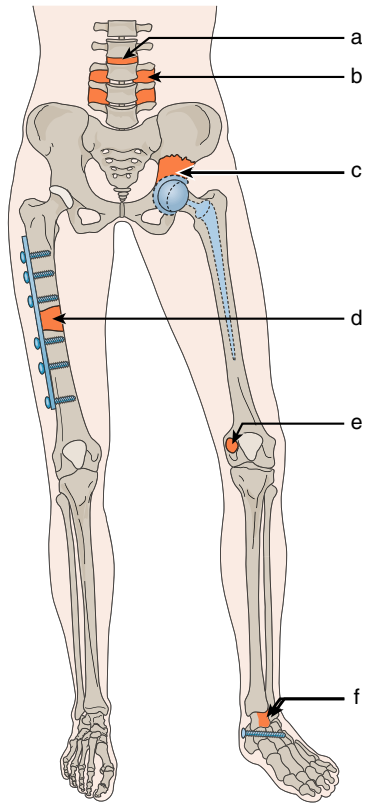


Fig. 21.3 Common defect areas augmented with and procedures utilizing autologous bone grafts: Spinal fusion (**a**, **b**), acetabular defects after hip replacement/revision surgery (**c**), segmental long bone defects (**d**), avascular necrosis (**e**), ankle joint arthrodesis (**f**)

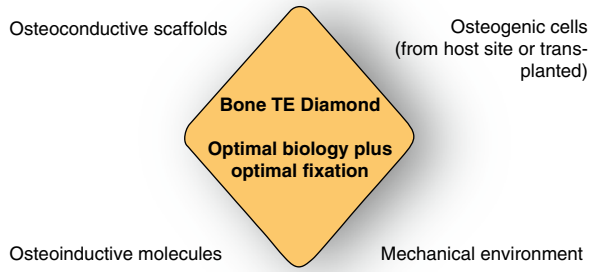


Fig. 21.4 The diamond concept of tissue engineering. When compared to the traditional triangular shaped models of tissue engineering (osteogenic cells, osteoconductive scaffold, and osteoinductive stimulus), the diamond concept of bone tissue engineering also includes the so far often neglected considerations in regard to the mechanical environment of the defect site

vascularized autografts are technically demanding and stress fractures are frequently observed, usually during the first postoperative year [14].

4. Allograft: Sections of donor bone (cancellous or cortical) are frozen, freeze dried, sterilized (gamma radiation, electron-beam radiation, ethylene oxide), and stored. Associated problems include implant devitalization and deactivation of bone specific proteins, and the risk of disease transmission [11].
5. Demineralized bone matrix (allograft or xenograft): With demineralized bone matrix (DBM) (after mild acid extraction), growth factors, noncollagenous proteins, and collagen remain, the mineral phase is reduced to about 40%, and cells are removed. In theory, the matrix provides a framework for cells to attach, proliferate, and eventually differentiate stimulated by noncollagenous extracellular matrix (ECM) proteins. The grafts are however of uncontrolled macrostructure and thus variable mechanical properties and the available clinical data assessing the performance of DBM is very limited.
6. Implants: These are based on metals or more recently, specially designed polymers and ceramic composites. Currently available metals have sufficient strength, but very high moduli, while the composites have suitable moduli, but insufficient strength for major load bearing. Metals have been the major solution for hip replacement and large bone grafts, while composites can only be used in a limited number of applications [37].

The biology of bone grafting, as well as advantages and disadvantages of different graft types, has been reviewed in detail by Khan et al. [47].

More recently, the concept of tissue engineering has emerged as a promising alternative therapeutic concept for bone regeneration to overcome the drawbacks associated with conventional bone grafting. Tissue engineering unites aspects of cellular biology, biomechanical engineering, biomaterial sciences, and trauma and orthopedic surgery. Its general principle involves the association of cells with a natural or synthetic supporting scaffold to produce a three-dimensional, implantable construct (Fig. 21.4).

The following book chapter gives a brief overview on bone engineering principles, describes the opportunities tissue engineering offers, its current limitations, and future directions.

21.2 Aim of the Discipline

At present, tissue engineering strategies can generally be classified into different categories. These include strategies which primarily aim at tissue conduction, induction, and cell transplantation. Tissue conduction relies on graft materials (hydrogels, microspheres/beads, scaffolds) to facilitate the attachment of cells providing an interconnected structure to support cell migration and blood vessel formation. While tissue induction describes the ability of a graft or substance (growth factors, lyophilized cell fractions, peptides, etc.) to induce progenitor cells to migrate, proliferate, and differentiate into functionally mature cell types, cell transplantation refers to the use of cells and cell substitutes to replace limited structural and/or biochemical functions of the target tissue (e.g., chondrocytes transplanted in combination with a periosteal flap, myocardiocyte injection into heart muscle, hematopoietic bone marrow cell transplantation, skin regeneration: cultured epidermal cell sheet) [43].

Other methodical approaches combine these basic principles and include the culture of specific cell types in 3D environments with the aim to mimic naturally found extracellular matrices as closely as possible. Such 3D environments can be created by establishing sandwich cultures, by cell encapsulation into hydrogels, and by cell seeding specifically configured cellular solids or scaffolds [82].

Applied to bone regeneration specifically, the following factors have to be considered when developing a tissue engineered implant [37]:

1. Cell technology
   - Cell sourcing.
   - Effective use of stem cell technology.
   - Cell seeding.
   - Manipulation of cell function (morphogenetic signals).
   - Growth and proliferation of cells, premature and mature tissue (static and dynamic/bioreactor culture) (Fig. 21.5).
2. Scaffold design and fabrication
   - Development and design of materials in 3D using both natural and synthetic molecules.
   - Manufacturing techniques.
   - Mechanical properties. Surface modification to facilitate cell attachment, growth, and proliferation.
3. Integration of scaffold in host tissue
   - Surface biocompatibility.
   - Structural compatibility.
   - Strength of bond between cells and scaffold.
   - Transplantation techniques.
   - Avoidance of foreign body reactions.
   - Vascularization and mass transfer of nutrients to cells deep within the scaffold.
   - Transplant assimilation and remodeling.

Currently, most of the bone tissue engineering efforts concentrate on research on adult stem cells, as scaffolds populated with these cells were found to undergo subsequent differentiation after in vivo transplantation. These concepts mainly rely on scaffold guided host cell and tissue ingrowth as well as transplanted cells as a part of an engineered device as the main factors

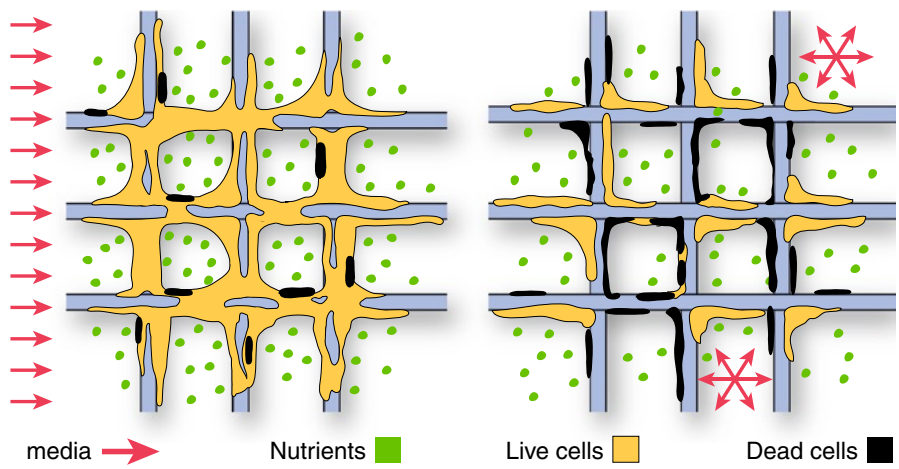


Fig. 21.5 The influence of static (*left*) and dynamic (*right*) culture systems on cell proliferation

regulating cell behavior and performance in vivo are local cells and nonsoluble factors within the ECM (collagens, glycosaminoglycans (GAGs), cytokines, hormones, nutrients, minerals, and waste products).

In summary, the main aim of bone tissue engineering is to utilize cells isolated from tissue samples of a specific donor site, to expand them in vitro and to deliver them within a scaffold or matrix in a configuration that generates new functional bone.

21.3 State of the Art

The combination of living cells, biologically active molecules, and a structural scaffold to form a tissue engineered construct (TEC) to promote the repair and regeneration of tissues is the most common concept underlying tissue engineering. The scaffold component is expected to support cell colonization, migration, growth, and differentiation guiding the development of the desired tissue. Concurrently with tissue neo-formation, scaffolds ideally undergo degradation processes producing biocompatible, metabolizable, and excretable by-products. While tissue engineering can be more broadly defined as a strategy to engineer a functional substitute tissue for the repair or regeneration of impaired organs, it is clear that scaffolds play a central role and therefore represent the main focus within this article.

It is important to emphasize that so far no definite criteria are identified to define an ideal scaffold-cell or scaffold-neotissue construct, not even for a specific tissue type. As some tissues perform multiple functional roles, it is highly unlikely for a single scaffold to serve as a universal foundation for the regeneration of even a single tissue. One of the definitions of material biocompatibility determines the suitability of a particular material in regards to its functional role following implantation [62]. Accordingly, scaffold design for bone engineering is a complex task and includes considerations in respect to material composition, architecture, structural mechanics, surface properties, degradation properties, and products. Moreover, the integration of biologically active components, and the changes of all above mentioned factors over time have to be accounted for.

For every application, tissue engineering constructs have to meet certain minimal requirements regarding biological, biochemical, and physical properties. Scaffolds may initially be required to provide sufficient mechanical strength and stiffness to substitute for lacking mechanical function of the diseased or damaged tissue. Scaffold stiffness and strength should be sufficient to permit requisite cell seeding in vitro without compromising scaffold architecture and to support and transmit mechanical stimuli in an in vivo healing site. For bone engineering specifically, the scaffold should be able to resist change in shape and internal pore architecture resulting from tractional forces by introduced cell. It should moreover withstand contraction, torsional and/or compressive forces invoked during tissue healing in vivo. However, after transplantation in vivo, external and internal fixation systems or other supports or restrictions on patient activity may reduce the importance of a scaffold's mechanical properties during early stage recovery. The maintenance of sufficient structural integrity however is critical, since cell and tissue remodeling is an essential requirement to achieve stable biomechanical conditions and vascularization at host sites. The degree of remodeling depends on the tissue itself (cancellous bone 3–6 months; cortical bone 6–12 months) and its host anatomy and physiology. The scaffold architecture has to facilitate initial cell attachment and subsequent migration into and through the scaffold matrix. It should allow mass transfer of nutrients and metabolites and provide sufficient space for the development and remodeling of neotissue. Due to scaffold degradation, the porosity and internal space within a scaffold increase over time providing more space for tissue formation or remodeling processes. In addition to these essentials of mechanics and geometry, a construct needs to possess surface properties which facilitate the attachment and migration of desired cell types. The dimensions and overall shape of the construct must also be taken into consideration, especially if constructs are customized for individual patients [17].

A holistic tissue engineering strategy also includes practical considerations of scaffold manufacturing. For clinical applications, scaffold fabrication at a reproducible speed and economic costs must be possible. Scaffold fabrication might have to accommodate the processing of biological components; it may be necessary to allow cell seeding or the incorporation of biomolecules that are soluble or heat labile. A review of the current bone engineering literature suggests that scaffolds need to fulfill a range of minimal requirements. The repair and regeneration of musculoskeletal tissues, particularly

bone, demands scaffolds with a high elastic modulus to provide temporary mechanical support without signs of fatigue or failure. The development of scaffolds that are retained in the space they were designated for and that provide the tissue with adequate space for growth remains demanding [21, 24].

In vivo, bone is able to remodel. This process is mediated by physiologically occurring loads. A bone engineering strategy that accounts for this biological process requires controlled scaffold degradation and resorption kinetics. The scaffold needs to retain its physical properties for at least 3–6 months (e.g., 1–3 months for cell culturing and 1–3 months in situ). The mechanical properties of a bioresorbable 3-D scaffold/tissue construct at the time of implantation should match those of the host tissue as closely as possible. It should possess sufficient strength and stiffness to maintain structural and mechanical function until in vivo tissue remodeling has replaced the slowly degrading scaffold matrix [38]. Thereafter, the scaffold's mechanical properties become less important and it should be metabolized within 12–18 months.

Mechanical loading may directly affect the degradation behavior. Under cyclic compressive loading, changes in surface deformation and morphology show up first followed by condensation and stiffening of the polymer matrix. The decrease in molecular weight is slowed down because of the reduction of surface area from hydrolysis, until the matrix architecture no longer accommodates the mechanical loading and begins to lose its integrity.

To evaluate the effects of different bioceramics on bone regeneration during repair of segmental bone defects, Gao et al. [27] implanted biocoral and tricalcium phosphate cylinders (TCP) in sheep tibial defects of 16 mm length. The defects were stabilized with overlapping auto-compression plates and cortical screws. When compared to TCP, groups with biocoral implants showed a significant increment in external callus amount and density after 3 weeks. Also, an increase in torque capacity, maximal angle of deformation, and energy absorption was measured after 12 weeks. In addition, microscopically assessed osseointegration seemed to be better for biocoral implants. Unfortunately, Gao et al. had used both male and female animals largely varying in body weight. Gender and body weight however are known to influence bone regeneration affecting both biomechanical environmental conditions and hormonal feed-back mechanisms.

Den Boer et al. reported an ovine segmental bone defect model of 30 mm stabilized by an interlocking intramedullary nail, The authors described the fabrication of biosynthetic bone grafts and their application [18]. The study groups included empty controls, autografts, hydroxyapatite, hydroxyapatite with recombinant human osteogenic protein 1 (rhOP-1), and hydroxyapatite with autologous bone marrow. At 12 weeks, healing of the defect was evaluated radiographically, by torsional biomechanical tests, and histological examination and revealed that torsional strength and stiffness were two fold higher for animals treated with autograft and hydroxyapatite plus rhOP-1 or bone marrow. A higher number of defect unions was described when hydroxyapatite plus rhOP-1 was applied rather than hydroxyapatite alone. It must be noted that animals treated with hydroxyapatite and bone marrow were of a different breed, had a higher average body weight, were held at a different holding facility, and therefore accustomed to unequal forage.

Bone healing in critical sized segmental diaphyseal defects in sheep tibiae was also investigated by Gugala and Gogolewski [30, 31]. Defects were bridged with a single porous tubular membrane or with anatomically shaped porous double tube-in-tube membranes. Membranes with different pore structures were applied alone and/or in combination with autologous bone graft. The diaphyseal defects were 40 mm in length and stabilized with a bilateral AO external fixator. Operated animals were 6–7 years of age. Of the six treatment groups, however, only in groups where the defect was filled with autogenous cancellous bone graft and covered with a single perforated membrane or where the bone graft was administered in a space between a perforated internal and external membrane, defect healing could be observed. The authors partly contribute the healing effect on defect healing to their membrane system; however a control group, where autologous bone graft is applied without membrane was not included in the study. After surgery, animals were held in suspending slings resulting in conditions poorly reflecting the actual physiological biomechanical environment.

The influence of resorbable calcium phosphate particles and paste forms on bone healing was investigated by Bloemers et al. [10] in a 30-mm segmental tibial defect fixed with a custom made AO unreamed interlocking titanium tibial nail. Twelve weeks after defect reconstruction, radiological, biomechanical, and histological analyses were performed. Radiographically,

the resorbable paste group performed best. Biomechanical tests showed a significantly higher torsional stiffness for the resorbable calcium–phosphate paste group when compared with autologous bone.

Insulin-like growth factor I (IGF I) exerts an important role during skeletal growth and bone formation. Therefore, its localized delivery appears attractive for the treatment of bone defects. To prolong IGF-1 delivery, Meinel et al. entrapped the protein into biodegradable poly(lactide-co-glycolide) microspheres and evaluated the potential of this delivery system for new bone formation in a noncritical 10 mm segmental tibia defect [59]. The defect was stabilized using a 3.5 mm 11 hole DCP. Administration of 100 μg of IGF-1 in the microspheres resulted in defect bridging within 8 weeks. Postoperatively, the animals were held in a suspension system for a period of 4 weeks.

Synthetic multiphase ceramic implants were evaluated in a 48 mm tibial defect model in [58]. The defect was stabilized with a 4.5-mm neutralizing plate that seemed, however, to be of insufficient strength. Good integration between the ceramic implants and the adjoining proximal and distal bone ends was observed. A progressive increase in new bone formation was seen over time, along with progressive resorption of the ceramic scaffold. On the basis of x-ray analysis, at the 1-year time-point, approximately 10–20% of the initial scaffold substance was still present, and after 2 years it was almost completely resorbed.

In yet another study in an ovine segmental defect model, Maissen et al. investigated the influence of rhTGFbeta-3 on mechanical and radiological parameters of a healing bone defect [57]. An 18-mm long tibial osteoperiosteal defect was fixed with a unilateral external fixator and treated with rhTGFbeta-3 absorbed by a poly(L/DL-lactide) carrier, with the carrier only or with autologous cancellous bone graft, or left untreated. Weekly in vivo stiffness measurements and radiological assessments were undertaken as well as quantitative computed tomographic assessments of bone mineral density in 4 week intervals. Animals were held in a support system to prevent critical loads on the fixator and its interface to bone. The 18 mm defect size was described as spontaneously nonhealing. In the bone graft group, a marginally significant higher increase in stiffness was observed than in the PLA/rhTGFbeta-3 group and a significantly higher increase than in the PLA-only group. The radiographic as well as the computed tomographic evaluation yielded significant differences between the groups, indicating that the bone graft treatment performed better than the PLA/rhTGFbeta-3 and the PLA-only treatment.

Sarkar et al. assessed the effect of platelet-rich plasma (PRP) on new bone formation in an ovine critical 25 mm diaphyseal tibial defect [75]. The defect was stabilized with a custom-made intramedullary nail (stainless steel, diameter proximal 12 mm, distal 10 mm). To reduce stress at the screw/bone interface, a custom made stainless steel plate was additionally applied medially. Defects were treated with autogenous PRP in a collagen carrier or with collagen alone. After 12 weeks, the explanted bone specimens were quantitatively assessed by x-ray, computed tomography (CT), biomechanical testing, and histological evaluation. Bone volume, mineral density, mechanical rigidity, and histology of the newly formed bone in the defect did not differ significantly between the PRP treated and the control group, and no positive effect of PRP on bone formation was observed.

Recently, Liu et al. reported on the use of highly porous beta-TCP scaffolds to repair goat tibial defects [56]. In this study, 15 goats were randomly assigned to one of three groups, and a 26-mm-long defect at the middle part of the right tibia in each goat was created and stabilized using a circular external fixator. In Group A, a porous beta-TCP ceramic cylinder loaded with osteogenically induced autologous bone marrow stromal cells (BMSCs) was implanted in the defect of each animal. In Group B, the same beta-TCP ceramic cylinder without any cells was placed in the defect. In Group C, the defect was left untreated. In Group A, bony union could be observed by gross view, x-ray and micro-computed tomography (micro-CT) as well as histologically at 32 weeks postimplantation. The implanted beta-TCP scaffolds were almost completely replaced by host bone. Bone mineral density in the repaired area of Group A was significantly higher than in Group B, in which scant new bone was formed in each defect and the beta-TCP had not been completely resorbed after 32 weeks. Moreover, the tissue-engineered bone of Group A had similar biomechanical properties as the contralateral tibia in terms of bending strength and Young's modulus. In Group C, little or no new bone was formed and nonunion occurred, demonstrating the critical nature of the defect.

Rozen et al. investigated whether blood-derived endothelial progenitor cells promote bone regeneration once transplanted into an ovine, critical sized, tibial defect [73]. Cells were isolated and expanded in vitro; 2×10^7 cells in 0.2 mL saline were transplanted 2 weeks after a 3.2-cm defect had been created ($n=7$). Defect fixation was achieved by a 4.5-mm stainless steel plate with four screws each proximally and distally. In the control group ($n=8$) only 0.2 mL saline were injected. Defect bridging was observed in 6 out of 7 animals in the experimental group. In the control group, 5 out of 6 defects analyzed via μCT showed discontinuous (2 animals) or minute bridging (3 animals) as stated by the authors. No reference to the remaining 3 animals of the control group was found throughout the manuscript. Therefore, the critical nature of the defect has to be questioned. Not resecting the periosteum and screw loosening as clearly evident in the published x-ray images might have contributed to defect bridging in the control group.

The regenerative capacity of xenogenic human and autologous ovine mesenchymal progenitor cells was assessed by Niemeyer et al. in an ovine critical-size defect model [63]. Human and ovine MSCs from bone marrow, were cultured on mineralized collagen and implanted into a 3.0 cm-long sheep tibia bone defect ($n=7$). Unloaded mineralized collagen served as control. The 3 cm mid-diaphyseal defects were fixed with a 7-hole LC-LCP (Synthes) and a carbon fiber reinforced poly-ether-ketone plate (snakeplate, Isotec AG, Altstätten, Switzerland). Animals were kept in suspending slings for 8 weeks post surgery. Nevertheless, implant failure occurred in one animal requiring immediate euthanasia. Wound healing related problems were reported for another animal. Bone healing was assessed up to 26 weeks. Presence of human cells after xenogenic transplantation was analyzed using human-specific in situ hybridization. Radiology and histology demonstrated significantly better bone formation after transplantation of autologous ovine MSCs on mineralized collagen compared to unloaded matrices and to the xenogenic treatment group. No local or systemic rejection reactions could be observed after transplantation of human MSCs although the presence of human MSCs could be demonstrated.

The results of some of these research activities in the field of scaffold-based bone engineering will be discussed from a clinical point of view in the subsequent section of this book chapter.

21.4 Clinical Application

Considerable progress has been made in the last two decades in the area of skeletal reconstruction; however, the treatment of osseous defects and nonunion fractures remains critical to numerous orthopedic and craniofacial treatment concepts. The lack of techniques and approaches in reconstructive surgery emphasizes the number of clinical applications that would benefit from tissue engineered bone. Although commonplace in orthopedic surgery, the current approaches, which include autografting and allografting cancellous bone, applying vascularized grafts of the fibula and iliac crest, and bone transport methods have a number of limitations as described earlier [47].

21.4.1 Orthopedic and reconstructive surgery

Vacanti et al. [83] reported the replacement of an avulsed phalanx with tissue engineered bone. Treatment resulted in the functional restoration of a biomechanically stable thumb of normal length. Periosteal osteoblastic progenitor cells were obtained from sections of the distal radius and were seeded onto a coral based scaffold. A calcium alginate hydrogel encapsulating the cells was used to saturate the coral implant. During follow-up, MRI examination showed evidence of vascular perfusion and biopsy revealed new bone formation with a lamellar architecture. In a first clinical study, Quarto et al. have reported the use of cell-based tissue engineering approaches to treat large bone defects in three patients suffering from various segmental defects (4 cm bone segment loss in the right tibia, 4 cm in the right ulna, and 7 cm in the right humerus) [70]. Prior to transplantation, bone marrow derived osteoprogenitor cells were isolated and expanded. The cells were then seeded onto a macroporous scaffold designed to fit the missing bone fragment. Defects were stabilized with an external fixator. The radiographs on follow-up showed abundant callus formation along the implants and good integration at the host bone interface. In all patients, recovery was reported. However, conclusions were drawn solely on the basis of radiographic evaluation; no confirming biopsies were taken. Due to the high radio-opacity of the

ceramic material, the assessment of bone formation within the ceramics might have been difficult as the gain in radiopacity due to new bone formation would have been overshadowed by scattering. It is furthermore unclear if the callus formation was induced by the implanted human MSCs or by bone-forming cells of the periosteum.

The second published clinical study describes the augmentation of the posterior maxilla in 27 patients, using matrix derived from mandibular periosteum cells on a polymer fleece (Ethisorb, Ethicon) [77]. In 12 patients, only radiographic and clinical assessments were performed. Limited conclusions can be drawn from the radiographic findings. The other 15 patients were treated in a biphasic approach. First, reconstruction of the host area was performed. After a healing period of 3 months, prior to dental implant placement, a biopsy was taken. In eight of these 15 patients a nonsatisfying outcome was observed; the tissue engineered bone had been resorbed and replaced with connective tissue. In cases of a positive biopsies (seven patients), the authors were unable to distinguish between bone formation induced by the implanted cells (osteoinduction) or by resident osteoblasts from the pre-existing bone (osteoconduction).

Bajada et al. [7] reported on the successful healing of a 9-year old's left tibial mid-shaft nonunion following a high-speed road traffic accident. The nonunion had been resistant to various surgical procedures including the application of a monolateral external fixation, functional bracing, and two programs of ring circular external fixation with autologous bone grafting. Using autologous BMSCs expanded in vitro to 5×10^6 cells within a period of 3 weeks combined with calcium sulfate ($CaSO_4$) in pellet form, the defect was reconstructed observing clinical and radiological convalescence for 2 months after implantation (Fig. 21.6).

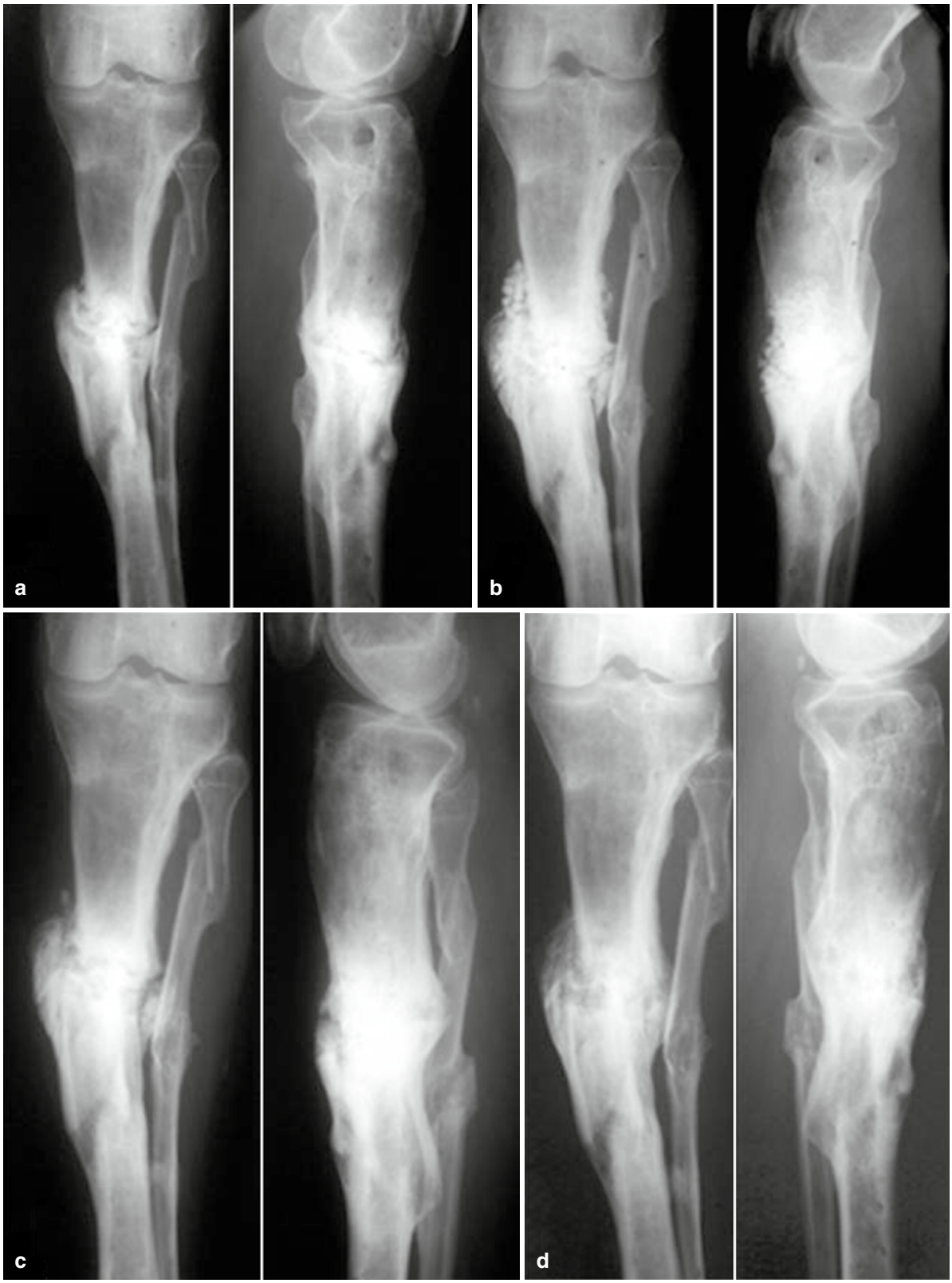
The overall goal of treatment concepts for femoral head necrosis in adults is the preservation of the femoral head and therefore to avoid total hip replacement surgery. Core decompression has been shown to decrease intraosseous pressure additionally providing the opportunity to deliver bioactive materials as well as different progenitor cells to enhance healing. In particular, the use of cell-based strategies has great therapeutic potential and could play an important role in the treatment of femoral head necrosis in adults in the future. Noth et al. [65] presented a therapeutic approach for patients suffering from femoral head necrosis stage ARCO II using bone marrow stem cells in combination with a beta-TCP matrix (Fig. 21.7). Kawate et al. [46] reported on three cases of steroid induced femoral head osteonecrosis stage Steinberg 4A (one patient) and C (two patients) treated with MSCs cultured with beta-TCP ceramics and with a free vascularized fibula. The average follow-up period was 34 months and the average patient age at the time of surgery was 28 years. All hips showed preoperative collapse and radiographic progression was observed in two hips postoperatively although osteonecrosis did not progress any further.

Recently Hernigou and Beaujean [34] published data obtained from autologous bone marrow transplantations combined with core decompression. They could demonstrate that surgery before collapse of the femoral head made hip replacement surgery necessary in only 9 of 145 cases compared to 25 of 44 performed hip replacements when operated after manifest collapse. As well, Gangji et al. [26] reported on successful treatment of 18 patients treated with bone marrow cells harvested from the iliac crest with an average follow-up of 24 months suggesting that the application of cell-based treatment concepts in case of femoral head necrosis might play a decisive role in future therapeutics.

Bone repair and regeneration with bone morphogenetic proteins (BMPs) have advanced as an alternative treatment option in orthopedic and trauma surgery. A number of animal studies and subsequent clinical trials have demonstrated the osteogenic potential of BMPs. The result has been the commercialization of two of the early BMPs, BMP-2 and BMP-7 (also called osteogenic protein-1 or OP-1).

OP-1 is an attractive adjunct in the treatment of fractures and atrophic long bone nonunions.

Fig. 21.6 Preoperative (**a**), postoperative (**b**), 8 week (**c**) and 24 month (**d**) anteroposterior and lateral radiographs of a 9-year old's tibial nonunion which was resistant to six previous surgical procedures. In vitro expanded autologous bone marrow stromal cells (BMSCs) combined with $CaSO_4$ in pellet form led to union clinically and radiologically 2 months after transplantation reprinted with permission


a
b
c
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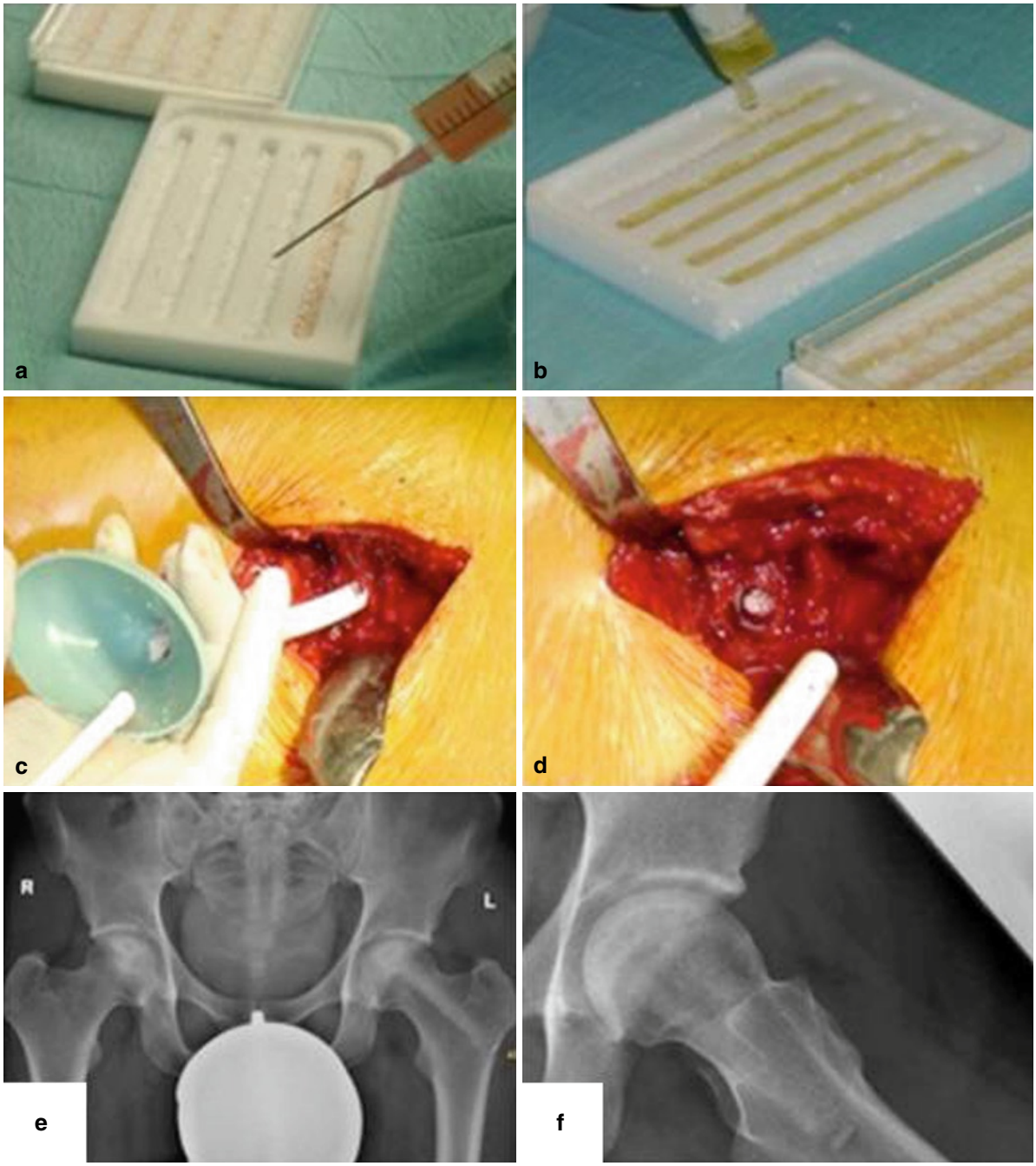

Fig. 21.7 Preparation and application of stem cell-TCP matrix. (**a**) The stem cell suspension is added to the β-TCP granules with a syringe. (**b**) Addition of serum isolated from patient blood via centrifugation. (**c**) The stem cell-TCP matrix is installed through the boring duct using a special funnel-formed applicator. (**d**) Completely filled boring duct. Imaging diagnostic 6 weeks post application of the stem cell-TCP matrix. (**e**) X-Ray of the pelvis. (**f**) The axial projection demonstrates the anterior and posterior boring duct. (**g**) The MRI (T1) shows the anterior boring duct directly reaching the necrotic area in the coronar plain. (**h**) In the horizontal plain (T2) the anterior and posterior boring ducts are presented adjacently (from: Nöth U, Reichert J, Reppenhagen S, Steinert A et al. [80]; With kind permission of Springer Science+Business Media)

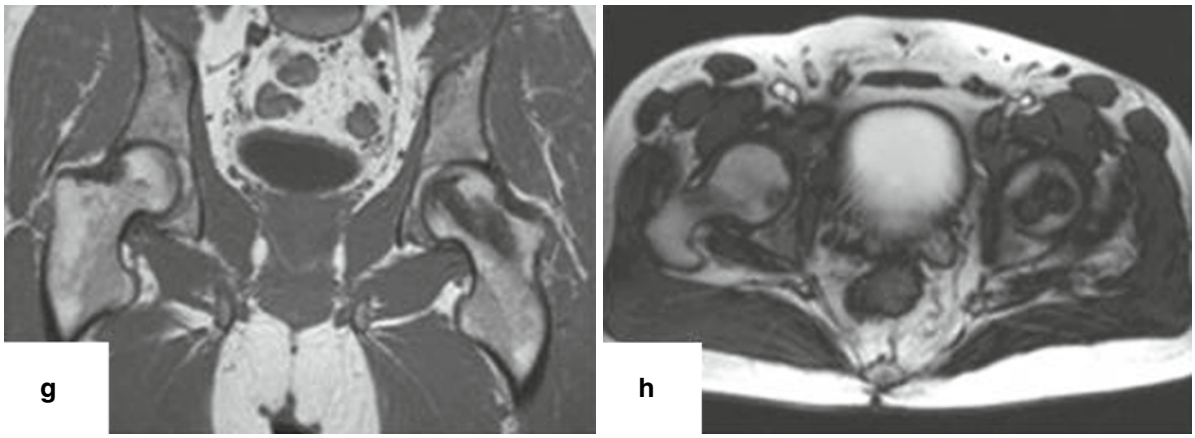

Fig. 21.7 (continued)

The use of OP-1 in the treatment of open tibial shaft fractures was evaluated by the Canadian Orthopedic Trauma Society. One hundred and twenty-four open tibial fractures (62 controls and 62 OP-1) were included in the study. After irrigation, debridement, and intramedullary nailing, at the time of definitive wound closure, patients were randomized to standard wound closure or standard wound closure with the addition of OP-1 to the fracture site. Patients were followed up radiographically, clinically, and serologically until union. Outcomes showed that the number of secondary interventions for delayed union and nonunion was significantly lower in the OP-1 group than in the control group (8 vs. 17; $p=0.02$). A significantly greater number of patients in the OP-1 group were able to bear the weight fully without pain at 12 months compared to the control group. No OP-1 related adverse effects were clinically evident. The study investigators suggested that the use of OP-1 is safe in open tibial shaft fractures, and its use decreased the number of secondary procedures for delayed or nonunion.

More recently, Ristiniemi et al. have evaluated OP-1 in the treatment of distal tibial fractures [72]. Twenty patients with distal tibial fractures were treated with external hybrid fixators and OP-1 and compared to 20 matched patients treated without BMP. Outcome measures included time to radiographic union, duration of application of the external fixator, the number of secondary interventions due to delayed healing, and length of absence from work. The mean time to union as well as to fixator removal was significantly shorter in the BMP group (15.7 weeks vs. 23.5 weeks, $p=0.002$; 15 weeks vs. 21.4 weeks, $p=0.037$). Revisions for delayed union were required in two patients in the BMP group and seven patients in the control group. Average time off work was significantly lower in the BMP group than in the controls.

The use of OP-1 in the treatment of tibial nonunion was studied by Friedlaender et al. in a randomized controlled, prospective clinical trial [25]. Clinical and radiographic results were compared to assess the efficacy of OP-1 vs. autograft in the treatment of tibial nonunions that had persisted for at least 9 months. One hundred and twenty-four tibial nonunions in 122 patients were randomized to either intramedullary nail and autograft or intramedullary nail and implantation of OP-1 at the nonunion site. Nine months after surgery, 81% of the OP-1 group and 85% of the autograft group had achieved clinical union. Radiographic analysis indicated that 75% of the fractures treated with OP-1 and 84% of the autograft-treated group had healed. There was no statistically significant difference between groups clinically or radiographically. More than 20% of the bone graft group complained of persistent donor site pain. The authors concluded that OP-1 was a safe and effective alternative to bone grafting in the treatment of tibial nonunions. This study led to multiple regulatory approvals worldwide. Numerous studies in the literature suggest that OP-1 is a safe and effective treatment option for fractures and atrophic nonunions not only of the lower but also of the upper extremities.

21.4.2 Oral and Maxillofacial Surgery

Tissue engineering represents one of the most exciting advances in regenerative medicine. However, little has been reported on the application of tissue engineering for the regeneration of periodontal tissues. Yamada et al. [86] have applied tissue engineering principles to periodontology. MSCs harvested from bone marrow aspirates of the iliac crest were cultured and expanded. As well, PRP from the same patient was isolated from peripheral blood. Full-thickness periodontal flaps were elevated and the root surfaces were scaled and planed. Using the expanded MSCs and PRP, a gel was prepared and applied to the root surface and adjacent defect space. Follow-up examinations revealed that the application of MSCs in combination with PRP at periodontal sites with angular defects resulted in a 4-mm reduction in probing depths and a 4-mm clinical attachment gain, while bleeding and tooth mobility disappeared. Radiographic assessments showed reduction of the bone defect in depth. Moreover, it could be observed that interdental papillae showed signs of regeneration. Kawaguchi et al. [45] demonstrated that transplantations of ex vivo expanded autologous MSCs can regenerate new cementum, alveolar bone, and periodontal ligament in class III periodontal defects in dogs. Morphometric analysis revealed a 20% increase in new cementum length and bone area in animals treated with MSCs. In a subsequent study the same group reported a similar approach in humans [44]. They transplanted 2×10^7 cells/mL autologous expanded bone marrow-derived MSCs mixed with atelocollagen into periodontal osseous defects. All patients showed a significant improvement. Warnke et al. [85] #22] reported the fabrication of a mandibular transplant for a patient suffering from a large resection of his mandible. The transplant consisted of a titanium mesh cage filled with bone mineral blocks which were infiltrated with a combination of autologous bone marrow from the iliac crest and rhBMP-7. The transplant was then implanted into the right latissimus dorsi muscle and left there for a period of 7 weeks. Skeletal scintigraphy showed bone remodeling and mineralization inside the mandibular transplant both before and after transplantation. Computed tomography provided evidence of new bone formation. Seven weeks posttransplantation, the transplant was excised with an adjoining part of the latissimus dorsi muscle containing the thoracodorsal artery and vein ensuring blood supply for the entire transplant. It was then transplanted into the mandibular defect for reconstruction. As a result, the patient showed an improved degree of mastication and a satisfactory esthetic outcome.

An alternative direction for bone tissue engineering does not involve the pre- or perioperative use of stem cells and/or angiogenic factors, but uses appropriate scaffolds that attract the patient's own precursor cells postimplantation. This would circumvent all disadvantages associated with cell-based approaches (MSC harvest and/or expansion prior to clinical use), and we previously were able to show [76] that such an in situ bone tissue engineering approach is feasible for skull defects up to 2.5 cm (Fig. 21.8).

21.5 Expert Opinion

The reconstruction of large bone segments remains a significant clinical problem. Large bone defects occur mainly as a result of extensive bone loss due to pathological events such as trauma, inflammation, and surgical treatment of tumors. Present therapeutic approaches include the application of bone graft transplants (autologous, allogenic, xenografts), as well as implants made of different synthetic and natural biomaterials or segmental bone transport. However, up to the present time none has been proven to be fully satisfactory. A considerable number of research groups worldwide work on the development of new bone grafting materials, carriers, growth factors, and TECs for bone regeneration and are therefore interested to evaluate their concepts in reproducible large segmental defect models (Fig. 21.9). The optimization of cell-scaffold combinations and locally or systemically active stimuli will remain a complex process characterized by a highly interdependent set of variables with a large range of possible variations. Consequently, these developments must be nurtured and evaluated by clinical experience, knowledge of basic biological principles, medical necessity, and commercial practicality. The area of tissue engineering which has its main focus on the development of bioactive materials depends on the use of animal models to evaluate both experimental and clinical hypotheses. To tackle major bone tissue engineering problems, researchers must rely on the functional assessment of biological and

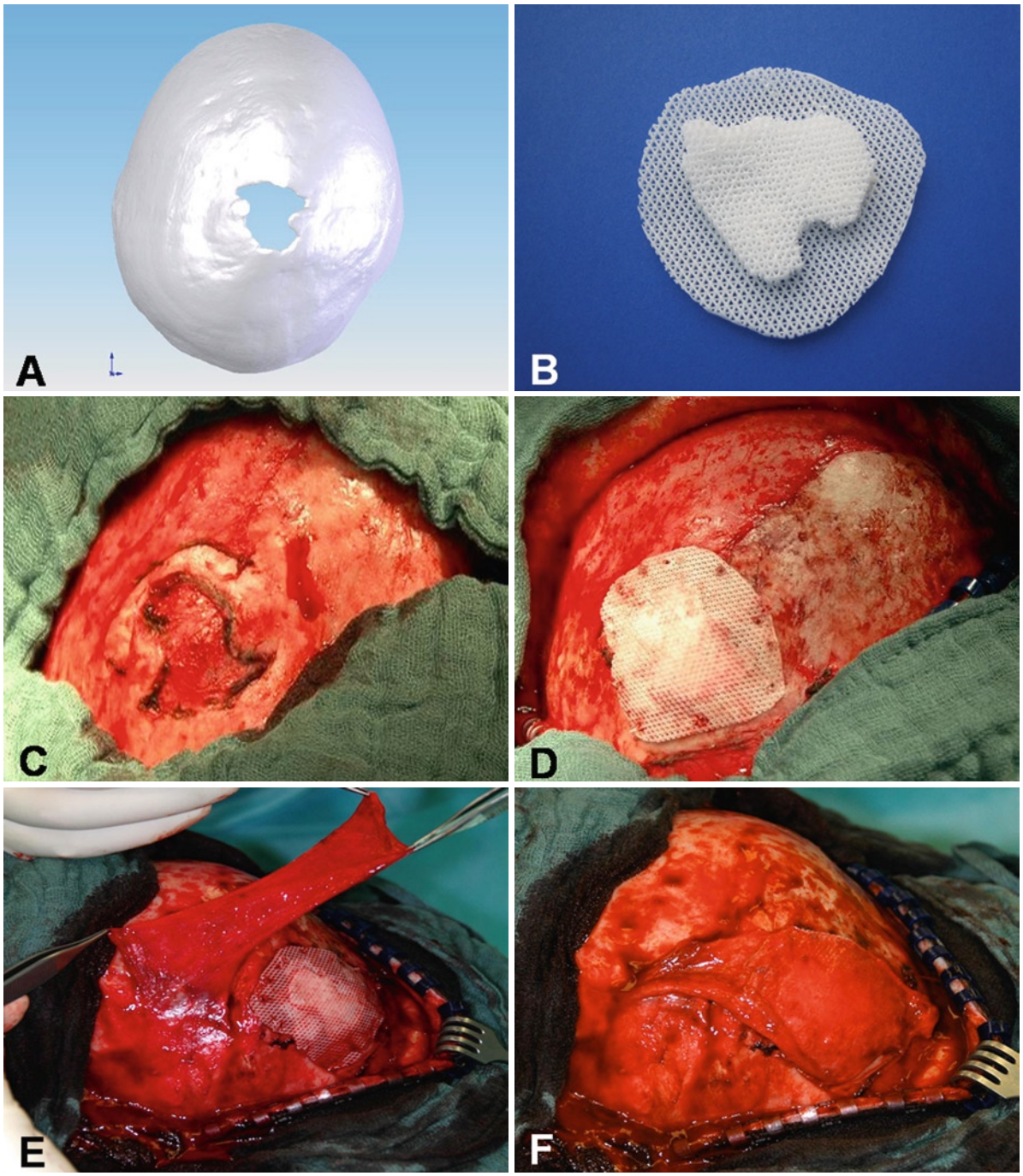

Fig. 21.8 The figure shows the 3D reconstruction of a skull defect (**a**). The defect (**a**, **c**) was reconstructed using acustom-made, prefabricated mPCL–TCP scaffold (**b**) and covered with a periosteal flap to supply osteogenic cells to the transplant (**e**, **f**) (reprinted with permission).

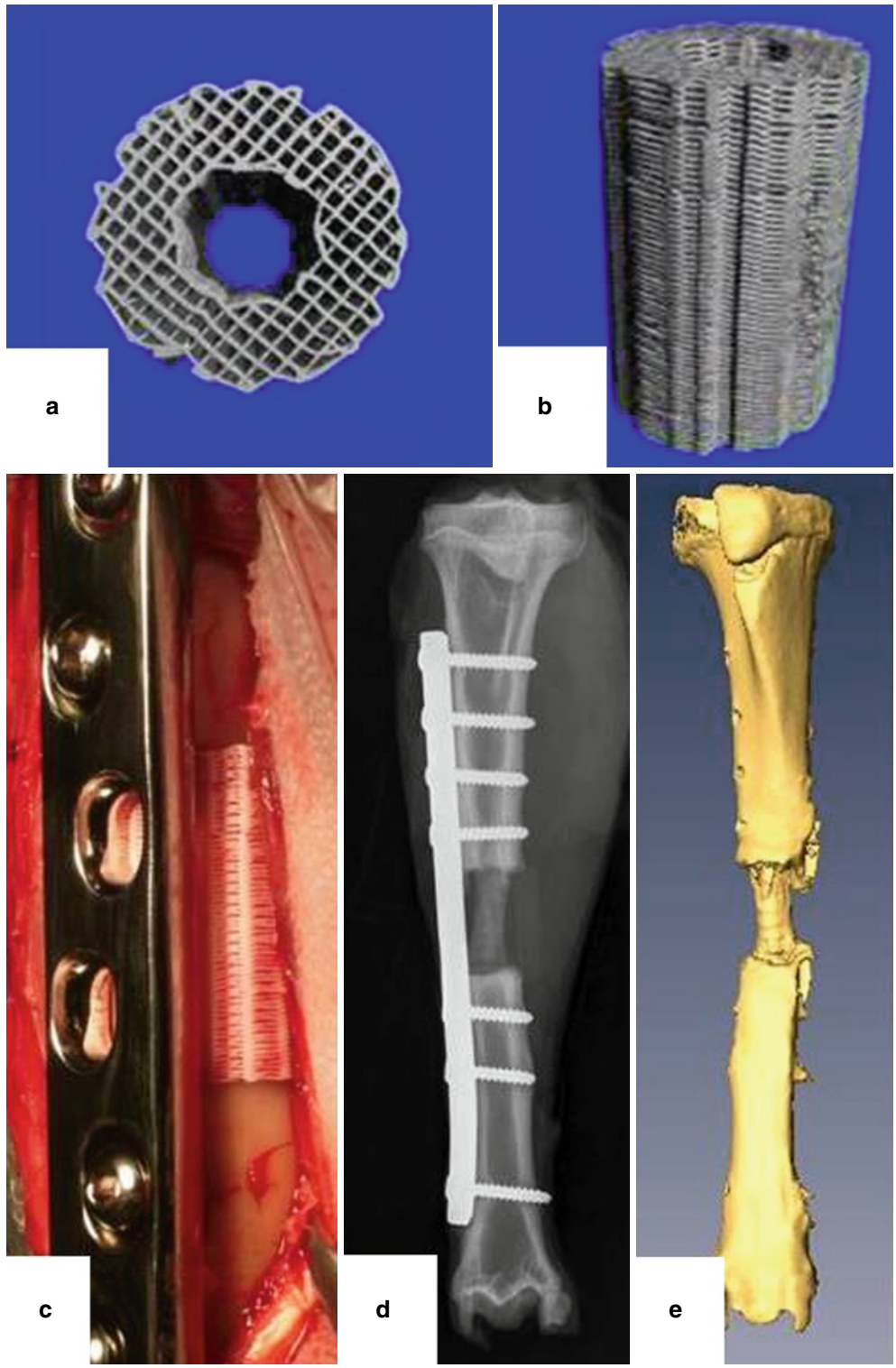

Fig. 21.9 Cylindrical medical grade polycaprolactone beta-tricalcium phosphate (mPCL-TCP) scaffold produced via fused deposition modeling (**a**, **b**) (dimension: height: 30 mm, outer diameter 20 mm). mPCL-TCP scaffold transplanted into an ovine 3 cm critical sized segmental bone defect (**c**). X-ray image (**d**) and 3D CT reconstruction (**e**) show defect bridging 12 weeks after scaffold transplantation

biomechanical parameters of generated constructs. However, to allow comparison between different studies and their outcomes, it is essential that in animal models and human clinical trials, fixation devices, surgical procedures, and methods of taking measurements are standardized to achieve the accumulation of a reliable data pool as a base for further directions to orthopedic and tissue engineering developments.

Therefore, bone tissue engineering standards need to be introduced if we are to build on promising research in the field and work under one governing body towards the end goal to provide today's surgeons with enhanced therapeutic concepts to repair bone. These standards should cover not only scaffold design and fabrication as well as implantation procedures, but also the analysis of the engineered constructs to facilitate comparisons between different studies.

The concepts of tissue engineering have matured over the last two decades and it can be concluded that the translation of tissue engineering strategies from bench to bedside is a difficult, expensive, and time-consuming process. Hollister recently addressed the different issues pertaining to the translation and commercialization of tissue-engineered constructs (TEC) [36]. He argues that the so-called "Valley of Death," which describes the lack between research and commercialization, is highly prevalent in the area of tissue engineering/regenerative medicine and originates from the high costs associated with technology development and the need of funding preclinical animal models and clinical trials for regulatory approval. However, to establish a tissue-engineering concept in a clinical setting, a rigorous demonstration of the level of therapeutic benefit in clinically relevant animal models is an essential requirement. We construe that the lack of translation in the field of bone engineering might further be related to difficulties in integrating individual technical discoveries in model tissue engineering systems, in manufacturing scale up, in funding, and in regulatory approval.

21.6 Five-Year Perspective

21.6.1 *In Vivo Bone Engineering*

In the near future, several approaches to improve the oxygen and nutrient supply will be further investigated. One approach is the stimulation of vessel growth by addition of angiogenic growth factors or endothelial cells to TECs. Especially when pursuing cell-based approaches, vessel growth needs to be stimulated as soon as possible after implantation of the TEC [62].

A method that bypasses the problems associated with orthotopic bone engineering by growing a bone transplant in a well-vascularized, muscular environment (ectopic bone formation) was described by Warnke et al. (see above) [85].

Although it was shown that the transplantation of osteogenic bioartificial bone tissues is technically achievable, and some of the currently available concepts represent alternatives to autologous bone grafts in defined clinical settings, the reconstruction of large bone defects remains challenging. Often, initially insufficient vascularization limits the survival of transplanted cells in the center of cell-based TECs as a result of insufficient oxygenation and supply of nutrition. Experiments with labeled osteoblasts have shown that the amount of cells significantly decreases within the first 7 days post transplantation [49, 50]. Therefore, the induction of vascularization in biomaterials prior to cell injection (i.e., the prevascularization of scaffolds) may help to increase the initial survival and engraftment of transplanted cells and may consequently optimize bone formation in bioartificial osteogenic bone tissues.

In plastic surgery, the prefabrication of multicomponent flaps is a commonly applied procedure [48, 78]. Several publications describe the implantation of vascular carriers into biomaterials for engineering of axially vascularized bone tissues [28, 54]. In 1979, Erol and Spira described the successful vascularization of a full-thickness skin graft using an arteriovenous (AV) loop in a rat model [22]. Morrison's research group. further improved the model by inserting the loop into isolation chambers [35, 60]. They demonstrated the successful induction of vascularization in polymer and gel matrices [13]. Furthermore, the superiority of the AV loop as a vascular carrier over the vascular bundle in terms of vascular density and capacity for generation of new tissue was demonstrated [81]. Kneser et al. were the first to describe the induction of axial vascularization in a solid porous matrix, namely in processed bovine cancellous bone [49, 50]. It was shown that axial prevascularization of porous matrices using an AV loop promotes survival and differentiation of transplanted autologous osteoblasts [2].

Cell survival is the most important requirement to achieve clinical success in cell-based bone tissue engineering. An alternative approach to prevent death of transplanted cells bypassing the deleterious effect of the hematoma and lack of early vascularization is a two-step implantation procedure in which the scaffold is transplanted first and approximately 7–14 days later the cells and/or growth factors. Cells additionally could be implanted at a time-point during the wound healing process at which the body would normally recruit stem cells to the defect site. However, a single approach, an on-the-spot repair, as currently achieved with autologous bone grafting, still appears optimal. Nevertheless, it is indisputable that multipotent mesenchymal progenitor cells are an essential requirement for the successful healing of bone defects [49, 50].

Bone marrow derived MSCs represent one of the promising cell sources for regenerative medicine and tissue engineering. However, the amount of marrow that can be obtained under local anesthesia is generally limited to no more than 40 mL and yields approximately 2×10^9 nucleated cells [6]. Obtaining a larger volume of bone marrow necessitates the use of general anesthesia, increases donor site morbidity [3, 64], and further dilutes the stem cell fraction with stem cell-free blood [6]. This extends the expansion time to generate cells in therapeutic numbers.

Therefore, adipose tissue represents another potential source of multipotent cells that could be procured in large amounts and hosts a reasonably high fraction of mesenchymal progenitor cells. These adipose derived multipotent stem cells are capable of differentiating into adipogenic, chondrogenic, and osteogenic lineages when subjected to appropriate induction stimulants [87].

Adipose tissue occurs in abundance in subcutaneous tissue, around the kidneys, in the mesenteries and omenta, in the female breast, in the orbit behind the eyeball, in the marrow of bones deep to the plantar skin of the foot, and as localized pads in the synovial membrane of many joints. It has a remarkable ability to undergo considerable changes in volume during the lifespan of an individual. Although relatively small increases in volume can be accommodated by changes in the amount of lipid stored in individual adipocytes (hypertrophy), larger changes are mediated by the generation of new adipocytes (hyperplasia) accompanied by coordinated expansion and remodeling of the adipose vasculature [33, 74]. The dynamism may be mediated by resident stem cells. These cells can be isolated enzymatically from adipose tissue and separated by centrifugation from buoyant adipocytes. A more homogeneous, multipotent cell population emerges in culture under conditions supportive of MSC growth. Under local anesthesia, a typical harvest of adipose tissue can easily exceed 200 ml and yield 2×10^8 nucleated cells per 100 mL of lipoaspirate [4].

21.6.2 Stimulation of New Candidate Pathways

There are myriad factors, which complicate the translation of breakthroughs in basic biology of osteoprogenitor differentiation to bone regeneration. Almost all mechanistic data on human-specific responses can be derived from in vitro experiments only; the source of cells used for these studies greatly influences the outcome and conclusions. Fortunately, research into the fundamental nature of the cells' source elucidates these discrepancies more and more. These descriptive studies can, therefore, complement functional experiments utilizing transgenic techniques in model organisms and in vitro. By pooling these data we will be best placed to develop the necessary concepts for clinical implementation, which then can ultimately prove our understanding of how to direct and guide bone cells [66].

Regenerative medicine differs from conventional bone graft treatment concepts in the physiological pathways that it aims at stimulating (or antagonizing) and in the nature of the applied stimulus**.** In more general terms, regenerative medicine aims to recapitulate the developmental processes of physiological genesis of a specific tissue to positively affect repair. The discipline therefore requires the activation of progenitor cells capable of proliferation and differentiation. In some instances, such as long bone fracture repair, the recapitulation of an embryonic process occurs resulting in perfect repair. Bone regeneration in vivo is generally preceded by an inflammatory reaction followed by angiogenesis. The recruited osteoprogenitors can be perivascular cells, cells derived from the periosteal cambium, the endosteum, or bone marrow. This shift in emphasis, in emphasis, away from material properties to the potential of combining a scaffold and cells, has led to new approaches cognizant of the capacity and needs of applied cells concerning stimulating factors and ECM [43, 62].

The gene expression profile of fetal and adult bone precursors and their differentiated descendants varies. Studies however suggest that despite these differences, both adult and fetal precursors similarly respond to stimulation with osteogenic factors: *Indian hedgehog* is re-expressed during long bone fracture repair in the adult mammal [23, 53].

Preliminary studies on adult osteoprogenitors also confirm the view that stimulation of the *hedgehog* pathway has regenerative potential as calvarial regeneration is enhanced by gene-mediated delivery of *sonic hedgehog* [19]. Mouse calvarial osteoblasts and human bone marrow-derived MSCs respond to hedgehog agonists in vitro by upregulation of alkaline phosphatase and deposition of mineralized matrix. During the development of the skeleton, there is interplay between *hedgehog* and both *Wnt* (wingless-type) and *BMP* signaling pathways. Costimulation of the *Wnt* pathway and *BMP* pathway produces pronounced osteogenic differentiation of multipotent cells in vitro, and promotes new calvarial bone formation in vivo. While causative sites of *Wnt* ligand expression are still sought, overwhelming genetic evidence has demonstrated their importance [66].

Osteoblastogenesis from precursors in the fetus is dependent upon active *Wnt* signaling; in its absence, chondrogenesis ensues, either in calvarial-derived osteoblasts or in the long bone rudiments. Application of *Wnt* ligand itself is hampered by the nature of the protein; it is hydrophobic, lipid modified, and consequently insoluble. However, small molecule screens are identifying novel compounds capable of *Wnt* pathway interference or stimulation. Again, as our knowledge of the optimal stimulation of osteogenic differentiation improves, we find, perhaps unsurprisingly, that it matches well the growth factors present in hypertrophic cartilage where *hedgehog, Wnt, BMP, FGF (fibroblast growth factor) an VEGF (vascular endothelial growth factor)* ligands are all expressed [51].

21.6.3 Design and Fabrication of Scaffolds

The major limitation of the first generation of bioabsorbable scaffolds was their relatively poor mechanical properties and brittle behavior. Therefore, several reinforcing techniques have been used to improve the mechanical properties (Fig. 21.10). These include polymer chain orientation techniques and the use of fiber reinforcements. The latest innovation for bioactive and fiber-reinforced bioabsorbable composites is to use both bioactive and bioresorbable ceramic and bioabsorbable polymeric fiber reinforcement in the same composite structure. Hence, from a materials point of view, a clear trend towards the development of composites can be detected [5, 41]. It can therefore be predicted that composites will be the second and third generation of scaffold materials to enter the clinical arena.[41]

An important aspect of design and fabrication of composite scaffolds that should be addressed in more detail is the interfacial properties between ceramic and matrix phase. It appears that this issue has been neglected in the context of bone engineering, although much effort has been made in the enhancement of the interfacial adhesion in conventional polymer matrix composites. Calcium–phosphate (CaP) particle size and their respective distribution have been recognized as important parameters affecting the mechanical behavior of polymer-ceramic systems. Apparently, a smaller particle size leads to stiffer composites. Furthermore, the stiffness of the composites is proportional to the CaP volume fraction. [41]

Ceramic particles increase material stiffness and enhance creep behavior. Higher particle contents however increase the number of interfaces between polymer and ceramic. This must be considered carefully as under mechanical loading, scaffold failure preferentially occurs at the interface between polymer and ceramic. Studies by Wang and Bonfield pointed out the low efficiency of hydroxyapatite (HA) particles as reinforcement agents for high density polyethylen (HDPE), due to their inherent low aspect ratio and low degree of chemical interaction with the HDPE phase [84]. Attempts to enhance the mechanical performance investigated the chemical coupling of HDPE-HA composites by means of silane agents and acrylic acid grafting. These attempts allowed for the enhancement of strength and ductility, but did not improve the stiffness consistently. The development of coupling methodologies that increase the adhesion of ceramic particles to a polymeric matrix is believed to be a possible route for the improvement of mechanical performance of these composites. An investigation by Sousa et al. [79] showed the effectiveness of silane coupling treatments to be dependent on factors such as the particle surface area, the particle size distribution, and the

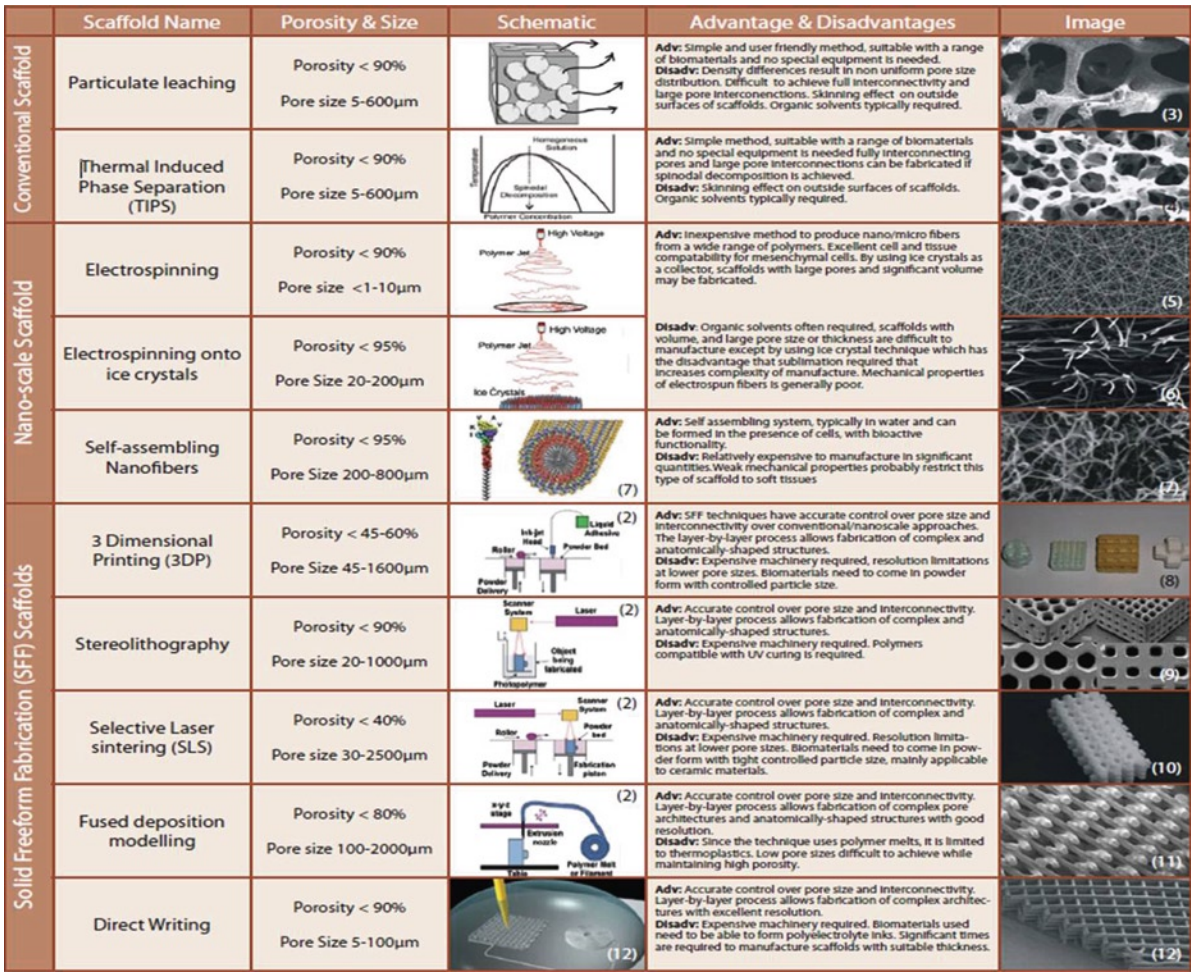

Fig. 21.10 Graphical Illustration of different scaffold fabrication techniques (reprinted with permison from 154. Dalton P, T W, Hutmacher D. Snapshot: Polymer scaffolds for tissue engineering. reprinted with permission"Biomaterials 2009 2009 Feb. (30(4):701-702.)

chemical reactivity of HA particles. Another study by the same group [29] investigated the use of alternative titanate and zirconate coupling agents and concluded that the positive effect of these agents on stiffness and strength results from their dominant effect as CaP dispersion promoters. These coupling agents proved to be noncytotoxic, which is a great advantage when compared to standard silane coupling agents.

The Davies laboratory reported the development of the so-called 'third generation' of scaffold materials [16, 55]. Their results demonstrate that the addition of a thin film of CaP to the surface of a macroporous poly(lactic-co-glycolic acid) (PLGA)-CaP composite yields an osteoconductive scaffold of higher compressive strength, which also prevents a chronic inflammatory response. This three-phase construct overcomes both biological and material limitations of previous scaffold material formulations to describe a unique, fully resorbable scaffold for use as an alternative to the trabecular bone graft.

21.6.4 Application of Bone Tissue Engineering Platforms to Study Mechanisms of Bone Metastasis

Animal models are important tools to investigate the pathogenesis of and to develop treatment strategies for bone metastases in humans. Although rodents (rats and mice) and other, large animals (dogs and cats) are known to spontaneously develop cancer, there are only

few animal models of bone metastasis [69]. Most of these experimental models of bone metastasis in rodents however require the injection of neoplastic cells into bones (e.g. tibia), or the left ventricle of the heart or cell transplantation to orthotopic locations [12].

Cells that undergo oncogenic transformation acquire the ability to proliferate independent of specific growth signals, to delay apoptosis, to stimulate neo-angiogenesis, and to invade other tissue types. They harness the tumor microenvironment recruiting normal surrounding host cells to facilitate their growth and progression. As a result, tumor and host cells undertake a complex bidirectional interplay.

Physiological bone remodeling involves the equilibrium of bone resorption and osteosynthesis. This balance between osteoblasts synthesizing bone and osteoclasts resorbing bone is distorted in bone metastasis [15] as a result of the interaction of tumor cells with the bone stroma. Notably, in all focal metastatic bone lesions, components of both new bone synthesis and osteolysis are evident. However, certain malignancies tend to favor one end of the spectrum.

Prostate and breast cancer are both likely to metastasize to bone at advanced disease stages; their metastatic profile however differs. As prostate cancer-induced bone metastases tend to demonstrate predominantly focal osteogenesis, bone metastases induced by mammary tumors are predominantly osteolytic [1].

The ability of malignant cells to detach from a primary tumor site, to invade the circulatory system, to adhere to the endothelium, and to transmigrate and establish a secondary tumor colony in the bone is referred to as "osteotropism". This spread of cancer to bone is largely restricted to breast and prostate cancers, and a small number of others, such as lung, thyroid, and kidney cancers, and multiple myeloma. A major barrier in identifying the mechanisms of breast and prostate cancers underlying osteotropism is the lack of suitable animal models fully reflecting the biological conditions favoring human breast/prostate cancer metastasis to bone and to induce skeletal metastases [9]. Hence, most human tumor models to study cancer metastasis to bone rely on introducing cancer cells directly to the circulation, either by injection into the tail vein or the left cardiac ventricle of immunodeficient mice. These models are useful to examine and identify factors involved in proliferation of breast cancer cells deposited directly into the bone environment. However, they do not replicate the early events of metastasis at the primary tumor site and, therefore, may not encompass the molecular mechanisms by which cancer cells are attracted to bone in patients. In addition, most of these models utilize highly evolved cancer cells that may not necessarily reflect the behavior of tumor cells in vivo. Recently, important advances have been made in creating animal models of human cancer metastasis to human bone [32, 40, 52].

21.6.5 Bone Chip Model vs. Tissue Engineered Bone

Human fetal bone has been transplanted subcutaneously to immunodeficient SCID (severe combined immuno-deficiency) mice to provide a site for metastasis and tumor growth of human prostate carcinoma cells injected into the left cardiac ventricle or adjacent to the viable bone substrate. This model demonstrated the cancer cells' preferential selection of human bone over mouse bone to home to and proliferate in. Similar results were also reported for adult human bone transplanted to SCID mice [52].

Human fetal bone and, recently, adult human rib derived bone have been implanted into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice, a model termed NOD/SCID-hu, and utilized to study prostate and lung cancer osteotropism. In these studies, human prostate cancer cells were administered via tail vein injections or directly introduced to the implanted bone. The administered human prostate cancer cells formed visible tumors only in the human bone implants and not in the mouse skeleton or in other human or mouse tissues implanted at the same ectopic site. Hence, this experimental model enables the study of human prostate cancer cell metastasis in a tissue-specific and species-specific manner. Kaplan and Rosenblatt have extended the NOD/SCID-hu model to characterize an osteotropic breast cancer cell line and to study the process of breast cancer metastasis to bone [61].

Despite refinements advancing the treatment of breast cancer (BrCa) in general, little progress has been made to treat bone metastasis, a complication that signals disease entry to an incurable stage. The identification of genes and gene signatures associated with metastatic processes in BrCa is progressing. In contrast, the knowledge on how bone stroma components contribute to metastasis formation is still rudimentary. With mounting evidence for a mutual recognition between BrCa and bone, various groups are now investigating

the phenomenon of osteotropism from both sides of the tumor-stroma interface. Rosenblatt et al. have created a novel "all human" model in which human bone was transplanted into immunodeficient (NOD/SCID) mice. Human BrCa cells were injected into the mammary fat pad. Metastases later appeared as metastases in the human bone, but not mouse skeleton [61].

The high frequency and mortality associated with breast cancer metastasis to bone have motivated efforts to elucidate tumor-stroma interactions in the bone microenvironment contributing to invasion and proliferation of metastatic cells. The development of engineered tissues has prompted the integration of engineered bone scaffolds into animal models as potential targets for metastatic spread. Silk scaffolds were coupled with bone morphogenetic protein-2 (BMP-2), seeded with BMSCs, and maintained in culture for 7 weeks, 4 weeks, and 1 day before orthotopic, subcutaneous implantation in a mouse model of human breast cancer metastasis. Following injection of SUM1315 cells into mouse mammary fat pads, tumor burden of implanted tissues was observed only in 1-day scaffolds. Scaffold development and implantation was then reinitiated to identify the elements of the engineered bone that contribute to metastatic spread. Untreated scaffolds were compared with BMP-2-coupled, BMSC-seeded, or BMP-2/BMSC-combined treatment. Migration of SUM1315 cells was detected in four of four mice bearing scaffolds with BMP-2 treatment and with BMSC treatment, respectively, whereas only one of six mice of the BMP-2/BMSC combination showed evidence of metastatic spread. Histology confirmed active matrix modeling and stromal cell/fibroblast infiltration in scaffolds positive for the presence of metastasis. These results show the first successful integration of engineered tissues in a model system of human breast cancer metastasis. This novel platform can now be used in continued investigation of the bone environment and stem cell contributions to the process of breast cancer metastasis [61].

Prostate cancer (PCa) is the most common cancer and the second leading cause of male cancer deaths. Despite its common occurrence, the underlying cause of this cancer and its progression to bone metastases remain poorly characterized. The ideal in vivo model would reproduce the genetic and phenotypic changes that occur with human cancer cells seeding in human bone as closely as possible. Recently developed mouse models indicate that PCa cells have a preference for human bone. While mouse tibia invasion models provide important data on bone–PCa cell interactions in vivo, they do not allow "homing" of PCa cells and are not considered metastatic models. Human fetal long bone chips implanted subcutaneously into the flanks of SCID mice provide a more appropriate human-specific bone microenvironment with intact anatomic and hematopoietic features. However, this model also has major limitations: firstly, the implanted human bone chips get poorly vascularized and hence the dead bone does not reflect the real clinical situation; secondly, it is very difficult to control size and shape of the bone core implants, which makes it difficult to establish a reproducible model; thirdly, it is more appropriate to use cancellous or cortical bone of adult humans. Therefore, it can be concluded that the bone chip model is not highly reproducible and has a significant failure rate. In contrast, Hutmacher's work has shown that tissue engineering of human bone in a nude mice and rat model is highly reproducible (a more than 90% take rate of the TEC). Depending on the cell type (osteoblast vs. BMSCs) and time of implantation, bone of different mineral densities can be engineered [40].

Hence, our interdisciplinary research program has created a novel "all human" model transplanting tissue engineered human bone into immunodeficient (NOD/SCID) mice. This model is compared to the standard bone chip model. Human derived PC3 cells are injected and we hypothesize that metastases later appear as metastases in the human bone, but not mouse skeleton. Such a model recapitulates the metastatic sequence occurring in patients much better than the bone chip model. We then will test the hypothesis that distinct "tool kits" are used by PCa metastasizing to human tissue engineered bone. In addition, we are identifying components within the bone stroma essential for metastasis, and osteotropism associated genes expressed by bone cells in response to the presence of PCa [40] (Fig. 21.11).

Current animal models to study prostate cancer are prone to complications and challenges in interpretation and relevance; there is a clear need for improved options to study the pathogenesis and progression of the disease in order to facilitate the development of novel therapeutic strategies. For these purposes, a novel 3D in vitro and an in vivo system based on tissue engineered human bone is proposed to advance the

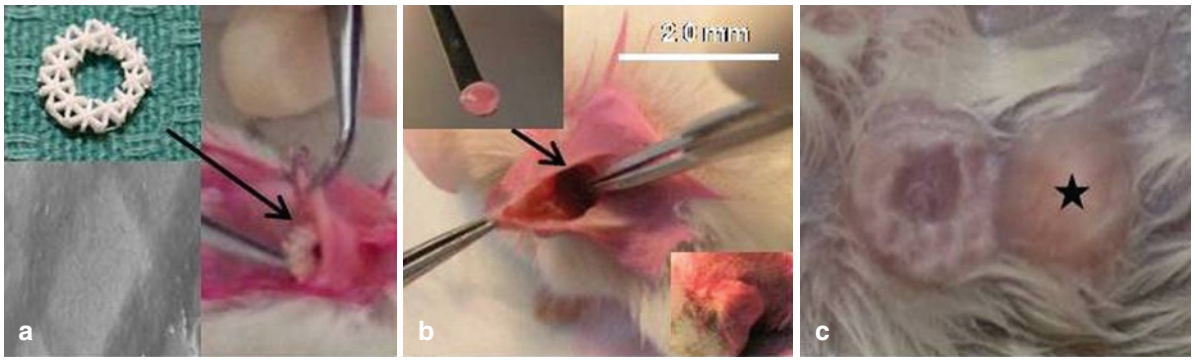

Fig. 21.11 The authors' interdisciplinary group is currently creating a novel "all human" model in which tissue engineered human bone is transplanted into immunodeficient (NOD/SCID) mice and compared to a standard bone chip model to study bone metastases related to prostate and breast cancer. (**a**) In vitro bone engineering by using mPCL-TCP scaffolds in combination with primary human osteoblasts. (**b**) Implantation of tissue engineered construct (TEC) into the flanks of NOD/SCID mice (note scaffold has a similar geometry as long bones with a hollow core which mimics bone marrow cavity). (**c**) Eight weeks post implantation of the TEC, a second surgery is performed to implant a biomimetic hydrogel seeded with breast cancer cells. Mice develop tumors 8 weeks later (*asterisk*) reprinted with permission

understanding of prostate cancer progression associated mechanisms, particularly the role of prostate-specific antigen (PSA) and the related prostatic kallikrein serine proteases (KLK) in bone metastasis [40].

21.7 Limitations/Critical View

The requirement of new bone to replace or restore the function of traumatized, damaged, or lost bone is a major clinical and socio-economic need. Bone engineering has been heralded as the alternative strategy of the twenty-first century to regenerate bone. In essence, the discipline aims to combine cells with scaffolds, or with appropriate growth factors, to initiate repair and regeneration. Despite some success by our group and others, it is widely recognized that bone engineering is not yet delivering a so called "block buster" in terms of clinically applicable concepts and product commercialization.

Up to the present time, the successful large-scale production of engineered tissues, which allows novel scaffold and cell-based bone engineering treatment concepts to enter the clinical platform, faces several challenges. Firstly, adequate sources of multipotent healthy expandable cells have to be determined while local and systemic stimuli driving cell migration, attachment, survival, proliferation, and differentiation have to be identified. Moreover, the optimization of scaffolds in respect to bulk material, architecture and porosity, mechanical properties, and surface chemistry plays a key role. In addition, the development and fabrication of bioreactors, which mimic the physiological environment of the body, can be of great benefit to form functional neotissues in a reliable fashion. Further challenges include the preservation of tissue engineered products and the successful prevention of tissue rejection. In other words, to develop a successful bone tissue engineering concept, four prerequisites are required: (1) sufficient numbers of cells with osteogenic capacity and/or growth factors; (2) an appropriate scaffold to seed cells onto; (3) suitable factors to stimulate osteogenic differentiation in vivo; and (4) sufficient vascular supply.

The first three prerequisites can be fulfilled by biomaterials bioengineering research, while prerequisite four is dependent on patient related factors, such as defect size, its pathogenesis, and preceding treatments. Lack of sufficient vascular supply resulting in immediate cell death after implantation is generally thought to be a major limitation of cell-based bone engineering strategies. The success of bone tissue engineering in small animal models (e.g. nude mice and ectopic rodent models) is explained by the far more favorable biological environment for implanted cells. Often only a few small samples are subcutaneously implanted in direct contact with the surrounding well-vascularized tissues. This shortens the diffusion depth and allows the seeded cells to be optimally supplied with oxygen

and nutrients. In addition, osseous defects in rodents are attractive sites for reconstruction. Defect sizes usually do not exceed the maximum distant depth of 5 mm, therefore allowing sufficient influx of oxygen and nutrition. Moreover, the remodeling speed in rodents is at least three times higher when compared to humans [20, 68].

New directions in research should therefore address diffusion/nutrition related problems or develop biomimectic scaffolds that recruit the appropriate endothelial and osteogenic cells after implantation as only a sufficient number of new blood vessels formed within a short period of time guarantee acceptable survival rates of transplanted cells. It has already been shown that improving vascularization of tissue-engineered constructs can advance in vivo cell performance [49, 50, 85].

Through translational and clinical research, the appeal to industry to commercialize new research products should be strengthened. Early stage development and associated product performance risks can be mitigated through a research continuum that moves from the bench to clinical practice, with industrial interests engaged along this path. New start up spin-out venture companies, and multicenter clinical studies should result from an applications focus that intensively involves clinical researchers right from the beginning of a bone engineering project [36, 71].

21.8 Conclusion/Summary

Tissue regeneration strategies based on scaffold/cell constructs, growth factor administration, and adult stem cell-based therapies have undergone significant development over the past two decades. Most notably, we are much closer to realize the engineering of bone for medium to high-load bearing defect sites with complex anatomies than we were 5 years ago. A major driving force has been the demand placed by the scientific community and clinicians to go beyond simple engineering of tissues and to demonstrate functionality in engineered tissues and functional recovery upon transplantation. Some recent advances include de novo engineering of bone and the prevascularization of entire constructs. Several challenges have to be overcome to establish regenerative bone engineering as a viable science of the future. One of the key issues will be the evolution of programs and policies that promote a close relationship between government agencies, the private sector, and academia, specifically between biomaterials scientists, biologists, and clinicians.

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Tissue Engineering of Organs: Brain Tissues

22

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22.1 Introduction

Anatomical organization and precise interconnectivity of neurons in the adult brain are critical for correct physiological function and integration of cognitive, sensory, and motor control. Individual neurons, the functional signaling units of the brain, are essentially highly integrated signal transducers. Inputs in the form of membrane potentials travel uni-directionally to the cell body of the neuron via membranous branches called dendrites, and then proceed down a larger elongated process, the axon, to join at closely apposed membrane junctions (synapses) with neighboring neuronal cell bodies. The highly precise spatial patterning of neuronal connections established during early brain development is the result of an orchestrated series of programmed differentiation events, regulated by controlled cell proliferation, migration, and differential adhesion. In particular, the establishment of connectivity relies heavily on the guidance of axons from their points of origin to their appropriate targets, over variable distances and in a precise spatial manner. This process is regulated by a host of environmental cues presented to the growth cone, a specialized ending at the tip of immature neurons, in specific spatial and temporal sequences. The spatial location of neurons, their relationship to other neurons, and the three-dimensional (3D) distribution of their axons within the nervous system are all crucial for normal brain function. The sheer complexity of adult brain anatomy and physiology, the adaptive and dynamic nature of the tissue, and the fact that many aspects of function are still being defined biologically, present major challenges for brain tissue engineering (BTE) and regeneration approaches.

The regenerative capacity of neurons in the brain following disease and injury is very limited and as a result, therapy is largely limited to rehabilitative measures at the level of behavioral entrainment of the patient. This limited regenerative capacity is due to the gradual loss of the intrinsic ability within the extracellular environment to promote cell division, axon outgrowth, and major structural remodeling at the tissue level, at the completion of corticogenesis (toward the end of fetal and neonatal development) [95]. There is, however, residual neuronal adaptability or "plasticity" in the adult brain that is modulated at the level of individual cells and synapses, which is evident in cases of enhanced and relocated cognitive function in patients following severe brain lesions [136]. Like all tissues, the central nervous system (CNS) also undergoes a staged inflammatory response following mechanical insults (e.g., compression or tearing) or other damage (e.g., ischemia or embolism). In the short term, localized fluid accumulation and leukocyte infiltration contribute to the formation of a cytotoxic environment at the injury site. In the longer term, the wound site is usually replaced by glial scarring, in the form of fibrotic tissue. Thus, in addition to the limited regenerative capacity, biochemical and biomechanical

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immunogenic factors at the injury site can act in unison to dramatically inhibit axon regrowth [70, 132].

Tissue engineering can be considered to be an application of engineering disciplines dedicated to the development of solutions, based on an understanding of biological systems, for the repair and restoration of dysfunctional tissue. Typically, solutions are envisaged as variable combinations of the following components: cells, scaffolds, and biomolecules. The optimal combinatorial treatment is dependent on the cell and tissue structures affected. In the context of brain repair, drug therapy represents a complex challenge in terms of demonstrating neuroprotective benefits, such as the modulation of the innate immune response [105]. In addition, the biological causes of many neurodegenerative brain conditions remain largely undefined. For these reasons, the topic of pharmacological induction of neuronal protection and/or regeneration will not be explored in depth in this chapter. Regardless of the cause of injury, under certain situations, the simple replacement of cells or scaffolds alone may provide a tissue engineering repair strategy that is sufficient to restore tissue function. Cell replacement therapies are particularly useful in this way, in particular for disorders that result in multifocal lesions such as multiple sclerosis (MS), where centrally administered cells can be targeted to focal sites of degeneration to promote repair. To these ends, there have been extensive studies into the efficacy of embryonic and adult stem cells as deliverable neural progenitor cells (NPCs) in experimental models, and more recently in clinical trials [8, 41, 57]. Similarly, scaffold biomaterials are also emerging as having therapeutically relevant uses in brain repair, although their clinical applications have not yet been fully realized. It is plausible that an optimized combination of stem cells and biomimetic scaffolds can further enable the bypassing of formidable barriers such as cytotoxic immunogenic microenvironments or local scar regions. *This chapter will focus on the design, fabrication, and biofunctionalization of scaffolds to enable neuronal repair in the diseased or injured brain, in particular at focal injury sites.* Some consideration of combinatorial approaches will also be made in due course.

The ultimate goal of BTE is to restore tissue structure and function to its original state. Loss of brain function often reflects the loss of connectivity between specific tracts or centers; hence, the main emphasis is on encouraging directed axonal regrowth. Recent efforts aimed at regenerating axons have exploited stem cells to recapitulate the events of embryonic development that promote axon extension and guided outgrowth to appropriate targets, and to eventually reinstate the reformation of neuronal connections in the mature brain tissue. During development, this is a complex physicochemical interplay between cell populations and their extracellular microenvironment in a 3D orientation. In this regard, BTE often involves the synthesis of implantable, 3D porous biomaterial scaffolds that act as supportive cellular microenvironments, a feature that is becoming increasingly important in terms of mature brain function, and induction of local tissue repair.

The concept of the cellular microenvironment (or niche) is central to BTE scaffold design, particularly in relation to biological aspects of neuronal regeneration in situ, where there exist overlaps with aspects of stem cell biology and specific cell–cell and cell–substrate interactions. There are intrinsic differences between the structure and function of the embryonic and adult brain that need to be considered; while the embryonic brain is involved in the establishment of neuronal networks, axonal fasciculation, and myelination, the adult brain is often engaged in a process of neuronal plasticity, conserved fasciculation, and maintenance of consolidated synapses without anatomical reorganization. As a consequence, compared to embryonic development, regeneration in an adult brain requires axons to traverse longer distances in order to reform connections within established neuronal pathways. In addition to intrinsic differences between the adult and embryonic brain, there are distinct differences within the different extracellular environments and their response to injury, such as the formation of physical scars and the local release of inhibitory factors by microglia and reactive astrocytes [51]. The multitude of guidance cues responsible for sculpting the neural connections of the brain are downregulated upon completion of development, but can still persist within the mature CNS to preserve the intricate neuronal circuitry by exerting inhibitory influences on axon outgrowth [159]. For example, certain signaling molecules, such as myelin-associated glycoprotein (MAG) can have variable effects on neurite outgrowth, transitioning from a promoter to an inhibitor of neurite outgrowth at birth [33, 69, 159]. Therefore, while it is important to fabricate niche microenvironments that encourage neuronal differentiation and guided neurite outgrowth, neuronal regeneration in an adult brain may not necessarily require identical conditions to those present during development. Regardless of the precise biological mechanisms involved at the

cellular level, BTE scaffolds represent the most adaptable materials engineering strategy for clinically relevant brain tissue repair due to their control of cell-biomaterial interactions, matching of structural and physical properties, and the provision of microenvironments at the cellular scale, as discussed in further detail in the following sections.

22.2 Aim of the Discipline

Disease and injury occurring in the brain often result in significant disruption of the normal physiological structure and function, with common occurrences of neuron death and/or demyelination. Loss of brain function often reflects the loss of connectivity between specific neuronal populations; hence, a key hypothesis in BTE is that entrainment of axonal guidance to designated targets can lead to restored function. Because of the multiple cell types involved in disease and injury, there is no single strategy to achieve this. However, common to many BTE strategies is the fabrication of synthetic cellular micro-environments, which consist of critical features to promote tissue regeneration by assisting combinations of cell proliferation, neuronal migration, and differentiation. Remnants of the permissive and instructive environment present during development persists within the adult brain, but are confined to distinct anatomical regions in the form of stem cell niches. Under certain injury conditions, endogenous neuronal stem cells (NSCs) in adult mammalian brains have been shown to imitate embryonic development to achieve regeneration [4, 20, 45, 46, 96, 148]. Thus, most approaches are on the basis of the belief that the molecular pathways responsible for directing appropriate cellular behavior during embryonic development are conserved in the adult brain, and participate in the process of regeneration. However, the confinement of neurogenesis to highly compartmentalized locations in the brain suggests that an additional role of the stem cell niche is to shield ongoing neurogenesis from inhibitory influences that naturally occur within the brain. Therefore, an additional unique complement of regulatory mechanisms must also be present for neurogenesis to proceed in the adult brain [118].

When exposed to the appropriate conditions, stem cells can be coaxed into committing to different cell lineages, producing specific cell types (e.g., neurogenesis and gliogenesis) that may be required for different BTE strategies. This is important because brain tissue consists of many cell types, including neurons, astrocytes, microglia, and oligodendrocytes; so tissue repair will also require the use of multiple cell types. Stem cell function is largely dependent on the niche conditions in which they exist. As the natural stem cell niche is a dynamic environment that is subject to alteration by external influences, this provides a gateway for intervention in stem cell behavior by engineering a synthetic niche bearing the critical properties required for tissue remodeling.

22.2.1 The Neuronal Niche

It has long been established that stem cells exist in niches, which are specialized microenvironments capable of maintaining the immature state of stem cell populations, in terms of self-renewal and multipotency, as well as partially dictating its differentiation pathway [109, 118, 126]. A stem cell niche is a dynamic environment that provides structural support, architectural features, and a range of support cells and signaling molecules, which are all instructive for directing stem cell behavior in response to the state of the tissue. The presence of NSCs within discrete locations of the adult brain is highly relevant to the development of therapeutic brain repair strategies, whether it is through stimulation of the endogenous populations, or by transplantation into affected areas. Both approaches require comprehensive understanding of how regulation of stem cell behavior is achieved normally in vivo. Adult NPCs have been isolated in two distinct regions of the human brain – the subventricular zone (SVZ) and subgranular zone (SGZ); these regions have been extensively studied to identify the critical components that define its unique ability to foster the maintenance, growth, and development of stem cells [3, 37, 97, 118, 126]. While much progress has been made in identifying individual components that form these niches, future success in recreating a synthetic niche environment will require a comprehensive understanding of their histological and extracellular composition, and how the different components interact and converge to give rise to precise control of stem cell behavior.

Physical, structural, and architectural cues within stem cell niches exist in the form of a specialized basal lamina (enabling cell anchorage) and various extracellular matrix (ECM) components that modulate the availability of cell signaling molecules by sequestering

relevant growth factors and cytokines [118]. Different types of support cells exist within the niche (e.g., astrocytes, ependymal cells, and endothelial cells), which generally function to integrate signals from within the niche environment, and influence resident stem cell behavior via the secretion of paracrine factors or contact-mediated cues accordingly. These signals are influenced locally by neurotransmitters secreted from synaptic junctions, and long-distance signalling from hormones and cytokines in the blood stream. Locally, niches can also be influenced by trophic factors, mitogens, and morphogens – secreted from neighboring populations of support cells and differentiated cells, which form part of a complex feedback mechanism [109, 118, 126]. The general view is that an acute release of factors from injury sites can induce differentiation in these niches, providing new differentiated cells for repair.

22.2.2 Engineering Cellular Microenvironments

The basis of most tissue engineering strategies is to first engineer a biomimetic of the extracellular microenvironment with favorable architecture to encourage cell adhesion and survival. The native extracellular microenvironment consists of a myriad of biomolecular factors that can affect cellular processes in vivo; these can be classified broadly into three main components [85]:

1. Insoluble hydrated macromolecules
   - Fibrillar proteins (e.g., collagens)
   - Noncollagenous glycoproteins (e.g., elastin, laminin, fibronectin)
   - Hydrophilic proteoglycans with large glycosaminoglycan side chains
2. Soluble macromolecules
   - Growth factors
   - Chemokines
   - Cytokines
   - Peptides
3. Cell-surface glycoproteins
   - Growth factor receptors
   - ECM receptors

The molecular structures that make up the cellular microenvironment (including the ECM) represent a complex array of signals manufactured by cells, to which they respond in a temporally and spatially coordinated manner. Ultimately, the highly organized nature of cell populations is critical in complex tissue dynamics such as tissue formation, homeostasis, and regeneration [85].

Scaffolds can be engineered to impart a range of biochemical and biomechanical cues that can be presented as signaling entities that guide neurite outgrowth. Such cues interact with the growth cone of a neurite and can act to either attract or repel outgrowth, enabling a growing axon to navigate through neighboring microenvironments toward specific targets [5]. Much progress is being made in understanding the roles of specific cues within the extracellular microenvironment, and how these cues can be translated and used in combination to have synergistic and hierarchical effects on specific cellular processes that directly affect the regeneration of brain tissue.

Physical cues can be incorporated into a scaffold by controlling the morphology and surface topography during fabrication. Aligned features, acting as conduits, can be in the form of electrospun polymer nanofibers or regular patterns of surface-etched grooves and ridges on planar surfaces, all of which can direct neurite outgrowth by a process known as contact guidance [73, 116, 142, 158]. The dimensions of such features are often at the micro/nano-scale, mimicking the structure of native tissue. Substrate stiffness is another relevant physical cue that is known to affect neuronal development by coaxing stem cells down a particular lineage [41], as well as influencing the extent of neurite outgrowth in cells that have committed to the neuronal lineage [6, 43, 67, 78, 151]. In addition, matching mechanical properties between the scaffold and the native brain tissue will play a role in controlling inflammation, which will facilitate scaffold integration. Therefore, during scaffold design, considerations must be made to ensure that scaffold properties (e.g., elastic modulus and rheological properties) are similar to those of the native brain.

There have been many attempts to measure the viscoelastic properties of the brain. However, reported values in the literature often have large variations, which can be attributed to inherent biological variability in tissue properties (e.g., age, sex, species, region of brain tested, etc.), and more critically on the measurement protocol (e.g., methodology and apparatus used) [22]. While in vitro testing presents a simple and practical way of measuring physical properties of the brain,

its accuracy in comparison to the properties of the brain in its natural state is questionable due to differences in hydration, blood perfusion, temperature regulation, postmortem tissue degeneration, and sample preparation artifacts [22]. In vivo testing is practically more difficult, and often ethically impossible in human subjects. Recent advances in magnetic resonance elastography (MRE) have made in vivo testing of viscoelastic properties in human and animal brains possible. However, its accuracy is limited because of attenuation of the propagating waves by tissue viscoelasticity, and variations in tissue stiffness within the intact structure. Nevertheless, the reported storage and loss moduli of human brain tissue, based on MRE, are in the range 1–12 kPa and 1–6 kPa, respectively [22, 53, 54, 149]. Because of the brain's neuroarchitecture, it is expected that regional differences in the physical properties and a certain degree of anisotropy will exist. In addition, the bulk physical properties of the brain are influenced by factors such as hydration and intracranial pressure; hence, the relevance of these measured quantities to tissue engineering at the micro- and nanoscale is yet to be determined.

The effectiveness of biochemical cues in facilitating neuronal regeneration depends on the type of molecule and the method of incorporation, as these will influence the molecule's orientation and presentation to cells, and hence its bioactivity. Many proteins can have multiple binding motifs within the same molecule (e.g., RGD, IKVAV, and YIGSR within laminin), each of which can exhibit specific bioactivities. The stability of attachment will also determine whether presentation of the biomolecule is sustained (e.g., covalent attachment) or time-dependent (e.g., soluble, where the biomolecule can eventually be internalized by cells or diffuse away). Patterning of substrates with chemical moieties can be used as an additional means of controlling cell behavior. "Stamping" of substrates to create tracks or grids of specific biomolecules has been used to define the spatial location of a population of cells [17, 56, 121, 122]. Neurites from dorsal root ganglia (DRG) cultured on 25 μm wide tracks of vitronectin align with the direction of the adhesive tracks after 24 h of culture [17]. Similarly, NSCs cultured on an interconnected array of poly-L-lysine (PLL) square patches (with area in the order of $10^4\,\mu m^2$, connected by lines 100–400 μm long and 15 μm wide) extend cellular protrusions along the interconnecting lines toward adjacent squares (Fig. 22.1) [121, 122]. Orientation of neurites on such patterned features is likely to reflect spatial confinement to regions of the substrate where outgrowth is favorable. This is supported by the observation that neurites growing outside of substrates patterned with protein retract back to their cell bodies [122]. Biomolecular patterns can also be deposited in the form of a static density gradient, which can potentially guide axon outgrowth along the gradient [71, 93]. This mechanism is known as chemotaxis, and is important for neurite guidance during development of the brain [36, 62, 134, 141].

An understanding of cell-scaffold interactions is also critical for the design of BTE scaffolds, particularly if a cell-based therapy is involved. One of the advantages of scaffolds is their ability to entrain three-dimensionality, which can simulate tissue structure outside the physiological environment, a capability that even optimized in vitro tissue models fail to achieve. Scaffolds generally provide a relatively stable and potentially more representative physiological environment supporting cell outgrowth, enhancing cell survival and maintaining pluripotent populations as an artificial niche microenvironment.

Current research has been directed toward understanding the mechanisms by which cells interpret such cues, and the optimization of individual cues required to direct neurite outgrowth. While the presentation of individual cues to growing neurites in vitro has shown great promise in guiding neurite outgrowth, their effectiveness in vivo remains a major challenge. It is expected that within the complex and dynamic physiological environment, the ability of individual cues to guide neurite outgrowth may be limited. Therefore, studying the use of a combination of cues within a scaffold, which can potentially act synergistically to guide neurite extension, will become an important step toward achieving neuronal regeneration in vivo. This is a promising approach as structures at different length scales can also be incorporated to control different aspects of cell behavior. While the guidance of neurites to appropriate targets will ensure that signal outputs from regenerated cells are directed toward the right area, signal transmission between cells will depend on the correct formation of synapses (point of functional contact) at the designated target. Similarly, neurons need to receive input from the "right" type of cells, i.e., dendritic processes must also have appropriate connections. The ability to control neuronal positioning will also play an important role in reforming

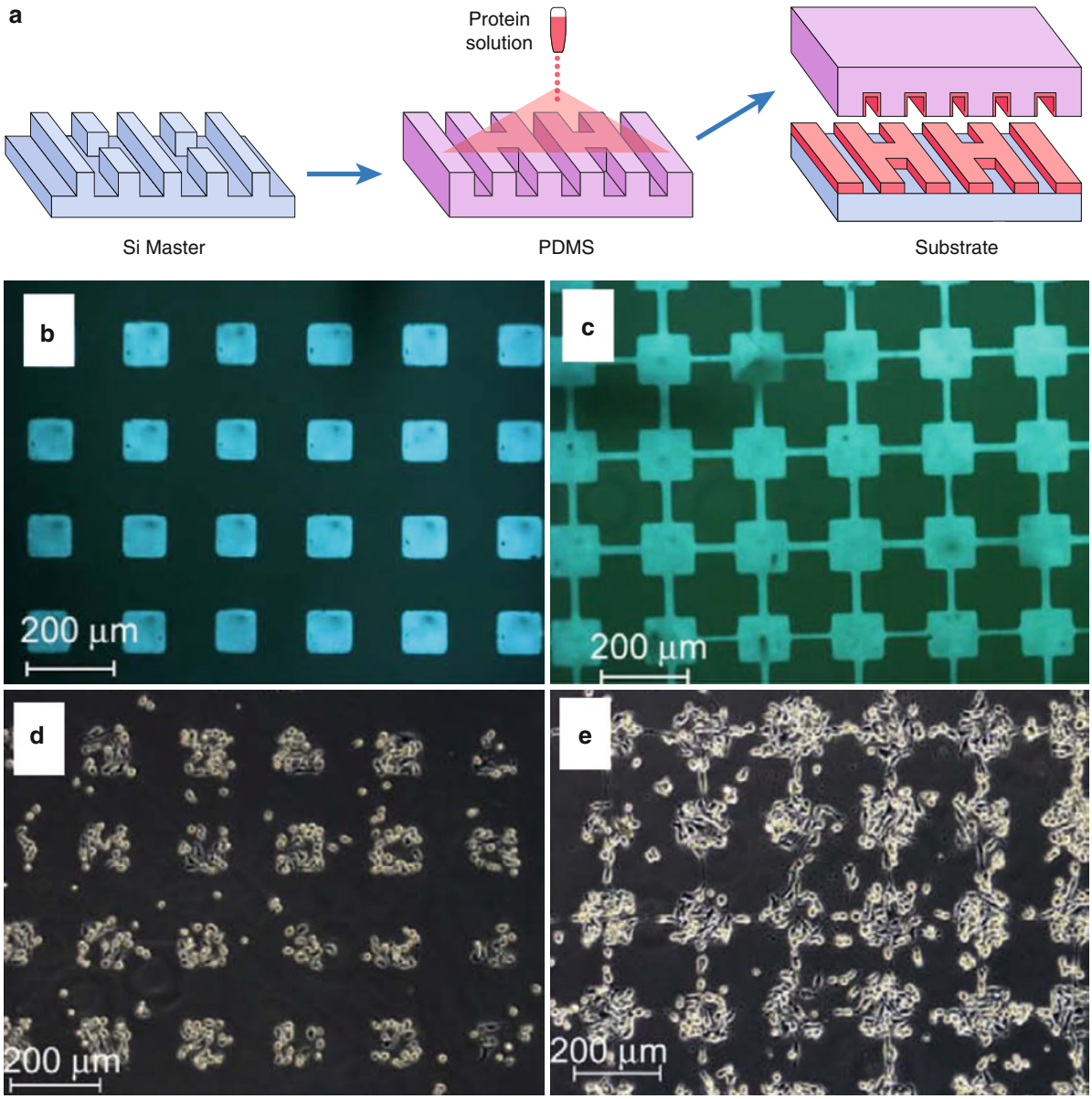

Fig. 22.1 (**a**) Schematic diagram illustrating the process of generating micropatterns by microcontact printing. (**b**, **c**) Fluorescence images of poly-L-lysine (PLL) patterns (light) microstamped onto PEO substrates (dark). (**d**, **e**) Fluorescence images of human umbilical cord blood neural stem cells (HUBC-NSCs) cultured on PLL-patterned regions (corresponding to (**b**) and (**c**)) of PEO substrate. (**a**) *Reproduced from Journal of Biomaterials Science, Polymer Edition, with permission of Brill.* (**b**–**e**) *reprinted from ref.* [122]. *Copyright (2008), with permission from Elsevier*

appropriate connections. Axon regenerations arising as a result of implants in the peripheral nervous system (PNS) have been shown to be capable of forming functional synapses at their designated targets, where electrical stimulation can trigger the release of neurotransmitters from axon terminals [73]. Despite differences between the CNS and PNS, neuronal circuits in the adult brain remain partially plastic (where neuronal connections are amenable to a certain degree of modulation), and continually refined throughout life, a property that enables learning [70]. Therefore, the potential for refinement of nascent synapses appears possible through rehabilitation. Despite the many challenges that still exist in the field of tissue engineering,

the foundations appear to be in place for functional recovery in the brain to be achievable.

22.3 State of the Art

As mentioned earlier, along the lines of the three well-established axioms of tissue engineering, it is envisaged that BTE strategies will consist of the following:

1. *Cells*
   - Exogenous neuronal progenitor cells that replenish specific cell types lost due to disease or injury through regeneration and proliferation
   - Other cell types, such as glia, may also be required to act as biological support that is representative of native niche microenvironments in the brain
2. *Scaffolds*
   - Biocompatible materials with appropriate morphology and surface chemistry to ensure cell survival (i.e., by shielding cells from inhibitory environment within the injured brain) promote cell growth, and help direct certain aspects of cellular development
3. *Biological signaling*
   - Biofunctionalization of biomaterial surfaces to encourage tissue integration in vivo
   - Use relevant biological signals to mimic in situ conditions, eliciting specific cellular responses that are critical during regeneration

On the basis of these requirements, each of these components will be described in greater detail below.

22.3.1 Exogenous Cell Source

Exogenous cells are required as a source of cell replacement, and stem cells are often the primary choice because they present a renewable source of multipotent cells. While embryonic stem cells (ESCs) can be a possible exogenous source, their use in cell-based therapies is currently limited by feeder-dependent growth (expansion), immunosuppression, and their propensity to form teratomas in vivo [12, 108, 135]. On the other hand, adult (somatic) stem cells present a ready-to-use cell source, which have not yet been associated with tumor formation or toxic side effects when used in experimental and clinical settings in vivo settings [108]. The mechanism by which various transplanted somatic stem cells (e.g., hematopoietic stem cells or HSCs, mesenchymal stem cells or MSCs) promote neuronal repair has been attributed to not only their ability to act as a source of cell replacement, but also their ability to constitutively secrete neuroprotective and immunomodulatory molecules, which can preserve surviving cells [108].

Following disease or injury in the adult brain, successful repair and regeneration requires the expansion of a quiescent stem cell population, cell migration to the lesion site, differentiation into appropriate phenotypes, and integration into existing neuronal circuitry. It is acknowledged that sensitivity to signaling molecules and the subsequent cellular response will vary depending on the progress of a cell developmental program; therefore, it is accepted that optimal cellular response can only be obtained when a spectrum of biochemical and biophysical cues are presented to them in a temporally and spatially coordinated manner. The type of cells used in BTE applications will ultimately affect the scaffold properties and biological signaling required. As individual stem cell niche components are better understood we believe that the incorporation of support cells within scaffolds will be of equal importance in neural tissue regeneration applications.

In the same way that specialized support cells are present in the stem cell niche to help sustain stem cell function, incorporation of support cells in synthetic scaffolds are also required to facilitate the regeneration process. While it is envisioned that exogenous stem cells will promote regeneration by replacement of lost cells and repairing neuronal circuitry, support cells are expected to aid in this process via secretion of diffusible factors (such as growth factors and cytokines) and contact-mediated cues. The ability of support cells to interact with stem cells, and secrete a wide range of molecules at physiologically relevant concentrations will result in a scaffold with greater degree of biomimicry. In fact, many studies have demonstrated the benefits of cocultures of glial cells (e.g., astrocytes) with neuronal cells in promoting overall cell survival and interaction with the scaffold [9, 115, 116, 129]. Thus, incorporation of glia into scaffolds not only acts as a better physiological representation of the native cortical tissue, but it also imparts an added dimension of biochemical complexity that, thus far, cannot be

replicated through control of the scaffold fabrication process alone. Furthermore, the inclusion of exogenous cells within scaffold microenvironments allows local sequestering of factors that are actively secreted by these cells.

22.3.2 Scaffold Materials

The success in any tissue engineering strategy requires the restoration of functional tissue, which usually depends on effective integration of an engineered-tissue construct with host tissue. The degree of tissue integration depends on both the interplay among various elements of the implanted scaffold with native functional tissue at the biomaterial interface, and matching of properties between implant and the host.

The design of a scaffold with suitable properties for tissue engineering can be considered as comprising of two main components – materials selection, and fabrication method. The fundamental role of scaffolds is to ensure cell survival, and to enable controlled proliferation and differentiation. In order to achieve this, the choice of material and fabrication method must take into account the primary requirements of a scaffold, which include the following:

- Biocompatibility
  - The materials used must not be cytotoxic or immunogenic
- 3D architecture and pore structure
  - High porosity will result in a large surface area-to-volume ratio for cell attachment and interaction at the material interface
  - Highly interconnected pores will enable cell infiltration and migration, as well as efficient mass transport and gas exchange of nutrients and metabolic waste
  - Pore size will affect cell infiltration, migration, and orientation
  - Appropriate structural morphology and topography will facilitate cell infiltration and help shape new tissue formation
- Controlled biodegradation
  - Products of degradation should be noncytotoxic and bioresorbable
  - Rate of degradation should match with the rate of new tissue formation

Given that success in BTE requires integration of regenerated cells with the existing functional neural circuit under in vivo conditions (i.e., following scaffold implantation), it is of primary importance that the foreign body response (FBR) is minimized. Any surgical implantation procedure in the brain will in itself elicit a multistaged inflammatory response that is mediated by microglia and astrocytes. The early phase of inflammation is considered a "destructive" response in which damaged or foreign tissue is removed by phagocytosis and cytotoxic molecules are secreted, which contribute to tissue necrosis. [2, 47, 138] Upon removal of all foreign material and damaged tissue, the inflammatory response transitions to a "cytotrophic" phase that aims to restore tissue integrity and cytoarchitecture through the secretion of anti-inflammatory cytokines, adhesive ECM molecules, and growth factors to facilitate neuronal regeneration [7, 132]. Disruptions to the events of inflammatory signaling between repairing cells can lead to chronic inflammation, where the initial cytotoxic phase is prolonged, leading to detrimental effects on the surrounding brain tissue. The continual presence of a foreign material, as in the case of an implanted scaffold, can upset this balance, leading to an extension of the FBR. In the context of brain repair, the ensuing encapsulation induced by the FBR is a detrimental process that will prevent integration of the implanted scaffold with the surrounding host tissue, impeding the re-integration of neuronal circuits.

It was originally proposed that the FBR was induced by biochemical reactions at the cell-scaffold interface because of nonspecific protein adsorption, acquired in vivo or in vitro, on the biomaterial surface [114]. This protein is detected by host immune cells and identified as foreign, which implies that the type of material and surface functionalization are critical factors determining the extent and severity of the FBR [88]. However, recent evidence suggests that pore size is a major contributing factor that promotes the healing of biomaterial implants by suppressing the effects of the FBR. Silicone elastomer and cross-linked poly(2-hydroxyethyl methacrylate) (PHEMA) were processed via sphere templating to create a scaffold with pores that were both highly uniform in size and highly interconnected. An optimal pore size of roughly 30 μm was found to significantly reduce fibrosis and enhance vascularization compared to scaffolds of other pore size, when implanted in the heart muscle

and percutaneously through the skin [113]. It is speculated that the attenuation of the inflammatory response is partly due to the ability of the pores to restrict macrophage migration, thus modulating their activity in the inflammatory response. In the delivery of a scaffold, the surgical implantation procedure will in itself elicit an inflammatory response. While inflammation is inevitable, it is actually an innate neuroprotective mechanism, aiding the healing process and providing a coordinated balance between damage control and tissue repair. Indiscriminate inhibition of cells mediating the inflammatory response (such as reactive astrocytes) can result in more damage by allowing widespread inflammation to run unabated in the initially unaffected neuronal tissue surrounding the injury site. [42, 94, 132] A key to successful tissue repair requires that such inflammatory response subsides gradually, without persisting as a chronic condition that will lead to a FBR. Recent studies within our group have investigated the inflammatory response associated with the implantation of poly-D-lysine (PDL)-modified xyloglucan hydrogels and electrospun poly(ε-caprolactone) (PCL) scaffolds, into the caudate putamen of rat brains [101]. These studies showed that the implants initially elicited an acute inflammatory response (peak response at 3 days post implantation), characterized by rapid migration of microglia and astrocytes to the implantation site. Staining for collagen III and IV (molecules secreted by reactive astrocytes that form part of the glial scar) showed no increase in the levels of these molecules at the implant-tissue interface. Furthermore, the presence and coexistence of astrocytes and neurites in the vicinity of the implant indicate the absence of scar tissue formation, and fibrous encapsulation (due to FBR), around the scaffold. In both cases, neurites from the surrounding host tissue were able to penetrate into the implanted scaffolds, the timing of which coincided with the attenuation of microglia cell numbers and a rise in astrocyte cell numbers. This signals the transition of the inflammatory response from a cytotoxic to cytotrophic phase, which may be essential in allowing the scaffold to present a permissive environment that encourages neurite infiltration and restoration of local cytoarchitecture (Fig. 22.2). Similar results have been observed in other types of scaffolds, such as hyaluronic acid (HA)-based [31, 59, 143] and poly[*N*-(2-hydroxypropyl)-methacrylamide] (PHPMA) hydrogels [38, 39, 153], poly(glycolic acid) (PGA) fibrous scaffolds [107], and self-assembled nanofibrous scaffolds [40], following implantation into animal brains.

Controlled biodegradation of the scaffold during in vivo conditions is important because the long-term biocompatibility of any implanted material in a biological environment is questionable. Ideally, the scaffold only acts as a temporary substrate to support and guide tissue outgrowth. Gradual elimination of the scaffold will make space for new tissue formation; therefore, it is vital that the degradation rate matches with that of tissue formation. This is particularly important because scaffolds often present a highly permissive environment for cell ingrowth, but not necessarily cell outgrowth beyond the material to integrate with the host tissue. Often, additional cues may be required to direct neurite extension beyond the permissive scaffold environment, or alternatively, controlled scaffold degradation can force cells to gradually integrate and reform connections within the host brain. This is best depicted by the reciprocal interactions between NSCs preseeded in a micro-fibrous PGA scaffold and host tissue after implantation into a brain cavity caused by hypoxic ischemia [107]. Transplanted NSCs adhered and migrated through porous scaffolds and were able to differentiate into all three neural lineages (neurons, astrocytes, and oligodendrocytes) in vivo. There was also evidence that the implanted cells interacted with the network of neurons and oligodendrocytes that had infiltrated the scaffold from the host tissue. The gradual degradation of the PGA scaffold is believed to assist with the integration of the NSC-PGA complex with the host tissue, and the spontaneous cell ingrowth from the endogenous tissue, and cell outgrowth from within the transplant. In addition to providing a permissive environment, implantation of the NSC-PGA scaffold was associated with a reduction in the inflammatory reaction and astroglial scarring, both of which are adverse secondary processes caused by CNS injury that will otherwise impede neuronal regeneration.

The predominant biomaterials used in BTE are polymer-based because they have mechanical properties that are comparable to the native brain tissue, as well as flexibility in processing that allows for the fabrication of sophisticated structures and tailored properties (Table 22.1). A wide range of biomaterials, both naturally-derived and synthetic in origin, have been used under in vitro conditions to investigate the effectiveness of different strategies in guiding cell behavior. However, the choice of biomaterial becomes more

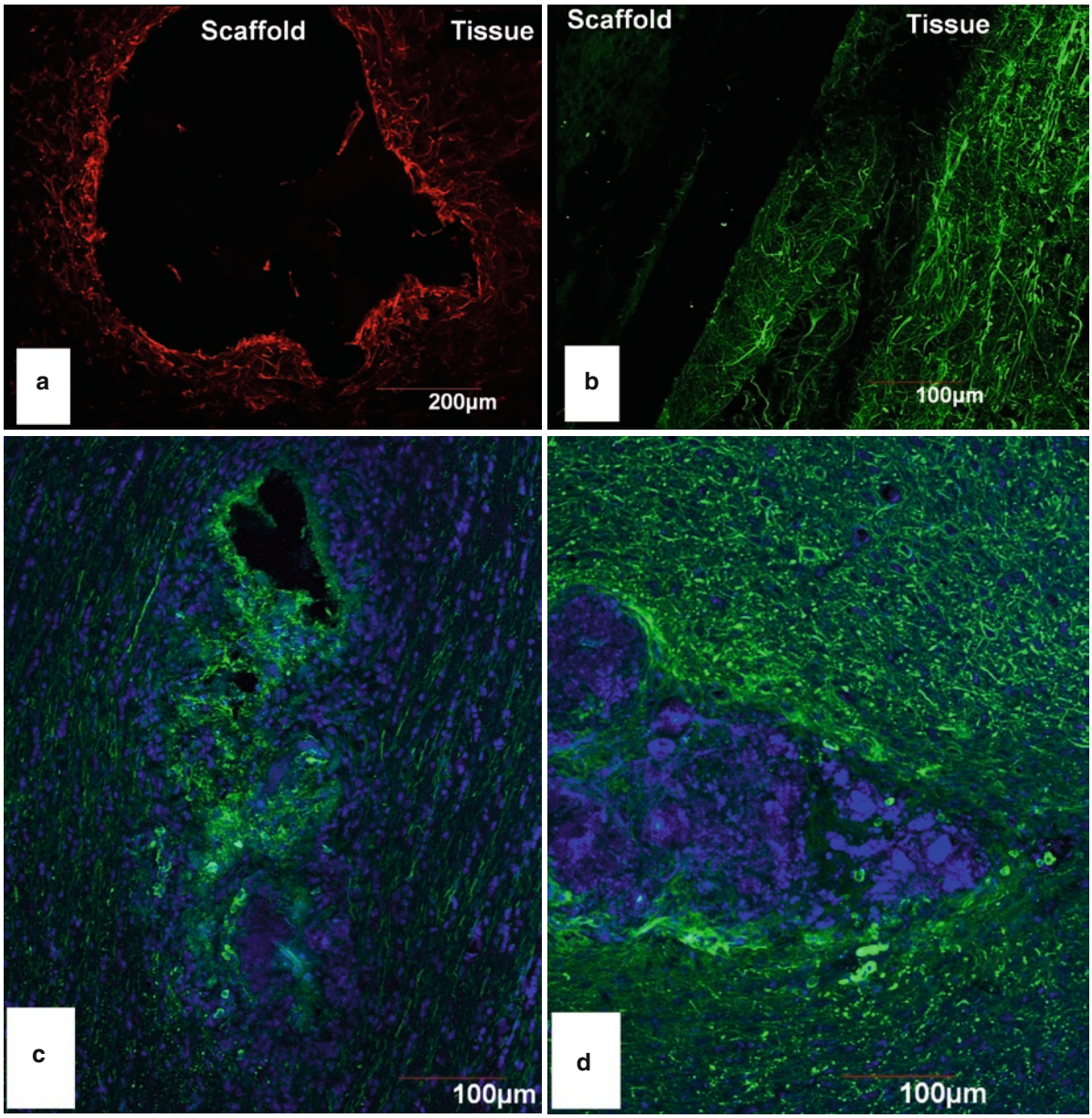

Fig. 22.2 Fluorescence images showing sections of electrospun PCL scaffolds (with randomly oriented nanofibers) (**a**, **b**) and poly-D-lysine (PDL)-modified xyloglucan hydrogels (**c**, **d**) 60 days after implantation into rat brains. The cross-section (**a**) and longitudinal section (**b**) of the implanted electrospun PCL scaffold (not immunostained) showing the coexistence of residual GFAP-positive astrocytes (*red*) and neurites (*green*) at the tissue-scaffold interface, indicating the absence of scar formation at the site of implantation. The longitudinal (**c**) and cross-section (**d**) of the implanted PDL-xyloglucan hydrogel, showing the infiltration of neurites (*green*) and other glia cells (DAPI-stained; *blue*) into the scaffold. Cells infiltrating into the scaffold are disorganized in nature, which can be clearly distinguished from the highly organized endogenous tissue

restricted for in vivo applications because the material becomes exposed to a more complex environment where scaffold biocompatibility and biodegradability become more important. With the wide range of processing technologies available, the chosen biomaterial can often be manipulated in an appropriate manner to yield a scaffold with the desired bulk mechanical and surface properties. The advantages associated with the use of naturally-derived polymers include the biological recognition that are sometimes intrinsically

Table 22.1 List of scaffold materials that have been used for in vivo brain repair and their corresponding effectiveness to induce neural regeneration

Material	Scaffold structure and properties	Implant site	In vivo response	References
Poly (glycolic acid) (PGA)	Woven array of PGA microfibers (fiber diameter of 10–15 μm) Scaffolds were preseeded with NSCs, which were maintained for 4 days prior to implantation	Cerebral infarct in mouse model of hypoxic ischemia (HI)	No glial scar formation at scaffold-tissue interface Neovascularization within scaffold Intricate reciprocal interactions between implanted scaffold and injured brain evident from the extensive neurite ingrowth (from host tissue) and outgrowth (from seeded NSCs)	[107]
Poly[*N*-(2-hydroxypropyl)-methacrylamide] (PHPMA)	3D hydrogel structure-Modified with *N*-acetylglucosamine (NacGlc) groups to improve interaction with cells	Frontal cortex of adult rats	No discernable macroscopic inflammationGood integration of scaffold with host brain tissue (esp. in NacGlc-PHPMA) Scaffold provided a permissive environment for cell infiltration and axon ingrowth When preseeded with fetal neurons, target reinnervation was partially achieved, but did not lead to functional recovery in corresponding cognitive functions	[38, 39, 153]
Hyaluronic acid (HA)	3D hydrogel structure-Modified with poly-D-lysine (PDL), laminin, and RGD to increase cell infiltration	Frontal cortex of adult rats	In all unmodified and modified HA scaffolds Transient localized inflammation only at lesion site No glial scar formation at scaffold-tissue interface Integration with host tissue Collagen deposition associated with cell infiltration into scaffold Angiogenesis around and within implant Biodegradable – complete resorption within 12 weeks post implantation Modification with PDL, laminin, or RGD led to greater amount of glial cell infiltration, compared to unmodified HA hydrogels Neurite regrowth observed only within HA hydrogels modified with laminin or RGD	[31, 59, 143]
Chitosan	Thermosensitive hydrogel formed in situ Modified with poly-D-lysine (PDL) to improve interaction with neuronal cells and promote neurite outgrowth	Striatum of adult rats	Completely engulfed by macrophages within 3 days post implantationPDL-modification had no effect on inflammatory response	[29]

(*continued*)

Table 22.1 (continued)

Material	Scaffold structure and properties	Implant site	In vivo response	References
Xyloglucan	Thermosensitive hydrogel formed in situ Modified with PDL to improve interaction with neuronal cells and promote neurite outgrowth	Caudate putamen of adult rats	Transient localized inflammatory response, but no glial scar formation around implant Presence of immobilized PDL had no significant influence on microglial response, where microglial cell numbers peaked 3 days post implantation, and subsided to normal physiological levels by 21 days Higher PDL content enabled astrocytes to infiltrate scaffold Neurites infiltrated PDL-modified xyloglucan scaffolds only, an increase in PDL content led to increased neurite density within the scaffold	[101]
Poly (ε-caprolactone) (PCL)	3D array of nonwoven electrospun nanofibers that are either aligned or randomly oriented	Caudate putamen of adult rats	Transient localized inflammatory response, but no glial scar formation around implants Neurites only infiltrated scaffold with randomly oriented PCL nanofibers, and coincided with the decline in activated microglial cells and maximum number of activated astrocytes Neurites crossed perpendicular to the direction of fiber alignment (i.e., perpendicular contact guidance)	[102]
RADA 16-I Self-assembling peptide nanofiber scaffold (SAPNS)	3D hydrogel-like scaffold consisting of a network of interwoven nanofibers (~10 nm) formed in situ	Superior colliculus (SC) (midbrain) of postnatal (P2) and adult hamsters	No scar tissue formation around implanted scaffold Seamless integration between scaffold and host brain tissue Axonal regrowth across the scaffold resulted in reinnervation of the SC, and return of functional vision within 6 weeks post implantation	[40]

present, as well as promoting certain cellular functions; such materials can potentially be enzymatically degraded in vivo giving noncytotoxic and resorbable degradation products. However, the use of naturally derived materials is often limited by their immunogenicity, poor mechanical properties, and batch-to-batch variations. In contrast, synthetic biomaterials can be processed in a more reliable manner, with a wide range of mechanical properties possible; however, such materials often require surface biofunctionalization to support various cellular functions. It is becoming increasingly common to find a middle ground between the extremes of biologically derived and synthetic polymers such as bioconjugates or blends. The materials selection process will also partially dictate the fabrication technique that can be used, and the subsequent types of morphologies that can be achieved. The scaffold morphologies commonly used in BTE can be broadly divided into two categories – *hydrogel* and *fibrous* structures.

22.3.2.1 Hydrogels

Hydrogels are 3D polymer networks that are chemically and/or physically crosslinked, forming an insoluble network of polymer chains that swell under aqueous conditions. Thus, hydrogels can be used as a synthetic analog of the hydrated network of insoluble macromolecules that naturally exist in the extracellular environment. Owing to the highly hydrophilic nature of the polymer chains, hydrogels have very high water content, as well as high permeability to oxygen and nutrients [98]. In addition, their crosslinked molecular architecture results in materials with mechanical properties similar to brain tissue. Hydrogels also have a very low interfacial tension, which aids in tissue integration by minimizing the barrier for cells to cross the scaffold-tissue boundary [98]. All these features make hydrogels an ideal choice of material to support brain tissue growth and survival both in vitro and in vivo.

Hydrogels can be formed via the addition of chemical crosslinkers that form a covalent network of polymer chains, or via the formation of physical junctions through secondary interactions, crystallite formation, or chain entanglements. The gelation behavior of many physically crosslinked hydrogels can be reversed in response to externally-applied stimuli such as temperature, pH, and ionic strength of the surrounding solution. This property is due to changes in the enthalpy and entropy associated with various interactions within a polymer solution (i.e., polymer–polymer, polymer–water, and water–water interactions). Many hydrogel materials can be modified in terms of monomer composition to allow gelation to occur during transition from ambient to physiological conditions. This is highly advantageous because such thermally sensitive hydrogels are injectable, resulting in a scaffold that can conform to the shape of any lesion, and can be implanted in a minimally invasive manner, even within deep brain structures.

Hydrogels present a unique microstructure to cells, which is characterized by its highly interconnected porous structure and high surface area. The precise microstructure depends on the conditions of gel preparation, including polymer concentration and speed of gelation. The most commonly observed macroporous hydrogel microstructures consist of an interconnected fiber-like morphology, laths or sheets that are interconnected by thin struts, and heterogeneous microstructures (Fig. 22.3), all of which can impart different mechanical properties to the material.

Hydrogels are often used as vehicles for encapsulation and subsequent delivery of therapeutic drugs, growth factors, and cells to sites of injury to promote regeneration. In cases where large volumes of neuronal tissue are lost, cells can be encapsulated into the polymer solution prior to implantation into the brain, thus acting as an exogenous source of cells. In addition, the hydrogel can be modified with specific biochemical properties and structural morphology to enable in vitro expansion of NPCs, followed by the direct implantation of the cell-encapsulated hydrogel, eliminating the need to remove cells from a substrate prior to implantation. The highly-hydrated nature, porous structure, and 3D micro-architecture of some hydrogels often present a permissive environment for cell growth when implanted in vivo, which is characterized by exuberant neurite ingrowth and glial cell invasion [38, 39, 153]. This extensive interaction between host cells and the hydrogel scaffold can be further promoted by adsorption of host-secreted adhesive ECM proteins such as fibronectin [39]. Despite this permissive environment, neurite outgrowth and subsequent target reinnervation are rarely observed [39], strongly suggesting that additional attractive cues are required to direct neurites beyond the hydrogel to reform appropriate connections. When hydrogel implants are used in combination with cell grafts to treat lesions within the septo-hippocampal pathway in the rat brain, partial reinnervation of the hippocampus has been observed at the tissue-scaffold interface [38]. This partial reinnervation was able to attenuate lesion-induced hyperactivity in the hippocampus, but was ineffective in reversing damages inflicted on behavioral and cognitive functions, which may be a reflection of the inadequacy in quantity and/or specificity of the new neuronal connections. Controlled degradation of hydrogel scaffolds is one approach to potentially improve reinnervation efficiency within a hydrogel by gradually exposing implanted cells to the host tissue.

Biodegradability and the rate of degradation can often be manipulated by incorporation of degradable units or crosslinkers into the synthetic hydrogel. Control over the degradation rate allows intimate control over the temporal evolution of the hydrogel's morphology in terms of its mesh size, which can in turn affect the proliferation of encapsulated precursor cells. Gradual degradation of a hydrogel material is important for tissue engineering applications because it will create free space for cells to proliferate, migrate,

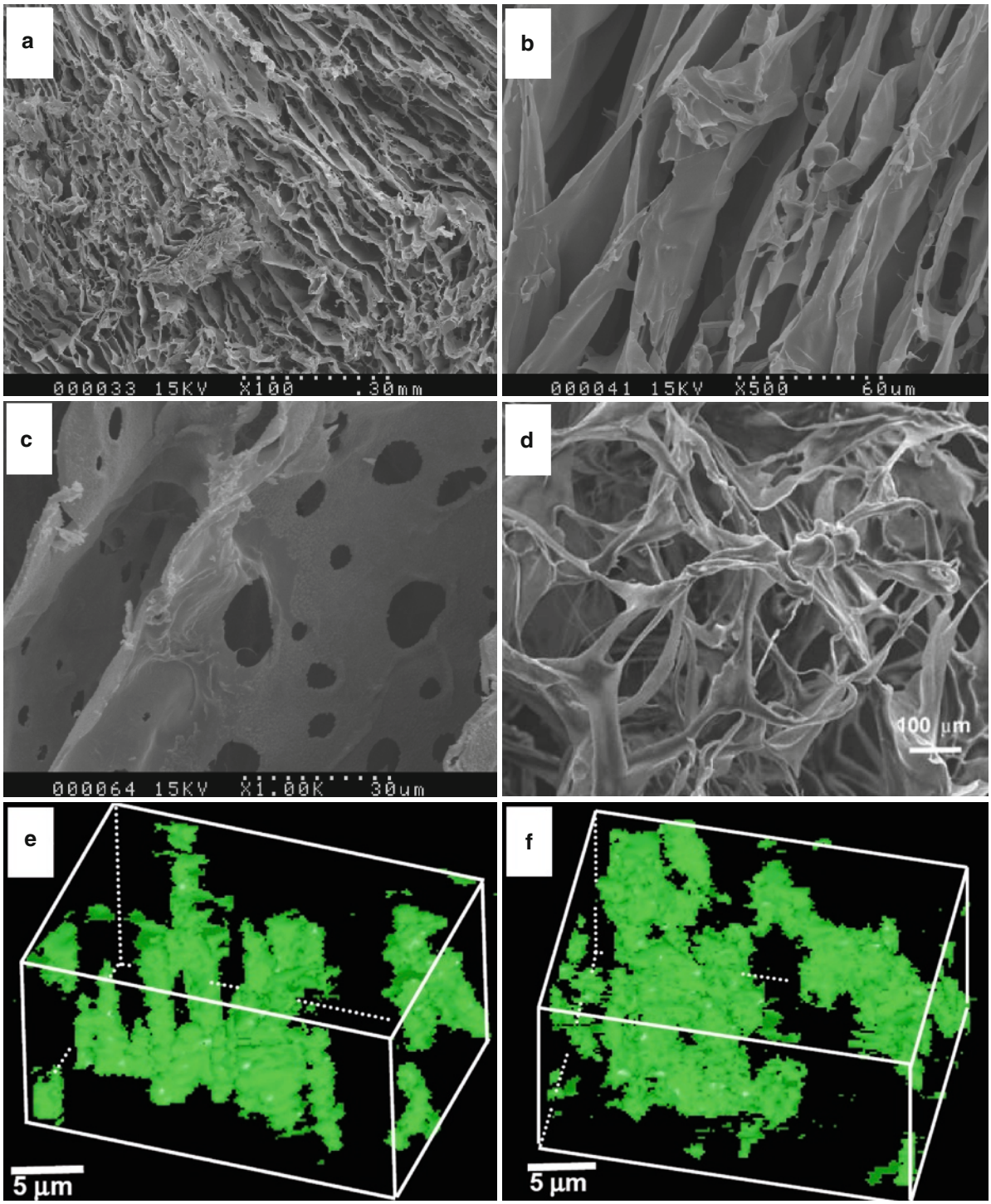

Fig. 22.3 SEM and LCSM images of different hydrogel materials, illustrating the different types of morphology that can be obtained, as well as the highly interconnected porous structure. (**a**–**c**) SEM images of xyloglucan hydrogels at various magnifications, showing sheet-like lamellar structures, and pores within individual sheets (**c**). (**d**) SEM image of hyaluronic acid (HA) hydrogels displaying flake-like morphology that are interconnected by fibrous structures; scale bar = 100 µm. (**e**, **f**) 3D visualizations of chitosan/GP hydrogels obtained using stacked LSCM images, where chitosan agglomerates to form either chain-like structures (0.25% w/v chitosan) (**e**) or polymeric aggregates (1.0% w/v chitosan) (**f**). (**d**) *Reproduced with permission from Springer Science+Business Media Cui et al.*[32], (**e**, **f**) *Reprinted from Biophysical Chemistry* [28], *Copyright (2005), with permission from Elsevier*

as well as an opportunity for cells to remodel and define their local microenvironment by depositing their own ECM molecules. Degradable hydrogels of poly(ethylene glycol) (PEG) were synthesized via the addition of methacrylate groups as crosslinking agents, and degradable functionalities were incorporated into the polymer in the form of PGA and poly(lactic acid) (PLA). NPCs encapsulated within the degradable PEG hydrogel survived the photo-polymerization process, and remained viable and proliferative after 16 days of culture. The time scale over which cell proliferation and spatial patterning of neuronal tissue formation occurred was controlled by the rate of degradation, which in turn was tailored by altering the relative composition of the degradable macromers [86]. Proteolytic susceptibility can also be engineered into hydrogel scaffolds such that degradation is dependent on the secretion of specific enzymes from cells [85, 111, 127, 150]. Proteolytic degradation is often localized to pericellular components of the ECM because of the involvement of membrane-bound proteases, which enables the structural stability of the bulk of the scaffold to be maintained for longer periods of time. Furthermore, such cell-mediated degradations allow scaffolds to behave in a dynamic and biologically responsive way, making them more biomimetic, and offering cells greater control over the remodeling process of their microenvironment.

Mechanical properties of the hydrogel such as elasticity are also tuneable by controlling crosslink density, which has been shown to be effective in controlling the rate of neurite outgrowth on different hydrogel materials including agarose [6], collagen [151], and DNA-crosslinked gels based on polyacrylamide [67]. There is strong evidence that cells can sense substrate stiffness via integrin-based adhesions with the substrate. Cellular mechano-transduction leads to rearrangement of the cytoskeleton, which may ultimately affect neurite outgrowth morphology. Substrate stiffness is a regulator of neurite branching and axonal regeneration, and can control neuronal and astroglial populations because of differing degrees of attachment as a consequence of substrate stiffness [68]. However, the effect of substrate stiffness on neurite extension is still under debate. Confounding factors in this regard may reflect the method in which substrate stiffness is manipulated; agarose and collagen gels of different stiffness were obtained by altering gel concentration. As both these materials are known to possess adhesive domains that can interact with cells, it is possible that neurite outgrowth was affected not only by gel stiffness, but also by the concomitant changes in ligand density associated with changes in gel concentration. Therefore, excessive adhesive interactions between cells and substrates could have slowed the rate of neurite extension. Recently, a novel method of designing gels of different stiffness without changing the surface ligand density was developed by using DNA as a crosslinker [67]. Increasing the gel stiffness up to 17.1 kPa decreased neurite extension and increased the number of primary dendrites, suggesting that distinct sensitivity and a threshold exists for dendrite and axonal extension [67]. It is also likely that the optimum substrate stiffness for neurite outgrowth will vary for different neuronal phenotypes.

HA hydrogels is one of a few materials that have been shown to minimize the FBR, which is the major reason behind their ability to integrate with the host tissue after implantation [31, 59, 143]. HA is an important natural component of the ECM in the brain as well as an important coregulator of human ESCs in vitro and in vivo [49]. While 3D hydrogels of HA are ineffective in promoting neurite outgrowth due to a lack of cell adhesion on the substrate, hydrogels consisting of copolymers of HA and PLL are effective in promoting neuronal repair by minimizing scar formation at the scaffold-tissue interface. When implanted into the rat brain, these materials only evoked a transient and localized inflammatory reaction, which began to subside after 3 weeks [143]. Chitosan hydrogels have also been shown to be a promising material for BTE applications, where properties such as degradation behavior can depend on the degree of deacetylation (DD). Chitosan has been considered a potential scaffold material for long-term implantation because of its biocompatibility with neurons [21, 52, 64–66], and at high DD they induce minimal inflammation with relatively slow degradation rates [144]. However, upon injection of an in situ forming tract of thermoresponsive chitosan into rat brains, it was found that the material was promptly engulfed by macrophages as part of the FBR [29]. Therefore, while scaffolds can be optimized with various physical and chemical properties to control cell behavior, it is equally important to determine the body's response to the implanted materials under relevant conditions to ensure that cellular responses achieved in vitro can be replicated in vivo.

Despite the flexibility in processing of hydrogels to generate tailored features, it is difficult to control the direction of neurite outgrowth, which is one of the most important requirements in neuronal regeneration. Advances in fabrication technologies have enabled the use of scaffolds with tailored micro- and nano-scaled features, emulating the intricate fibrillar architecture of natural ECM components, as a tool for directing neurite outgrowth.

22.3.2.2 Fibrous Structures

The ability of fibrous scaffolds to support cell survival may be at least partially due to the high surface area-to-volume ratio for optimal cell attachment and interaction with the material. However, there is strong evidence that the fibrous architecture itself is an important feature in supporting cell viability, and influencing subsequent cell morphology. The three main techniques used to fabricate 3D fibrous structures for BTE include thermally-induced phase separation (TIPS), electrospinning, and self-assembly.

In TIPS, a homogeneous polymer solution is cooled in order to induce phase separation via spinodal decomposition, where the resultant morphology consists of fibrous structures, with high porosity and interconnected pores. The thermodynamic equilibrium phase of a polymer solution can be determined from a phase diagram. For a typical polymer/solvent binary system, a binodal curve exists, above which the equilibrium phase is a homogeneous solution, and below which it is a mixture of solvent-rich and polymer-rich phases. When a polymer solution is cooled to within metastable regions below the binodal curve, phase separation will occur via nucleation and growth mechanisms, leading to a microstructure where component phases are present as discontinuous structures. In contrast, when a polymer solution is cooled to within an unstable region below the binodal curve, phase separation occurs via spontaneous demixing (spinodal decomposition), resulting in a microstructure where polymer and pores are cocontinuous in nature, leading to pore interconnection. Using such a method, nanofibrous PLLA structures have been fabricated, with fiber diameters in the range of 50–350 nm, and porosity of approximately 85% [157] (Fig. 22.4). Feature dimensions were manipulated by systematic variations of polymer concentration (in the range of 2–9% w/v). Such scaffolds were able to support the attachment of neonatal mouse NSCs, and their subsequent differentiation into neurons, which migrated into the porous scaffold [157]. Despite the simplicity and versatility of this technique, aligned features are not easily incorporated.

Electrospinning is another simple and inexpensive method for producing polymeric scaffolds with highly interconnected porosity and fibrous structures with fiber diameters in the range of nano- to micro-meters (Fig. 22.5). Fiber orientation can be developed by using different collection methods [140]. Aligned fibers have been obtained by collecting electrospun fibers on a rotating mandrel [158], dual rings [32], or across an insulative gap between two conductive strips [79]. Fibers form a parallel array across an insulative gap because of the electric field associated with such a configuration, and electrostatic interactions between depositing fibers.

Electrospun polymeric materials are capable of supporting the attachment, growth, and proliferation of various neuronal stem and progenitor cells in vitro [23–26, 50, 99, 129, 154, 158]. It has been shown that fiber diameter alone is capable of influencing cell behavior. Rat NSCs cultured on electrospun polyethersulfone (PES) scaffolds with increasing fiber diameters (in the range 280–1,450 nm), showed an associated decrease in cell proliferation [26]. Cells on 283 nm-fibers displayed a more rounded morphology compared to larger diameter fibers, as well as moderate spreading. In contrast, cells cultured on larger diameter fibers exhibited lower migratory activity and tended to form large cellular aggregates. The lower cell viability on larger diameter fibers is a result of poorer cell attachment and limited migratory ability, which may be mediated by poor cell spreading on the substrate, and the subsequent cytoskeletal arrangement due to lack of actin fiber formation.

Cell-scaffold interactions can be further enhanced by optimizing surface properties of the electrospun fibers. Cell attachment and subsequent proliferation can be increased by presenting amine moieties on the surface of electrospun PCL scaffolds [101]. The incorporation of bioactive ECM and ECM-like components, including laminin, collagen, and gelatin, as a blend in the electrospinning polymer solution, is an effective method of improving attachment and subsequent proliferation and survival of various cell types, including NSCs, DRG explants and PC12 cells, in vitro [50, 74, 129]. As laminin and collagen are major components

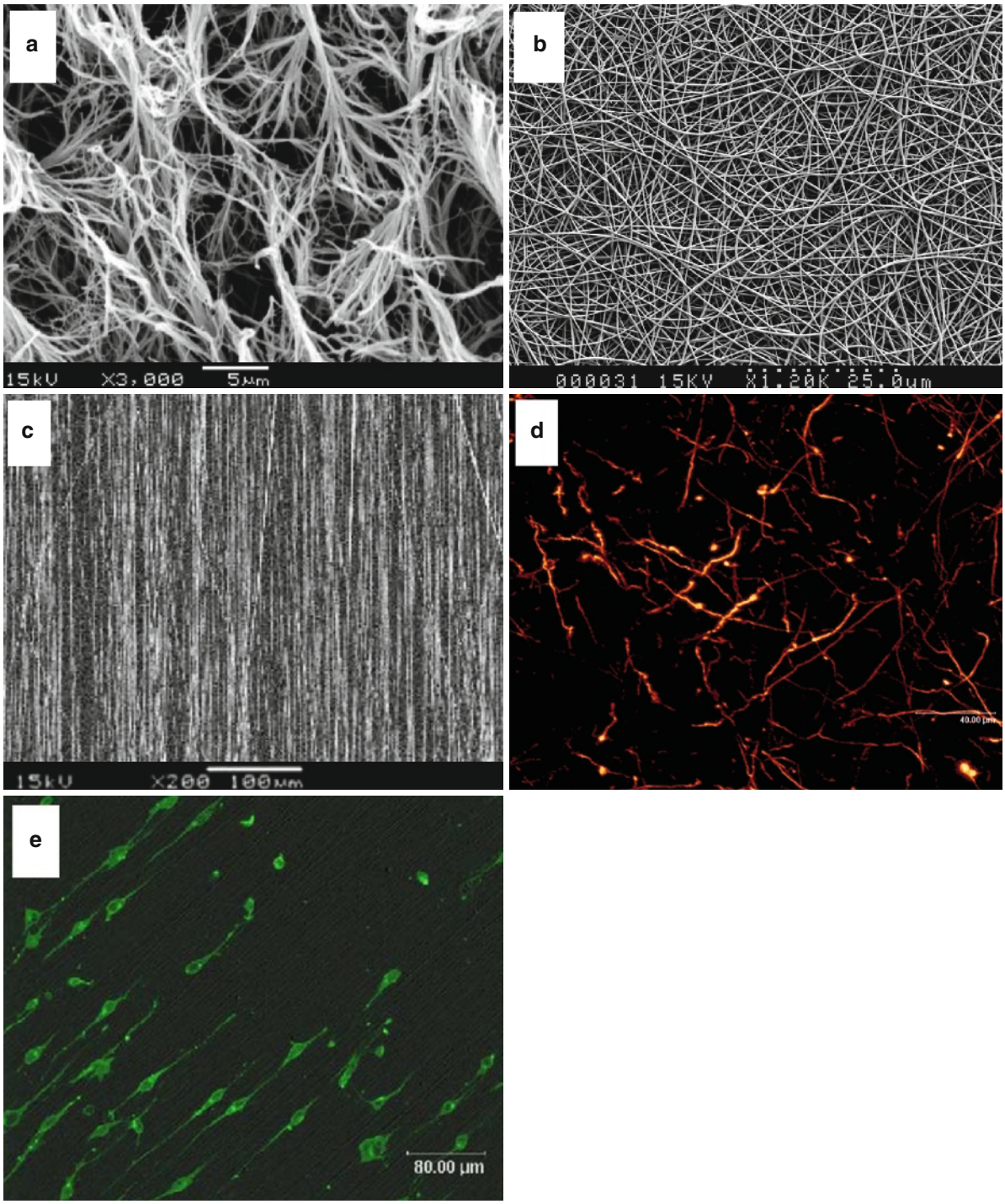

Fig. 22.4 SEM images of nanofiber morphology obtained via thermally-induced phase separation (TPS) (**a**) and electrospinning (**b**, **c**). Electrospun nanofibers can be collected as a nonwoven array of randomly oriented (**b**) or aligned (**c**) polymer fibers. Fluorescent images of (**d**) primary cortical neurons cultured on randomly oriented electrospun PCL nanofibers, and (**e**) neonatal mouse C17.2 neural stem cells (NSCs) cultured on aligned electrospun PLLA nanofibers, showing the ability of fiber alignment to provide contact guidance cues to guide neurite outgrowth in the direction of fiber alignment. (**a**) *Reprinted from Biomaterials, Yang et al.* [157], *Copyright (2004), with permission from Elsevier.* Fig (**c**, **e**) *Reprinted from Biomaterials, Yang et al.* [158], *Copyright (2005), with permission from Elsevier*

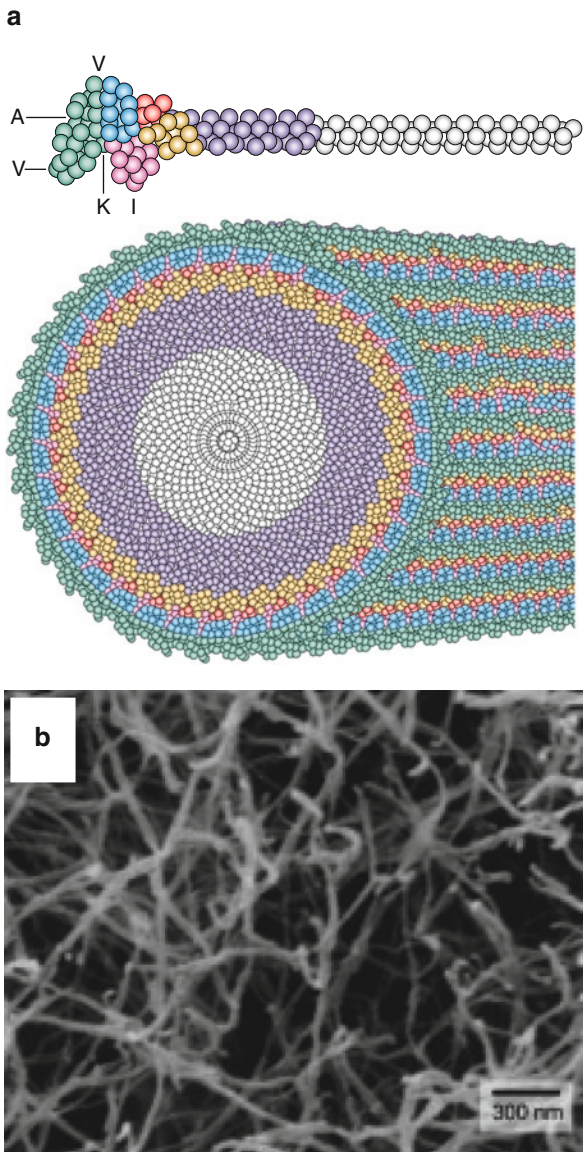

Fig. 22.5 Schematic molecular structure of self-assembled nanofibers from peptide amphiphilic molecules (**a**), and the corresponding structural morphology of the self-assembled nanofibers as depicted from SEM imaging (**b**). Reprinted from [130] with permission from AAAS

of the brain ECM, both are readily recognized by neuronal cell receptors for attachment; hence, improved cell viability is likely to be related to the ability of cells to interact via such cell adhesion molecules on the fiber surface.

The diameters of electrospun fibers can have potent effects on the differentiation of cultured NSCs. The differentiation specification of rat NSCs varies depending on the average diameter of electrospun fibers [26]. Compared to cells on control tissue-culture plastic surfaces (TCPS), there was a three-fold increase in oligodendrocyte differentiation on 273 nm-fibers, and the highest fraction of neuronal differentiation was observed on 749 nm-fibers. The migratory ability of differentiating cells enabled the electrospun fibers to guide the extension of cell processes, determining cell morphology and possibly supporting differentiation into different cell types. Electrospun PLLA nanofibers with average fiber diameters in the range of 250 and 300 nm strongly supported the differentiation of mouse NSCs into neurons compared to micron-sized fibers [158]. However, only markers for NSC differentiation toward neurons were used; hence, it is possible that cells had a similar differentiation profile.

Fiber alignment also influences the differentiation of cultured NSCs. The differentiation profile of murine ESCs cultured on electrospun PCL nanofibers (average fiber diameter approximately 250 nm) varied depending on whether fibers were aligned or randomly orientated [154]. While it was shown that culturing ESCs on nanofibrous PCL scaffolds induced differentiation into various neural lineages (including neural precursors, neurons, oligodendrocytes, and astrocytes), a significantly greater proportion of ESCs differentiated into astrocytes on randomly oriented PCL nanofibers compared to aligned PCL. Interestingly, Yang et al. reported that the rate of neuronal differentiation appeared to be independent of fiber alignment on both electrospun PLLA nano- and micro-fibers [158].

Collectively, the results of recent studies indicate that fiber diameter and alignment of electrospun fibers can influence the differentiation characteristics of NSCs. For instance, Nisbet [101] reported the preferential differentiation of rat brain-derived NSCs into oligodendrocytes, with no neuronal differentiation on electrospun PCL fibers with average diameters of 750 ± 100 nm; the differentiation profile was not affected by surface aminolyzation [101]. These ambivalent results may be a reflection of differences in materials and/or cell types used in the separate studies. For instance, each scaffold may display different surface chemistry and substrate stiffness, all of which can have varying effects on cell-material interactions and subsequent differentiation. Also, variability in fiber diameters within electrospun scaffolds may mask the true effect of fiber diameters on

cell behavior. Such preliminary studies provide evidence that differentiation specification may be mediated by variable cell adhesion onto fibrous surfaces, which can affect cell morphology, cytoskeletal arrangement, and the subsequent cell signaling that ultimately affects cell fate. While the exact mechanism by which fiber diameter and alignment can affect differentiation remains to be determined, it is clear that these are only two of the many cues that can be used to modulate cell behavior.

One of the main advantages of using nanofibrous scaffolds in BTE is their potential to guide neurites toward target locations utilizing aligned fiber morphologies. Many studies have shown the effectiveness of aligned electrospun nanofibers in not only guiding process outgrowth, but also supporting and enhancing the rate of process elongation [23, 24, 50, 73, 101, 129, 154, 158]. In general, cells cultured on aligned nano/ micro-fibrous substrates display a bipolar morphology, with cell body elongation occurring in the direction of fiber alignment. This suggests that aligned nanofibers can act as a positive guidance cue for directed neurite outgrowth. This process is more broadly known as "contact guidance," where direct contact-mediated interactions between the advancing growth cone and aligned fiber surfaces can direct neurite outgrowth. Experimental results have shown that neurites initially protruding from the neuronal cell body do not necessarily follow the direction of immediately adjacent fibers (or any other aligned topographical features) [87, 116, 129]. However, when the growing neurite comes into contact with a nanofiber that is part of an oriented assembly, it is capable of making sharp turns in order to continue following the underlying fiber. It is interesting to note that guidance of process outgrowth by aligned fibers is not restricted to neuronal cell types, but also applies to glial cells such as Schwann cells and astrocytes. In fact, in cell cultures containing both glia and neuronal cell types, glia cells are often in direct contact with the underlying fiber substrate, with neurons growing on top of the glia cells, such that any physical orienting effects from the substrate is mediated through contact between neuronal and glia cells [9, 116, 129]. There is also evidence that the guidance provided by aligned electrospun nanofibers is related to other factors such as fiber diameter and interfiber distance. Observations of the interactions between growing cortical neurons and randomly oriented nanofibers revealed that neurite outgrowth does not necessarily follow the underlying fibers. The direction of neurite outgrowth relative to the nanofibers was dependent on the interfiber distance; neurites were more inclined to follow a fiber if interfiber distances were relatively large (>15 μm), traverse perpendicular to the polymer nanofibers if interfiber distances were relatively small (2–15 μm), and completely avoided regions of high fiber density (interfiber distance less than 1 μm) [100].

The precise mechanism via which fiber diameter, alignment and spacing affect various aspects of cell behavior is yet to be elucidated. Difficulties in fabricating electrospun scaffolds with highly defined fiber diameters and interfiber distances mean that any observed changes in cell behavior cannot be solely attributed to one scaffold parameter alone. In particular, variations in fiber diameters or alignment also lead to concomitant changes in the pore structure of the scaffold in terms of interfiber distance and pore shape. However, recently, ice crystals were used as a removable void template for electrospinning nanofibrous scaffolds with variable interfiber distance, which can potentially enable the effects of fiber diameter and interfiber distance to be decoupled [131]. Furthermore, the use of different materials in different studies makes direct comparison of cell behavior difficult. Nevertheless, current experimental evidence suggests that the morphology of electrospun scaffolds can have beneficial effects on the manipulation of various progenitor cells for BTE. Moreover, it appears that scaffold structure can influence cell adhesion, and subsequent development of cell morphology and cytoskeletal arrangement, which may in turn affect the cells migratory ability, differentiation specification, process outgrowth, and maintenance of viability.

Self-assembly is a bottom-up nanofabrication approach that can be used to generate 3D fibrous structures with fiber diameters in the order of 10^0–10^1 nm. This method exploits the spontaneous association of certain molecules, under thermodynamic equilibrium conditions, to form ordered structures via noncovalent bonds. The building blocks of self-assembled nanofibers are often synthetically designed amphiphilic oligopeptides, consisting of specific sequences of amino acids [161]. Therefore, self-assembled nanofibers are often biodegradable, with noncytotoxic by-products. Self-assembled nanostructures are often designed to form under physiological conditions, enabling injection

into the brain as a solution to form a hydrogel-like material in situ. Self-assembled nanofibrous scaffolds typically consist of interwoven nanofibers with diameters in the order of 10 nm and pore sizes of 5–200 nm, and exist in a highly-hydrated state (water content up to 99.5% w/v) [161]. In addition to supporting neuronal differentiation and neurite outgrowth in vitro [58], self-assembled nanofibers are also capable of encouraging neuronal regeneration such as across transected optic nerve fibers in the superior colliculus, enabling restoration of functional vision in rodent models [40]. Integration of the self-assembling peptide nanofiber scaffold (SAPNS) at the sites of induced-lesions was also correlated to the degree of reinnervation in the superior colliculus, suggesting sufficient reinstatement of neuronal connectivity. Maintenance of a well-organized tissue structure at the injury site may have suppressed inflammation by controlling the infiltration of inflammatory cells to the scaffold-tissue interface. The presence of high levels of the RAD sequence (similar to the RGD motif found in adhesive ECM proteins) in the scaffold suggests that neuronal cell attachment, neurite outgrowth, and tissue remodeling are highly regulated by defined cell–substrate adhesion mechanisms.

Despite the ability of self-assembled nanofibers to support neuronal differentiation and neurite outgrowth both in vitro [58, 130] and in vivo [40, 147], the lack of alignment within the fabricated structure means that, by itself, it may not provide sufficient physical cues to direct neurite outgrowth. However, one of the major advantages of this fabrication method lies in its ability to efficiently present high densities of biologically-active ligands on the surface for cell recognition, which will be discussed below.

22.3.3 Biofunctionalization

The ability of biomaterials to influence cell behavior is initiated by interactions occurring at the cell-scaffol interface, and hence the importance of surface biofunctionalization. The motivation for biofunctionalization is to design biomimetic surfaces that encourage tissue integration with the scaffold, and to regulate cellular behavior. This often involves the immobilization of cell-signaling molecules onto the surface to better control cell behavior by targeting receptor-mediated cellular mechanisms. Table 22.2 presents a list of different bioactive molecules that have been used to biofunctionalize scaffolds for BTE applications. Surface biofunctionalization seeks to control the presentation of proteins to cells in such a way to enhance selective aspects of cell behavior and in some instances, limit nonspecific protein adsorption. Due to synergistic substrate and growth-factor effects, two goals of biofunctionalization are:

- Promote and maintain cell survival and proliferation via ligand signaling
- Enable cells to physically interact with the substrate such that subsequent cell growth and migration can be guided by the underlying structural/physical features

It is often desirable to present bioactive molecules on a scaffold in an immobilized form, as opposed to soluble form, because it will not be internalized by cells or dissipated because of diffusion. Therefore, immobilized biomolecules are capable of having a sustained and localized effect on cells. They influence cell behavior via activation of signaling pathways through specific ligand–receptor binding; whereas soluble factors can also potentially influence other cell functions including gene expression and apoptosis after they have been internalized and delivered toward the soma via retrograde transport [71]. Regardless of the optimal delivery method, functionalization of scaffold surfaces with biomolecules provides a flexible means of ligand presentation directly to the cell surface to elicit precise cell responses.

22.3.3.1 Bioactive Molecules

Cell-adhesive molecules such as laminin and fibronectin are utilized in many biofunctionalization strategies owing to their importance in physical axon guidance. Furthermore, their purified or synthesized adhesive domains (Table 22.2) have also been incorporated to improve cell attachment. The advantages associated with immobilizing short peptide motifs, as opposed to the parent protein, includes their enhanced stability during immobilization, affinity for specific ligand–receptor binding because of the reduction of steric hindrance, high density presentation on a substrate, and their ability to target specific cell types and selective signaling pathways. However, the use of whole proteins are often necessary because certain protein functionalities are dependent on structural conformation, which is often lost in purified peptides.

Table 22.2 List of biologically-active molecules used to biofunctionalize scaffolds in neural tissue engineering, especially for brain repair

		Effects on neuronal cell behavior	References
Extracellular matrix (ECM) molecules	Laminin	Provides a permissive substrate for cell and growth cone migration Enhances neuronal cell adhesion Promotes and accelerates neurite outgrowth	[56, 59, 74, 84, 110, 116]
	Collagen	Structural protein of the ECM containing specific sites for cell adhesion, thus enhances cell attachment	[129]
	Fibronectin	ECM protein that promotes cell adhesion	[162]
	Vitronectin	ECM protein that promotes cell adhesion	[17]
Peptide motifs	RGD	Cell-adhesive peptide sequence present in many cell-surface receptor proteins and ECM proteins, including laminin, fibronectin, and vitronectin	[31, 162]
	YIGSR	Peptide sequence within laminin that enhances neuronal cell adhesion	[35, 55, 84, 145]
	IKVAV	Peptide sequence within laminin that enhances neurite outgrowth	[1, 30, 55, 84, 124, 130, 145, 147]
Growth factors	Nerve growth factor (NGF)	Critical for neuronal survival during development and in the adult nervous systemCan act as a tropic factor, where neurite outgrowth is guided along the concentration gradient of NGF	[19, 71, 93, 133, 160]
	Brain-derived neurotrophic factor (BDNF)	Promotes survival of cortical neuronsImportant for cell survival and proliferation during oligodendrogenesis Promotes NSC proliferation	[60, 83]
	Neurotrophin-3 (NT-3)	Promotes neuron survivalEnhances neurite outgrowth Can act as a tropic factor, where neurite outgrowth is guided up the concentration gradient of NT-3	[83, 93]

In addition to adhesive molecules, other biologically-active molecules such as growth factors can be incorporated to target certain cell functions (Table 22.2). In particular, neurotrophins are a family of growth factors that provide target-derived trophic support through receptor-mediated signaling, controlling neuronal outgrowth and connectivity during brain development, and maintenance of neuronal populations in the adult CNS. At different stages of development, neurons depend on more than one neurotrophic factor-receptor signaling pathway to survive. The family of neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4). In addition to promoting growth, gradients of neurotrophins are capable of steering the growth cones of primitive and mature neuronal cell types in vitro [60]. Precise control over cell behavior can be achieved by careful manipulation of biomolecular presentation to cells in terms of the type of biomolecule, its conformation, surface density/concentration [89], and spatial patterning.

22.3.3.2 Incorporation of Bioactive Molecules onto Scaffolds

The main techniques that have been used to incorporate bioactive molecules within scaffolds include blending, physical adsorption, and covalent attachment. Blending involves mixing of biomolecules with the polymer solution during the fabrication process, such that they become physically entrapped within the polymeric structure postfabrication. This is a facile and efficient method of incorporating biomolecules within the bulk and surface of the material at high efficiency. Blending has been used for the introduction of adhesive molecules, such as collagen and laminin, into electrospun nanofibers [74, 129] resulting in improved cell attachment and enhanced neurite outgrowth. For example, the incorporation of collagen into aligned electrospun PCL nanofibers imparts a stronger orientation effect on neurite outgrowth [129]. Blending of neurotrophic factors such as NGF and NT-3 into hydrogels [71, 93] is an effective method of guiding neurite outgrowth, and improving cell penetration into poly (2-hydroxyethyl methacrylate) (PHEMA) scaffolds. However, the threshold concentration gradient required to elicit neurite guidance was significantly greater for physically entrapped neurotrophic factors compared to those presented in soluble form. Importantly, when concentration gradients of two immobilized neurotrophic factors, NGF and NT-3, were presented at the same time, the threshold concentration of the individual growth factors required to elicit neurite guidance was lower compared to when growth factors were presented individually, illustrating the synergistic effect of NGF and NT-3 in guiding neurite outgrowth [93]. The spatial distribution of ligands can also partially dictate the effectiveness of a particular protein or peptide motif on cell behavior [61, 89].

Bioactive proteins can be physically adsorbed onto a biomaterial surface via nonspecific (noncovalent) bonding. The type of interactions that lead to physical attachment of biomolecules onto the surface can include hydrogen-bonding, hydrophilic or hydrophobic interactions, and electrostatic forces. The bond strength for physically adsorbed molecules is relatively weak; hence, bound molecules are prone to displacement from the surface under in vitro and/or in vivo conditions. Therefore, their biological activity will decrease with time as they diffuse away from the scaffold or become internalized by cells. However, bond strength can be increased by pretreatment of the substrate surface to introduce functional chemical residues, such as carboxyl groups, that enhance physical interaction with the biomolecule. This technique is used predominantly for adsorbing proteins, rather than short peptides, because of stronger interactions between proteins and the material surface. Physically adsorbed adhesive proteins such as laminin are effective in improving cell attachment, proliferation, and neurite outgrowth [74, 112, 115, 116]. While physical adsorption presents a simple method of functionalizing surfaces with relevant proteins, it only allows limited control over conformation and exposure of the relevant motif; hence, the resultant bioactivity may not be optimal.

Immobilization of bioactive molecules onto biomaterial surfaces can also be achieved via covalent bonding. Recent studies within our research group have shown that BDNF can be covalently attached onto the surface of electrospun PCL nanofibers. The immobilized BDNFs in combination with the PCL nanofibers were able to enhance adhesion and proliferation of E14.5 cortical progenitor cells, as well as enriching the differentiation of cells into neuronal and oligodendrocyte lineages [59]. Using a similar strategy, Lu not only covalently attached NGF onto the surface of chitosan but were able to control the spatial distribution of the NGF via a photo-patterning method [160]. The chitosan surface was first primed with a bifunctional crosslinker, after which selective surface regions were photo-activated using a UV laser coupled to a confocal microscope to achieve precise spatial control. The concentration of photo-reactive moieties can be manipulated by varying the UV exposure time, which will subsequently determine the concentration of NGF coupled onto the activated surface. Thus, through UV irradiation a gradient pattern of NGF, or other growth factors, can be established. Improvement in cultured superior cervical ganglion cell attachment to the photo-immobilized NGF-patterned chitosan surfaces demonstrated that the NGF was stably bound to the surface, bioactivity was conserved, and that neuronal behavior was dependent on the concentration of NGF presented [160].

Spatial patterning in the form of regular arrays (tracks or squares) [17, 121, 122] of adhesive molecules, and growth factor concentration gradients [1, 34, 63, 71, 72, 93] have been used in vitro in an attempt to control cell positioning and direction of process outgrowth. Thus, incorporation of neurotrophic factors

such as NGF into scaffolds for BTE is an appealing strategy for promoting better neuron survival, differentiation, and directed axonal outgrowth. Morphogens can also be incorporated to help regulate tissue patterning; they display tropic effects where cellular response is dependent on the morphogen concentration. Morphogens that are known to play a role in regulating tissue patterning during development of the nervous system include sonic hedgehog (SHH), Wnt, epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor β (TGFβ) [3, 118].

A common technique for patterning 2D arrays of biomolecules onto substrate surfaces is via microstamping. Micro-stamping involves the use of a microfabricated elastomeric stamp to print regular patterns (e.g., strips) of proteins onto the surface of a substrate. It is commonly used to approximate a continuous gradient by systematically varying the spacing and density of the printed pattern [72]. This involves the "inking" of a prefabricated poly(dimethyl siloxane) (PDMS) stamp in a protein solution, and subsequently physically transferring the protein onto the substrate surface. Microfluidic devices have also been used to generate similar micropatterns, where a microchannel network utilizes laminar flow and diffusive mixing to generate a gradient [72]. The disadvantage of such diffusive gradients is that the it is often not stable over long periods of time, and requires constant replenishment of feeder reservoirs to maintain. Incorporation of biomolecular gradients within such three-dimensional structures have to date only been achieved in hydrogels. However, both approaches have shown promising results in terms of their ability to direct neurite outgrowth toward increasing concentrations of the deposited trophic factor [19, 72, 93]. The ability to generate chemical concentration gradients within 3D scaffold structures, which can also be used in conjunction with structural features, will prove useful for guiding neurite outgrowth. To this end, inkjet printing presents a convenient strategy for not only depositing biomolecules onto 3D scaffolds, but also controlling concentration gradients of biomolecules. These patterning methods can be coupled with the previously described methods of surface immobilization techniques to expose cells to stable concentration gradients.

Self-assembly methods can be used to fabricate biomaterials with a high density of bioactive ligands as well as to have greater control over the peptide conformation. Self-assembled nanofibers consisting of a high density of IKVAV peptides are effective in promoting selective differentiation of mouse NPCs into neurons and supporting long neurite outgrowth in vitro [130], as well as promoting regeneration of motor and sensory nerve fibers of a severed corticospinal tract in vivo [147]. When injected into a damaged mouse spinal cord, the solution of oligopeptides, consisting of the IKVAV peptide amphiphile, resulted in the formation of a self-assembled nanofibrous hydrogel that filled the cavity. This led to an increase in the number of oligodendrocytes surrounding the defect, concurrent with decreased cell death and reduction in scar formation, altogether promoting long-term nerve fiber regeneration within the 11-week study. The effectiveness of the self-assembled nanofibers in promoting nerve regeneration, as compared to the use of the parent protein (laminin) or the peptide sequence tethered to a 2D surface, has been attributed to the amplification of epitope density presented to cells by a factor of approximately 10^3 [130]. Studies into the use of self-assembling peptides for brain repair have been limited, but promising results have been obtained to date [40]. It is envisaged that self assembling peptides will provide excellent scaffolding material in the brain.

22.3.4 Summary

Different types of materials with variable microstructures offer a wide degree of flexibility in controlling neuronal regeneration for BTE approaches in controlling neuronal regenerative ability. There are advantages and disadvantages inherent to each type of scaffold that will dictate its efficacy in BTE. One of the main advantages of using in situ gelling hydrogels is the ability to completely fill large cavities and irregular voids, thus creating an intimate interface between host and implant. This type of juxtaposition can be beneficial, but not imperative, for allowing cells from host tissue to enter the highly-hydrated microenvironment. More rigid scaffolds (such as nano- and micro-fibrous scaffolds), which do not conform to lesion cavities the same way as in situ gelling hydrogels, are still able to achieve comparable results in terms of supporting the ingrowth of host tissue as well as survival and growth of seeded cells. It is presumed that such scaffolds act

as suitable physical and biomolecular templates to trigger tissue regeneration. Regardless of the types of microstructures used, any beneficial effects of scaffold implantation into an injury model involve the ability of the biomaterial to perform the following functions:

- Provide a permissive microenvironment for cell growth and a template to induce appropriate cell architecture
- Avert adverse secondary effects of inflammation that occur naturally in the injured brain (which can be detrimental to the regenerative process), such as infiltration by monocytes and reactive astrocytes, which ultimately leads to scar formations
- Shield new cells from the inhibitory brain microenvironment, while allowing adequate interaction with the host tissue for functional integration

Different fabrication techniques can be used to obtain a variety of morphologies with the capacity to mimic different structural and physiological aspects of the native extracellular microenvironment. Despite these differences, they are able to promote certain cell regenerative events that may ultimately lead to restoration of neuronal pathways, and in the best case, functional recovery. Although surface biofunctionalization strategies can increase the specificity with which cells interact with the scaffold, the importance of scaffold microstructure (e.g., nano-scaled features and microporous structure) in allowing regeneration to occur cannot be understated.

A detailed understanding of the stem cell niche is also very important for BTE as many of the regenerative strategies revolve around mimicking the niche microenvironment to recapitulate developmental events. While advances in nanofabrication techniques have enabled the engineering of more complex, molecular-scale, biomimetic scaffold structures, the level of detail and complexity that can be achieved pales in comparison to the natural biological version. Therefore, when undertaking scaffold design for this purpose, such differences and limitations should be considered. It is unreasonable to expect any synthetic/engineered scaffold to fully recapitulate every aspect of the embryonic development process, or the stem cell niche environment. Instead, biological support cells should be harnessed and integrated within the scaffold design to take advantage of their inherent ability to impart certain critical aspects of biological signaling that cannot be otherwise included synthetically.

22.4 Clinical Application

Current clinical treatment strategies for brain disorders and injuries are largely limited to cell- or drug-based therapies alone. Drug therapies are primarily aimed at alleviating symptoms or slowing disease progression. However, with the realization of the limited benefits that can be achieved by drug treatment alone, more emphasis is being placed on cell-therapies, with the intention of regenerating cells and their concomitant function(s) lost due to disease or injury.

The treatment strategy will depend on the type and nature of the disorder or injury. The anatomo-pathological features (i.e., whether CNS lesion sites are "*focal*" or "*multifocal*") and the types of cells lost will likely dictate the modality of treatment. For example, the focal nature of certain diseases, such as Parkinson's disease (PD) and stroke, makes the direct transplantation of cells into lesion sites a viable strategy for facilitating regeneration. On the contrary, the multifocal nature of neurodegeneration in diseases such as MS and epilepsy means that direct cell transplantation into lesion sites is an impractical approach in assisting regeneration [108]. Therefore, potential clinical treatments to stimulate repair of damage to distinct neural pathways caused by disease or injury will be discussed, using PD as a case study to demonstrate how tissue engineering can bring about a better therapeutic outlook.

22.4.1 Parkinson's Disease

PD represents an important case study for strategies used in the regeneration of focal brain lesions because it involves the loss of a distinct cell type (e.g., dopaminergic neurons) and degeneration of highly defined neural pathways. PD is a neurodegenerative disease that is characterized by selective loss of dopaminergic (DA) neurons in the substantia nigra that projects to the striatum in the basal ganglia (Fig. 22.6). The loss of nigrostriatal neurons and subsequent depletion of the neurotransmitter dopamine in the corpus striatum have severe consequence on the control of movement, which typically presents as, but not limited to, bradykinesia (slowed initiation and execution of movements), tremor at rest, cogwheel rigidity (intermittent increase in resistance to passive movement), and postural abnormalities

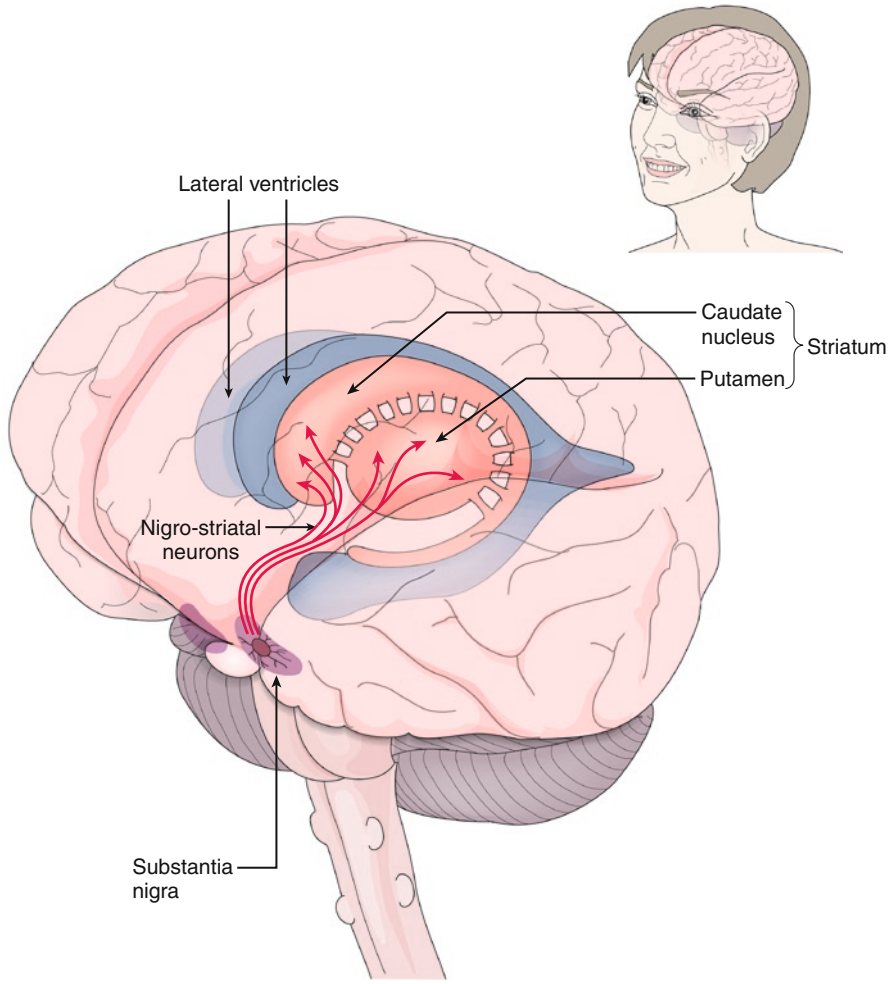

Fig. 22.6 Schematic diagram highlighting the degenerate neuronal pathways within the basal ganglia of the brain. Dopamine neurons, with cell bodies located in the substantia nigra, have axonal projections toward to the caudate putamen, where dopamine is released. Selective degeneration of these neurons results in motor symptoms observed in PD-affected patients. *Reproduced with permission from Terese Winslow ©2001*

[90, 146]. The release of dopamine in the striatum is part of a complex feedback loop, where DA inputs from the *substantia nigra pars compacta* (SNpc) effectively acts to reduce inhibition of thalamocortical neurons, thus facilitating movements initiated in the cortex. Early diagnosis and treatment of PD is often difficult because clinical symptoms only appear when approximately 50% of the DA neurons in the SNc and 80% of striatal dopamine are lost [146]. As such, the etiology of the disease still remains elusive, although certain genetic and environmental factors have been implicated in the cause of PD, and appears to involve mitochondrial dysfunction and/or oxidative stress in DA neurons. The presence of Lewy bodies is often viewed as one of the pathological features of PD. Lewy bodies are protein aggregates consisting of α-synuclein, neurofilament, crystallin, and proteins of the proteasome [90], which are often used as indicators of disease progression, rather than the cause. The composition of the Lewy bodies has led to the speculation that such inclusions are a reflection of the cell's inability to clear abnormal proteins, which may be an implication of cell dysfunction [90].

The current modalities of therapeutic treatment for PD include pharmacological and surgical approaches. Pharmacological treatment often involves delivery of an exogenous source of dopamine precursors (levodopa or L-DOPA), or dopamine receptor agonists, to alleviate motor symptoms brought about by the dopamine deficiency in the striatum [90, 146]. However, drug treatment strategies only provide limited relief of motor symptoms, and the beneficial effects often wear off after approximately 5 years, as patients become refractory to the effects of dopamine

treatment [15, 146]. Surgical therapy is an alternative treatment modality that aims to relieve motor symptoms by destruction (via surgical ablation or electrical stimulation) of specific deep brain structures to remove pathways that are partially responsible for the aberrant signaling [146]. These two types of treatment modalities only have limited efficacy in terms of the extent and duration of symptomatic relief that they can offer, and are often associated with many severe side effects. More importantly, at best, these are purely symptomatic treatments that are incapable of stopping, or reverting the course of the disease, and they cannot restore the loss of function incurred.

Cell-based therapies were developed as a potential long-term treatment for PD that aims to replace the DA neurons lost to the degenerative disease. Direct transplantation of fetal midbrain tissue (consisting of DA neurons) into preclinical models of PD had shown great promise, where the transplanted cells were able to reinnervate the damaged striatum, leading to significant functional (motor) recovery [10, 11, 81, 128]. Despite such promising results in preclinical studies, the results of clinical trials in which human fetal mesencephalic tissue was transplanted into the brains of PD patients have been mixed [44, 75, 80, 92, 103]. While cell replacement therapies appear to bring about short-term improvement of motor symptoms, there is scarce evidence for the long-term benefits of cell transplantation both clinically and in post-mortem analysis of the brain. It has been postulated that the limited efficacy may be partially due to the crude tissue preparation that consists of a heterogeneous population of cells, of which only approximately 10% are DA neurons [18, 152]. Axonal projections of transplanted DA neurons have been shown to make appropriate connections with target cells in the striatum, but the dendritic spines often do not receive input from its natural targets. This results in the unregulated release of dopamine from the transplanted DA neurons, as well as inappropriate release of serotonin from other cells present in the transplant [90, 92]. Significantly, recent postmortem analysis of some PD patients, who had survived the transplantation for more than 10 years, shows that the grafted neurons also developed Lewy body pathology [75, 80]. This provides strong evidence that grafted cells can also become affected by the PD pathology through prolonged interaction with the diseased host tissue. There is also strong evidence that neuronal degeneration in PD is not limited to DA neurons in the nigrostriatal pathway, but is also seen in neurons within the corticostriatal and thalamostriatal pathways at different stages of the disease [16, 137]. Thus, therapy based on restoration of the nigrostriatal pathway alone is likely to provide only temporary relief of motor symptoms, albeit longer than both drug and surgical therapy. As the related pathways and cell types that are affected in PD become elucidated, and more updated models describing PD progression are developed [16], it becomes apparent that effective treatment strategies must take into account the widespread degeneration of neurons in other parts of the nervous system.

While restoration of the nigrostriatal pathways may not provide a cure for PD, it is likely to remain a major component in the ultimate treatment regime. Therefore, current limitations to cell-based therapies, namely poor viability (only 3–5% of surviving transplanted cells are DA neurons, representing approximately 0.3% of total grafted cells [106, 139]) and heterogeneous nature of transplanted fetal tissue, must be addressed. Cell replacement therapies require fetal midbrain tissue to be obtained from multiple (up to 6–8) embryos and cell expansion in culture to reach therapeutically relevant quantities [82, 104, 146]. In addition, implanted fetal midbrain cells have poor viability, which in combination with the low percentage of relevant cell types in the transplanted tissue, further limits the efficiency of such cell-based therapies. Various tissue-engineered scaffolds, in particular hydrogels, appear capable of alleviating such limitations by providing a permissive environment for cell survival in vivo. Issues such as ectopic termination of dendritic and axonal processes, and persistence of irrelevant cell types, both of which are active topics of research in BTE, can be potentially resolved through the use of well-designed scaffolds and stem/progenitor cells. Physical and biochemical cues can be embedded within the scaffold to direct differentiation of exogenous stem cells into DA neurons, as well as guide process outgrowth such that the nigrostriatal pathway can be reconstructed.

Because of their capacity for self-renewal and differentiation into multiple cell types, stem cells have been heavily studied for use in cell replacement strategies. However, the differentiation of stem cells into a stable DA phenotype, even under the relatively stable in vitro conditions, is not a trivial task; as such there is still much debate regarding the type of stem cells

(embryonic or neural), and the state of differentiation of cells to be used for transplantation. Transplanted ESCs have shown a propensity to develop into teratomas [12, 135], whereas transplantation of differentiated DA neurons results in poor survival and subooptimal integration in the host brain [75, 76, 80, 91, 92]. Tissue engineering principles can be applied by combining a suitable scaffold, stem cells, and appropriate signaling molecules to improve clinical outcomes. In essence, the scaffold will function to improve the cell viability during the transplantation procedure by presenting a permissive microenvironment for cellular growth, while also shielding cells from any external inhibitory conditions. Such a scaffold can also be tailored with bioactive functionalities to meet the following objectives:

- Promote cell proliferation to generate an adequately large population to replace the cells lost to the degenerative process
- Specifically direct cell differentiation to attain relevant cell types only (i.e., DA neurons)
- Direct both axonal and dendritic processes to make connections with the appropriate cell types within the host brain

Optimization of each of these scaffold components is still required. However, the prospect of implanting such a scaffold directly into the lesion site of PD patients to bring about greater long-term relief from motor symptoms awaits further studies in vivo. The same principles can be adapted to regenerate other pathways that are known to be affected by PD.

22.4.2 Summary

For many brain disorders, such as PD and MS, the ultimate cause is mostly undetermined, which presents limitations to the effectiveness of any treatment aimed at alleviating symptoms, restoring function, and eventually curing the disease. Without an understanding of the upstream cellular dysfunctions, treatment downstream of the disease origin will provide limited relief of symptoms only. Nevertheless, because of the debilitating nature of any disorders of the brain, any partial relief that can be achieved will bring about significant benefits for the patient. Current existing treatments for CNS disorders revolve around drug therapy, surgical treatment, and cell-based therapy. Cell-replacement therapies are receiving greater attention with the expectation of regaining lost functions via restoration of affected cellular pathways. To this end, tissue engineering holds the potential to provide treatments that are superior to any of the currently existing treatment strategies. This potential lies in the capacity to approach treatment strategies in a more targeted and delicate manner, achieving regeneration or symptomatic relief with greater efficiency and fewer side effects. With the abundance of tools available for tailoring scaffolds with specific physical, structural, and biochemical properties, and the selection of stem/progenitor cells, a logically designed scaffold can be custom-made for individual patients with particular disorders. Thus, it is envisioned that tissue engineering principles will provide improved treatment strategies for various disorders of the brain.

22.5 Expert Opinion

22.5.1 Challenges in Tissue Engineering for Brain Repair

Tissue engineering in the brain requires a highly complex and multidisciplinary set of skills. The development of a single scaffold and cell therapy for this purpose can draw upon many fields including computational and experimental neurosciences, stem cell biology, clinical neurology, electrical engineering and signal propagation, materials engineering, nanotechnology, and surface science to name a few. Overall, the common goal is to restore physiological brain function through facilitating appropriate interactions between the CNS and the scaffolds developed for this purpose.

Recently, there has been considerable progress made toward understanding the barriers to regeneration within the adult mammalian brain, as well as different mechanisms that allow for regeneration and function recovery. These advances have paved the way for many different therapeutic avenues, including BTE. However, the recent progress toward axonal regeneration in vivo has mainly been restricted to the PNS using autografts, allografts, and xenografts. For nerve regeneration in the PNS, autografts are considered to be the best form of clinical treatment, as there is no need for immunosuppressive therapy post surgery. However, such strategies are problematic and impractical in the brain.

Any scaffold used to repair damaged tissue within the brain must not exacerbate the innate inflammatory response, or any appreciable form of FBR or cytotoxic response. In addition, the mechanical properties of the scaffolds should have a modulus similar to that of the surrounding tissue not only to avoid structural collapse during normal human activities but also to limit local tearing of surrounding tissue. Recently, considerable progress has been made toward generating biocompatible and compliance-matched biomaterials for nerve regeneration [6, 27, 48, 59, 68, 151]. However, in addition to these requirements it is also ideal for BTE scaffolds to have a porous structure and be biodegradable under physiological conditions. Ideal scaffolds will also have major roles in the following areas:

- Presenting cell adhesion and signaling molecules
- Delivering or presenting growth factors at specific stages of the regeneration process
- Incorporation of different support cells while facilitating their viability and migration

Fundamentally, the incorporation of all of these requirements into a single scaffold represents the holy grail for BTE, as it would replicate steps present during CNS development by providing a physical and biochemical platform for regeneration to occur, which would otherwise be absent or suppressed in the adult mammalian nervous system. Such regeneration would also require long-term histological analysis in preclinical models to determine structural integrity of repair sites, as well as functional repair using local innervations studies or whole animal scanning modalities. Once such regeneration is achieved, it will be critical for the patient to undergo extensive rehabilitation to facilitate adequate signal transduction down the regenerated pathways and synaptic remodeling before symptoms can be fully eradicated.

To date, despite extensive research, scaffolds or conduits only have the ability to incorporate several, but not all, of these critical requirements; hence, efficient and effective brain regeneration remains on the horizon.

22.5.2 Current and Future Scaffolds for Brain Repair

Recent efforts have focused on the formation of scaffolds that are designed to guide axonal regeneration from one location to another. These can be produced from a variety of different techniques, but thus far the majority of research has focused on electrospun and hydrogel scaffolds.

Electrospun polymer nanofibers have shown considerable promise because they provide the nano-dimension necessary for intimate interactions between regenerating brain tissue and the 3D environment provided by the scaffold. Neurites, as nano-scaled structures, extend parallel to the fiber direction when cultured on aligned nanofibers, inferring that contact guidance is providing a dominant cue for directing regeneration. However, a greater understanding of the interplay between physical and chemical cues will be essential for the progression of nerve regeneration within the brain.

Both synthetic and natural hydrogels have also shown considerable promise for nerve regeneration. They can be engineered to exhibit a thermo-responsive character and can be easily injected, and chemically modified to present desired growth factors to neuronal cells. One of the disadvantages of using hydrogels is that they are isotropic structures and therefore unable to provide directional cues. Consequently, exogenous delivery of neurotrophic factors, either homogeneously or as an immobilized gradient, is often required; however, such modified hydrogels have had limited success for neuronal regeneration over large distances.

The fabrication of a scaffold that simulates some features of the 3D niche is important to support the maintenance of implanted stem/progenitor cells. The scaffold must also provide guidance cues for the regenerating axon to reach its target and to control the relative positions of neurons and glia. It is already apparent that the characteristics needed to meet these two requirements are probably mutually exclusive. Thus, the use of multiphase scaffolds appears to be the next logical progression in scaffold design for BTE. For example, electrospun nanofibers with layered architectures of both aligned (providing contact guidance and controlling cell migration) and randomly orientated nanofibers (providing stem cell niches) can be envisaged. Different designs that consist of a mixture of composite material and microstructure, for instance incorporating aligned nanofibres within an isotropic hydrogel, in combination with biochemical support for regenerating axons may be more appropriate than current designs. Such biochemical support may be offered by growth factors (either soluble or immobilized to the fibers/hydrogel), and cellular support provided by stem cells and glial cells to the endogenous

tissue. However, the major pitfall of the use of guidance channels in the brain, as in the case of autografts, is the invasive surgical techniques that are required for implantation.

Current trends in surface micro- and nanotechnology are beginning to explore alternative methods of surface structuring at the cellular scale. To this end, inkjet printing of biomolecules, or even cells, represents a unique and high-throughput method, which may prove to be more economically viable and timesaving than soft lithographic approaches. Similar to the previously described micro-patterning techniques, spatial organization of cells can be achieved by inkjet printing of biomolecular patterns on a cell nonadhesive background [63, 120, 123]. Alternatively, cells can be directly deposited onto predetermined positions on the scaffolds via inkjet printing [13, 14, 117, 125, 155, 156]. Primary embryonic rat motoneurons, as well as hippocampal and cortical cells that were deposited by inkjet printing not only survived the printing process, but also exhibited typical neuronal morphology and electrophysiological characteristics [155, 156].

In addition to controlling cell positioning within a scaffold, inkjet printing can be used as another method of depositing concentration gradients of chemotropic factors and presenting chemotactic cues to direct cell migration and neurite outgrowth. Inkjet printed gradients approximate truly continuous gradients via the use of halftones, by varying the size and spacing between deposited droplets. Current commercial inkjet printers are capable of delivering drop sizes as small as 1–5 pL, producing spot sizes on substrates of approximately 20 μm [77]. Given that a typical growth cone has a radius of approximately 10 μm, and estimated concentration gradients generated via inkjet printing can have a resolution in the order of 10 μm, inkjet printed gradients should have sufficiently high resolution for directing neurite outgrowth especially with short range neurotrophins.

All equipment and components for inkjet printing are inexpensive and commercially available, and can be modified with relative ease to accommodate specific printing systems. Thus, inkjet printing has been used to print micro- and nano-arrays of proteins, nucleic acids, and even cells [63, 102, 119, 120, 125, 155, 156]. Despite exposure to high temperatures and high shear rate during the printing process, printed cells can remain viable, and printed proteins can remain bioactive. For example, a modified thermal inkjet printer has been used to print concentration gradients of fibroblast growth factor-2 (FGF-2) and ciliary neurotrophic factor (CNTF) onto thin films of polyacrylamide-based hydrogels. FGF-2 was used to maintain NSCs in a proliferative progenitor state, while CNTF was used to induce rapid differentiation of NSCs into astrocytes. Although concentration gradients of these factors were approximated by varying drop density, NSCs cultured on the printed gradients of CNTF demonstrated a linear increase in number of astrocytes. In thermal inkjet printing, the timescales involved in the drop ejection process (in the order of 100 μs) are sufficiently small to limit the diffusion of heat to the bulk of the liquid [156]. Therefore, it is possible that the majority of printed proteins will not be denatured because of the elevated temperatures associated with thermal inkjet printing.

22.6 Five-Year Perspective

While the long term aim of BTE strategies is brain repair, there are some intermediate goals that need to be more thoroughly understood before the ultimate aim can be realized. The molecular, cellular, and structural processes that guide the formation of white matter (axonal tracts) and grey matter (cell bodies), and the manner in which neurons and neurites assemble into interconnected communication channels, as well as the timing of these events must be considered in their entirety. Biologically, all these occur in 3D, yet in most circumstances these processes have been studied in vitro using 2D cell cultures, because of technical hurdles in modeling the complexity of the brain for long periods. Therefore, an immediate aim should be to develop scaffolds that can better integrate in vitro cell culture models, explants, or organotypic models, with in vivo animal models to address the current pitfalls that exist for cell replacement therapies and in vivo axonal extension. To this end, the already multipldiciplinary field must further expand and develop its interdisciplinary skills to produce structural and functional nano-materials that will encourage the formation of nuclei and neuronal tracts in 3D.

Such 3D culture tools would allow for a greater understanding with regard to the interactions between cells and their physical environment, while also offering some insight into chemical cues (signaling

molecules) and how they organize white matter tract and nuclei formation. For instance, the ability to manipulate important biological processes such as contact guidance, cell–cell interaction, and chemotaxis, to achieve meaningful outcomes in BTE is not yet fully understood. Three-dimensional culture tools may prove critical in answering these fundamental questions. In the next 5 years, these studies are vital in order to gain an understanding of the normal function and repair processes within the adult mammalian brain.

It follows that the critical criteria for developing a scaffold aimed at mimicking features of the natural ECM for brain repair must satisfy the following requirements:

- Provide discrete 3D *niche* environments that can selectively promote either cell body or neuronal tract formation
- Enable determination of the relative contributions of trophic factors, physical factors, and cell–cell interactions in inducing axon guidance
- Work toward an understanding of the temporal nature of chemical signals in tract formation

3D in vitro models are essential to study the complex interplay of the ECM environment, including chemical and mechanical stimulation, with cells. To achieve this, the next generation of nano-biomaterials should replicate the multifaceted features of the native 3D cellular niche. Recently, it has been shown that neurons cultured on 3D nanofibers or hydrogels adopt more in vivo-like morphologies compared to cultures on 2D substrates of the same material [99], highlighting the importance of providing architectural features that are similar to the native ECM. Furthermore, mechanical stimulus (e.g., substrate elasticity) can influence focal-adhesions and cytoskeletal organization, and has been shown to be a powerful regulator of stem cell differentiation. Substrate elasticity in 2D formats can also influence neurite outgrowth; hence, the optimization of this parameter for tissue engineering within the brain is also relevant. It is likely that the ability of cells to sense substrate stiffness in a 3D environment will be highly relevant to the final outcome of neuronal regeneration. Even though its effects remain largely unknown, the optimized combination and synergies of elasticity and 3D architecture may prove to be a dominant and powerful regulator of neuronal expression.

22.7 Limitations/Critical Review

Despite promising progress in our understanding of both molecular and cellular mechanisms underpinning development, regeneration, and disease processes, translation of tissue engineering principles into the clinical setting remains a challenge. One of the main issues that limit self-repair mechanisms within the brain following disease and injury is the naturally inhibitory CNS environment. In addition to this inhibitory environment, the presence of highly confined neurogenic niches may represent an evolutionary mechanism that reflects the importance of maintaining stable “hard-wired” neuronal networks for normal adult brain function. Excessive growth and introduction of new neurons into highly precise, existing circuits may interfere with brain function. While suppression of such (neurogenic) events is essential in a healthy brain, it poses severe limitations for brain tissue remodeling and regeneration in response to disease and injury. Indeed, therapeutic suppression of a natural and neuroprotective immune response may be detrimental, by prolonging a chronic state of disrepair that may lead to long term side-effects.

Tissue engineering in the brain strives to restore normal brain function following disease or injury by enabling cells to regenerate and reform any lost connections. The general approach to this problem involves the use of an optimized combination of scaffold, exogenous source of cells, and signaling molecules to recreate an environment with the capacity to trigger a sequence of cellular events that promote regeneration. However, interactions between cells and their natural extracellular environment are highly complex, where cells receive and integrate an intricate sequence of information from multiple sources (ECM, nearby cells, distant cells, growth factors), as well as routine signaling during normal neural circuit processes. It appears that the spatial and temporal coordination of signaling events provides multiple opportunities for modulation and fine control of cell response, while maintaining robustness and preservation of tissue function. This level of complexity is very hard to mimic, and detailed studies of the temporal, spatial, and cellular (phenotype) evolution of signaling events are required to elicit appropriate cellular responses such as proliferation, migration, and differentiation.

Traditional cell replacement therapies utilized in degenerative diseases such as PD are often viewed as a crude approach in addressing a meticulous problem of restoring neuronal circuitry. They highlight the advantages of engaging tissue engineering approaches to achieve greater efficacy in treatment of brain disorders because of their potential ability to precisely control cell behavior both before and after implantation. This ultimately means that transplanted cells will have a better chance of achieving functional integration into the existing host circuitry. However, in order for tissue-engineering based treatments to become prominent in the clinical setting, the long-term performance of therapeutic interventions using scaffold-based approaches need to be established. Many in vivo studies have often shown that the observed restoration of degenerated neuronal pathway does not always correlate to the degree of functional recovery. Therefore, the link between local brain regeneration and improvement in clinical outcomes needs to be further investigated. It is equally important in the development of repair and regeneration strategies to consider the cause/etiology of a disease, and not just to focus on replacing cells lost as part of the disease process.

22.8 Conclusion/Summary

The primary requirement of any scaffolds for tissue engineering is to provide a permissive environment that is conducive to cell growth and survival. Thereafter, BTE places significant emphasis on recreating a specialized niche microenvironment endowed with physical, biochemical, and biological cues to instruct cell behavior. While an arsenal of techniques have been developed to control various aspects of cell behavior – including survival, adhesion, proliferation, migration, differentiation, and process outgrowth – the precise mechanisms via which individual cues exert influences over cell behavior are still being uncovered, and the optimal combinations and possible synergy that can be achieved through a combination of BTE interventions are still also evolving in parallel with these new biological insights.

Even though the design of synthetic scaffolds should endeavor to replicate certain conditions that occur during embryonic brain development, recapitulation of all such cellular events is both impractical and not necessarily required for regeneration to occur. However, it is anticipated that scaffolds utilized for BTE should embrace the concept of biomolecular signal presentation to cells in a spatially and temporally coordinated manner, with a concomitant sequence of cellular responses.

Of the currently available fabrication technologies, hydrogels, electrospun and self-assembled nanofibers to exhibit desirable architectural and structural features to support cell adhesion and survival. Properties of the electrospun scaffolds, such as average fiber diameter, fiber alignment, and interfiber distance can be systematically altered to modulate various aspects of cell behavior. However, progress in BTE is likely to require innovative designs, combining elements of different scaffolds that are known to promote desirable cell behavior. The use of scaffolds with component structures that are optimized for different cell behaviors (e.g., neurite outgrowth and supportive niche conditions) is likely to become an important next step in BTE. Biofunctionalization of scaffolds with growth factors, adhesive ECM proteins, and peptide motifs has also become a prerequisite for selectively enhancing cell response through specific and ordered ligand–receptor mediated signaling cascades. The importance of biological support cells within scaffolds is also becoming more apparent, improving interaction between cells and the substrates, as well as providing a biochemical milieu that is required for cellular regeneration, which cannot be generated by synthetic means.

In order for more effective therapeutic treatments to be developed, a comprehensive understanding of the roles and interactions of cells and bioactive molecules in the normal and diseased brain needs to be elucidated. Of particular importance is how neurogenesis and gliogenesis are regulated in the adult brain and how they can be harnessed in the regeneration process via tissue engineering principles. This includes an improved understanding of the significance and functions of different cell types, as well as cells in different stages of their developmental program. It is hoped that the wealth of technical expertise in the fabrication of biomaterial scaffolds for BTE will continue to mesh synergistically with stem cell and regenerative medicine, and resources from existing therapies, to expedite bench-to-bedside efforts for brain repair.

Suggested Readings

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Engineering Cartilage Tissue

23

Robert L. Mauck and Jason A. Burdick

23.1 Introduction

Although there are many types of cartilage in the human body, including hyaline cartilage in articulating surfaces and in the nose and ribs, elastic cartilage within the ear (auricular), and fibrocartilage within the intervertebral disks and meniscus, the intent of this chapter is to focus on the specific state of the art for the engineering of articular cartilage found in joints. Promising developments have been made in the engineering of each of these types of cartilage, with research focused on recapitulation of the differing general structures and cell types of each (shown in Fig. 23.1 for articular and auricular cartilage). These differences likely arise due to their different functions and loading environment during development and in the adult. Articular cartilage will be addressed in detail throughout this chapter and is important due to the clinical need for new therapies for patients with cartilage damage or disease. Unlike many of these other types of cartilage, the load-bearing nature of articular cartilage also makes it challenging to engineer with the appropriate properties to enable it to immediately act as a replacement tissue.

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23.1.1 Adult Articular Cartilage: Composition, Mechanical Properties, and Physiologic Loading

As the load bearing material of diarthrodial joints, articular cartilage lines the boney surfaces and functions to transmit the high stresses that originate with motion. Articular cartilage consists of both a solid matrix and a fluid phase [9]. The solid matrix is composed of a dense network of type II collagen fibrils enmeshed in a solution of charged, aggregated proteoglycans (PGs: aggrecan core protein plus glycosaminoglycan (GAG) side chains). Collagen content ranges from 5 to 30% by wet weight and PG content from 2 to 10%, with the remainder of the tissue made up of water. Chondrocytes make up less than 10% of the tissue and produce extracellular martrix (ECM) to balance continual degradation and remodeling by matrix metalloproteinases (MMPs, collagenases and aggrecanases) [61]. The composition, architecture, and remodeling of cartilage are uniquely adapted to function over a lifetime of repetitive use.

The mechanical properties of cartilage are crucial for its ability to operate in the demanding joint environment without wear or structural damage. A summary of the various mechanical properties of cartilage tissue and how they are modulated with developmental status/age are outlined in Table 23.1. A normally active adult takes 1–2 million steps per year [164] with the resulting forces acting on cartilage of extrapolated weight 2.5–4.9 times the body weight, with stresses reaching 2–10 MPa [9]. Loading is cyclical/intermittent and contact areas move as joint surfaces slide relative to one another with velocities of 20–250 mm/s [26]. The dense PG-rich collagenous matrix of cartilage resists

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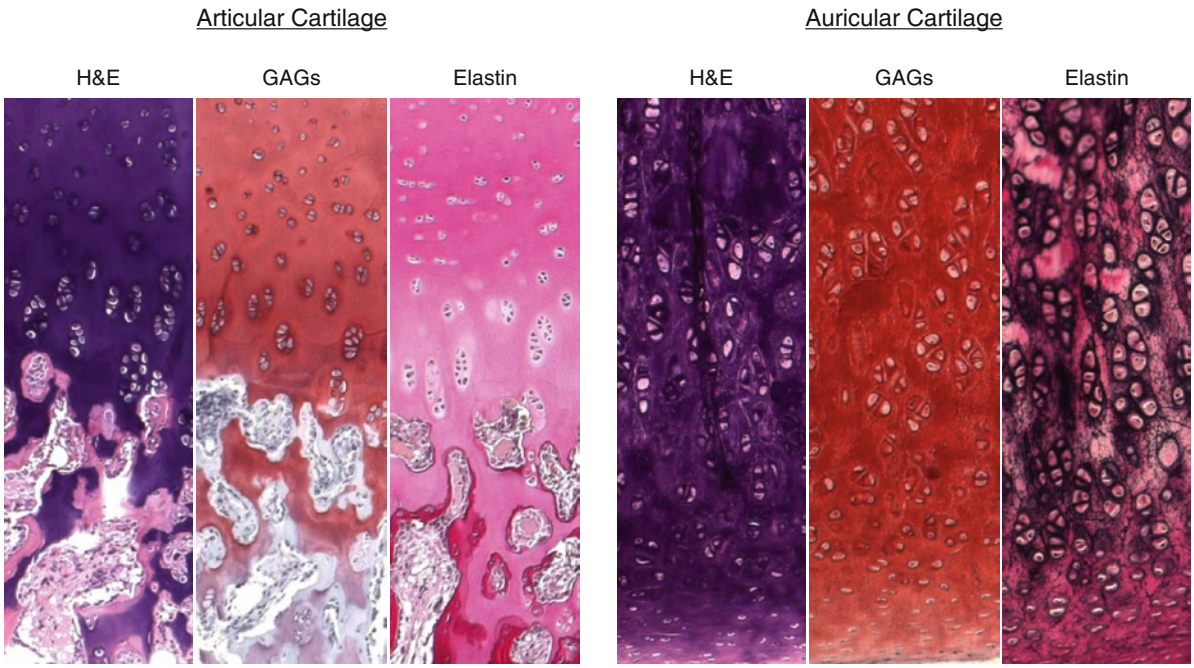

Fig. 23.1 Histomorphology of cartilage tissue. Histological staining for hematoxylin and eosin (H&E), glycosaminoglycans (GAGs), and elastin for both articular (joint) and auricular (ear) cartilage. Note the differences in the cellular morphology with depth, the interface with subchondral bone tissue in the articular cartilage, and the presence of elastin in the auricular tissue. These differences in cartilage structure and matrix content and distribution necessitate a range of tissue engineering strategies to fabricate each specific type of cartilage

Table 23.1 Cartilage biochemical and mechanical properties

Cartilage Property	Developmental stage		
	Fetal	Juvenile	Adult
Water content (%)	88	82	80
Chondrocyte density ($\times 10^3$ cells/mL, middle zone)	240	170	80
Bulk GAG content (% wet weight)	2.1–2.2	2.1–2.2	1.8–2.2
Bulk collagen content (% wet weight)	4–5	6–8	10–14
Bulk compressive Young's or aggregate modulus (MPa)	0.15	0.28	0.30
Local compressive modulus (superficial zone, MPa)	0.03	0.15	
Local compressive modulus (middle/deep zone, MPa)	0.13	0.65	
Dynamic compressive modulus (at 1 Hz, MPa)		7.0	
Tensile modulus (MPa)	0.5–1.0	4	5
Friction coefficient (instantaneous)		0.010	
Friction coefficient (equilibrium)		0.243	

Data extracted or estimated from refs [74, 81, 87, 161, 166, 167] for bovine cartilage

this loading environment with its high equilibrium compression modulus of 0.2–1.4 MPa [162] and even higher tensile modulus ranging from 1 to 30 MPa [2] in the plane of the tissue. Other minor structural elements of cartilage, including type IX collagen [170] and cartilage oligomeric matrix protein (COMP) [31], play significant roles in the cross-linking of the type II collagen network. The organization and prevailing direction of collagen fibers (the split line direction) are of particular importance for the tensile properties which are highest at the surface and in the split line direction [69]. Split lines have a different organization in loaded and unloaded regions of joints [55] that is similar between patients, consistent with the idea that physiologic use defines structural organization [14].

An additional higher-order characteristic of cartilage is its marked inhomogeneity through the depth, with a depth-dependent compressive modulus varying from <100 kPa at the surface to >2 MPa in the deep zone [144, 161]. With this dense specialized matrix, cyclical compressive loading at physiologic frequencies (0.1–2 Hz) causes the interstitial fluid pressure to increase, resulting in a higher dynamic modulus than equilibrium modulus [127]. This is enhanced by the disparity between the tensile and compressive moduli [150, 151]. The enhanced fluid pressurization at the point of contact supports >90% of applied stress, shielding the solid matrix from excess deformation [150]. Furthermore, this fluid pressurization, coupled with boundary lubricants such as lubricin/SZP located at the articular surface, helps maintain an extremely low coefficient of friction [76, 87], thereby reducing the potential for wear to occur under repeated sliding contact. Remarkably, with its high contact stresses, cartilage thickness in vivo changes by <6–20% with use [35]. It is precisely this combination of tensile and compressive properties, promoting fluid pressurization, which enables cartilage mechanical function and must be recapitulated in any successful repair.

23.1.2 Articular Cartilage: Formation and Maturation

While much is known regarding the function and composition of adult cartilage, it is also important to understand how cartilage develops such unique properties. As illustrated in Fig. 23.2, the first appearance of a cartilage-like tissue (dense organization of PG and type II collagen) is in the limb bud [53]. Limb bud cells aggregate and undergo chondrogenesis under a complex array of morphogenetic and transcription factors, and other signaling cues [53]. While this material is "cartilage," it is transient in nature, and most of it is eventually replaced through endochondral ossification. At sites of future joint formation [126], early signaling events result in the expression of such factors as GDF-5, PRG4, Wnt14, and the transcription factor ERG [126]. These molecules distinguish this "interzone" from the remainder of the cartilaginous

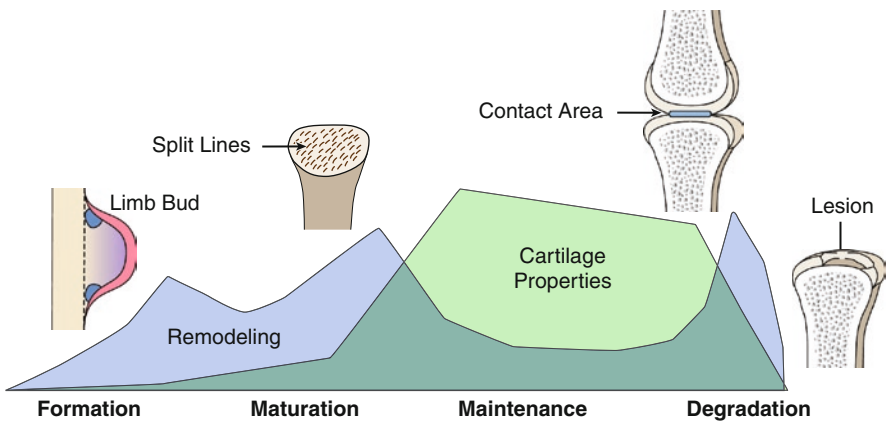

Fig. 23.2 Life cycle of articular cartilage. The development of articular cartilage tissue starts with a formation stage in the embryo, continues with a maturation stage through development, leads to a maintenance stage in adulthood, and potentially through to a degradation stage with aging or after a traumatic event. This dynamic life cycle, including developmental biology considerations, may be useful in optimizing efforts toward cartilage tissue engineering

anlagen. While the precise sequence of events has yet to be definitively mapped, the interzone appears to be invaded by cells located on the anlagen periphery [86]. These cells differentiate into all of the fibrous joint structures, and occupy at least the superficial and middle zones of the eventual articular cartilage. In addition to biological factors, mechanical forces are essential for cartilage joint formation [109]. For example, inhibitors of muscle contraction in vivo lead to incomplete joint formation and decreased properties [107, 108, 133].

After birth, articular cartilage undergoes dramatic maturational changes, with collagen fiber organization transforming from an isotropic arrangement to a mature configuration with superficial fibers arranged into split lines parallel to the surface [8]. Compared to the initial anlagen formation, less is known about the cells in the articulating cartilage, and the signaling events that define their maturation. Unlike cells within other skeletal structures, articulating cartilage cells never undergo hypertrophy. Pacifici et al. recently reviewed the extant literature on these articular chondrocytes, and concluded that articular cells that retain a permanent cartilage phenotype are distinguishable from the transient chondrocytes that form the initial anlagen and growth plate [125]. While these different cell types likely share some signaling mechanisms, it is not yet clear whether these factors act in the same fashion. Nevertheless, what is clear is that the maturation of cartilage results in dramatic increases in tissue mechanics (particularly tensile properties) [10, 77]. In mice, MMPs-2,-3, and -9 are absent at birth and peak 2 weeks later, suggesting an intense early ECM remodeling [51]. In the cow, mechanical and biochemical properties increase rapidly in juvenile compared to fetal cartilages [166], with increases in tensile properties correlated to increases in collagen content and cross-linking. Similarly, fetal cartilage shows a relatively uniform compressive modulus through the depth, while the marked inhomogeneity arises in animals 1–2 weeks post natal [81]. At the same time, tissue cellularity decreases markedly, from greater than 200 million cells/mL in fetal calf tissue to less than 80 million cells/mL in adult tissues [74]. When embryonic and juvenile bovine cartilage explants are removed from the joint environment, tensile properties decrease [168]. While one cannot overstate the importance of biologic factors in the in situ milieu, these findings also suggest that the demands placed on cartilage, coincident with use, help define organization and properties (e.g., collagen split lines), allowing the tissue to achieve its mature load bearing capacity. Thus, a developmental cascade of events (see Fig. 23.2), from formation in the embryo through maturation in the adult, results in the unique properties of the native tissue.

23.2 Aim of the Discipline

23.2.1 Overview

The overall objective of this field is to develop a tissue replacement that can be used to restore native function to damaged or diseased cartilage tissue. Two main clinical areas for engineered cartilage have been identified, each with its own requirements. First, focal cartilage lesions that result from trauma present a well defined point of intervention, and are typically surrounded by an otherwise healthy cartilage expanse. Second, in osteoarthritic joints, slow degenerative and catabolic cascades result in entire joint surfaces that have lost their native structure and functional capacity; such widespread damage necessitate strategies that address issues of joint congruity and cartilage topography. In focal defects and entire joint surfaces, integration of engineered cartilage to native joint structures (be it remaining healthy cartilage or subchondral bone) must be considered for effective repair. Moreover, these engineered cartilage implants must be able to operate in the demanding joint environment (described above) either immediately, or after some period of in vivo maturation and integration. These challenges, along with cost and efficacy relative to total joint replacement (with metal and plastic prostheses) remain significant hurdles in cartilage tissue engineering strategies, and must be addressed for any successful repair modality.

23.2.2 Cartilage Tissue Engineering Strategies

As existing surgical methods have yet to provide long-term functional cartilage repair, new strategies are being investigated. In cartilage tissue engineering,

chondrocytes (or a chondrogenic cell source) are combined with a scaffolding material to sequester cells and capture accumulated ECM, forming a cartilage-like replacement tissue. This general approach is outlined in Fig. 23.3. One approach that has been investigated extensively is the seeding of porous scaffolds (foams and fibrous meshes) fabricated from poly(α-hydroxy esters) including poly(glycolic acid), poly(lactic acid), and their copolymers [140, 143, 159]. Examples of biomaterials that have been used for cartilage tissue engineering are listed in Table 23.2. Additional work has shown that foams and meshes based on natural materials (Type I, II collagen, PG/collagen composites) support cartilage growth as well [116, 171]. Chondrocytes cultured on these porous scaffolds form ECM and increased mechanics with culture. An image of chondrocytes interacting with a biomaterial surface is shown in Fig. 23.4. However, uniform seeding throughout the scaffold expanse is a challenge, cells flatten and line the pore spaces, collagen contents remain lower than in native tissue, and directionality of formed matrix has not been achieved.

Alternatively, hydrogels are attractive biomaterials for cartilage regeneration and many natural (e.g., collagen and alginate) and synthetic polymers (e.g., pluronics and PEG) have been investigated (see Table 23.2). Specific examples in the literature include alginate [141], agarose [104], fibrin [122], type I and II collagen [72], peptide gels [79], and PEG [19, 22, 38], to list but a few. This focus is motivated by the observation that in hydrogel culture, chondrocytes can be well dispersed and can take on their natural round shape and phenotype. This shape is particularly important, and it has long been demonstrated that de-differentiated chondrocytes can regain their cartilage ECM producing capacity when seeded in even simple hydrogels [15]. Furthermore, these gels efficiently entrap the cartilage-like ECM produced by the cells [24], and can rapidly assemble a neo-cartilage-like matrix with functional properties. In addition to the scaffolding material, several studies have shown that increasing the initial cell number within the construct can lead to more rapid and/or greater cartilage-ECM formation and mechanics [30, 104]. Further work has shown that inclusion of anabolic growth factors normally found in

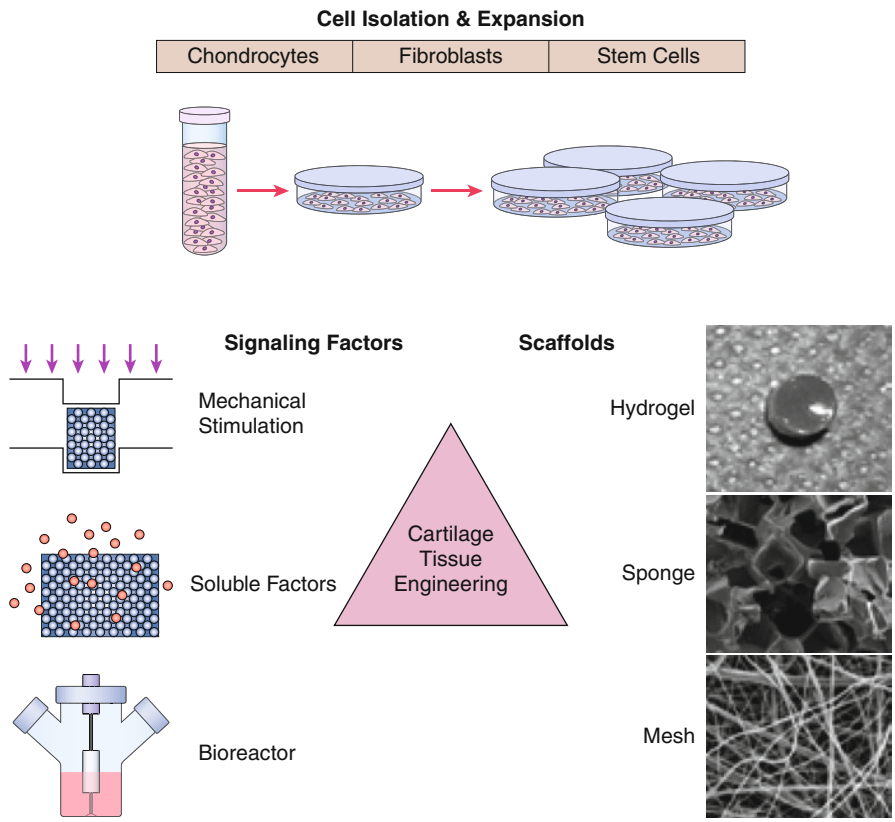

Fig. 23.3 Cartilage tissue engineering components. Schematic of the cartilage tissue engineering approaches that combine cells (chondrocytes, fibroblasts, stem cells), signaling factors (mechanical loading, soluble molecules, bioreactors), and scaffolds (hydrogels, sponges, meshes) in an attempt to grow tissue to replace cartilage lost to damage or disease

Table 23.2 Types of biomaterials used in cartilage tissue engineering

Class	Examples
Natural polymers	Agarose Alginate Cellulose Collagen Chitosan Chondroitin sulfate Fibrin glue Gelatin Hyaluronic acid Silk fibroin Elastin-like peptides Self-assembling peptides
Synthetic polymers	Poly(α-hydroxy esters) Poly(ethylene glycol/oxide) Poly(NiPAAm) Poly(propylene fumarate) Poly(urethane) Poly(vinyl alcohol)

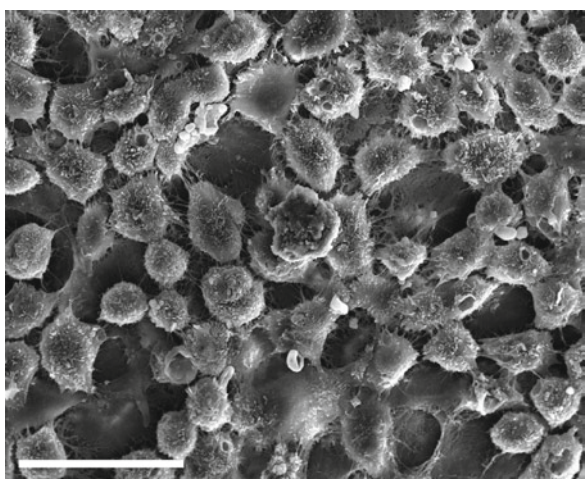

Fig. 23.4 Chondrocyte morphology when interacting with biomaterials. Scanning electron micrograph of articular chondrocytes interacting with the surface of a biomaterial. Note that cells exhibit a range of morphologies, from rounded (chondrocyte-like) to spread (fibroblast-like). Cell shape has been implicated in maintenance of the chondrocyte phenotype (scale bar = 30 μm)

the maturing and mature synovial fluid (such as IGF-1, transforming growth factor (TGF) beta family members, and FGF) can further improve cartilage-like tissue development in engineered constructs [25, 102, 130].

23.3 State of the Art

As stated above, there are many types of materials that have been used in strategies for cartilage tissue engineering, but hydrogels have several distinct advantages and will be a focus of our discussion of state of the art for cartilage repair. Additionally, although there are a range of choices for the encapsulation of cells in hydrogels, photoinitiated polymerizations are becoming a popular choice. As a cell source, there are many choices to be made and this section will focus primarily on the use of adult mesenchymal stem cells (MSCs) as a source for applications in the regeneration of cartilage tissues.

23.3.1 Photopolymerizable Hydrogels

Although there are many classes of hydrogels for biomedical applications, photopolymerizable hydrogels have many advantages in medicine and specifically, for cartilage tissue engineering (TE) [7, 165]. These macromolecular assemblies form by the covalent coupling of polymer chains with light exposure in the presence of a photoinitiator. Schematics of the use of photopolymerization in cartilage tissue engineering for the encapsulation of cells in vitro or for in vivo cell delivery are shown in Fig. 23.5. Benefits of such gels include the ability to form directly in a defect with a complex geometry, the ability to polymerize at any temperature without the need for toxic solvents, and the potential for adhesion and integration with surrounding tissue. Additionally, a wide variety of chemistries and network modifications have been developed. Finally, the photopolymerization process provides both temporal and spatial control over the polymerization, something that is limited with other materials. Elisseeff et al. [37] first used a transdermally polymerized hydrogel formed from dimethacrylated poly(ethylene oxide) and semi-interpenetrating linear poly(ethylene oxide) chains. This work illustrated both the ability to photoencapsulate viable chondrocytes in a hydrogel network with light transmitted through dermal tissue and the production of neocartilage with both PG and collagen present. Bryant and Anseth later showed that photocrosslinked scaffolds could be fabricated that span the thickness of native cartilage found in vivo while maintaining PG production [19].

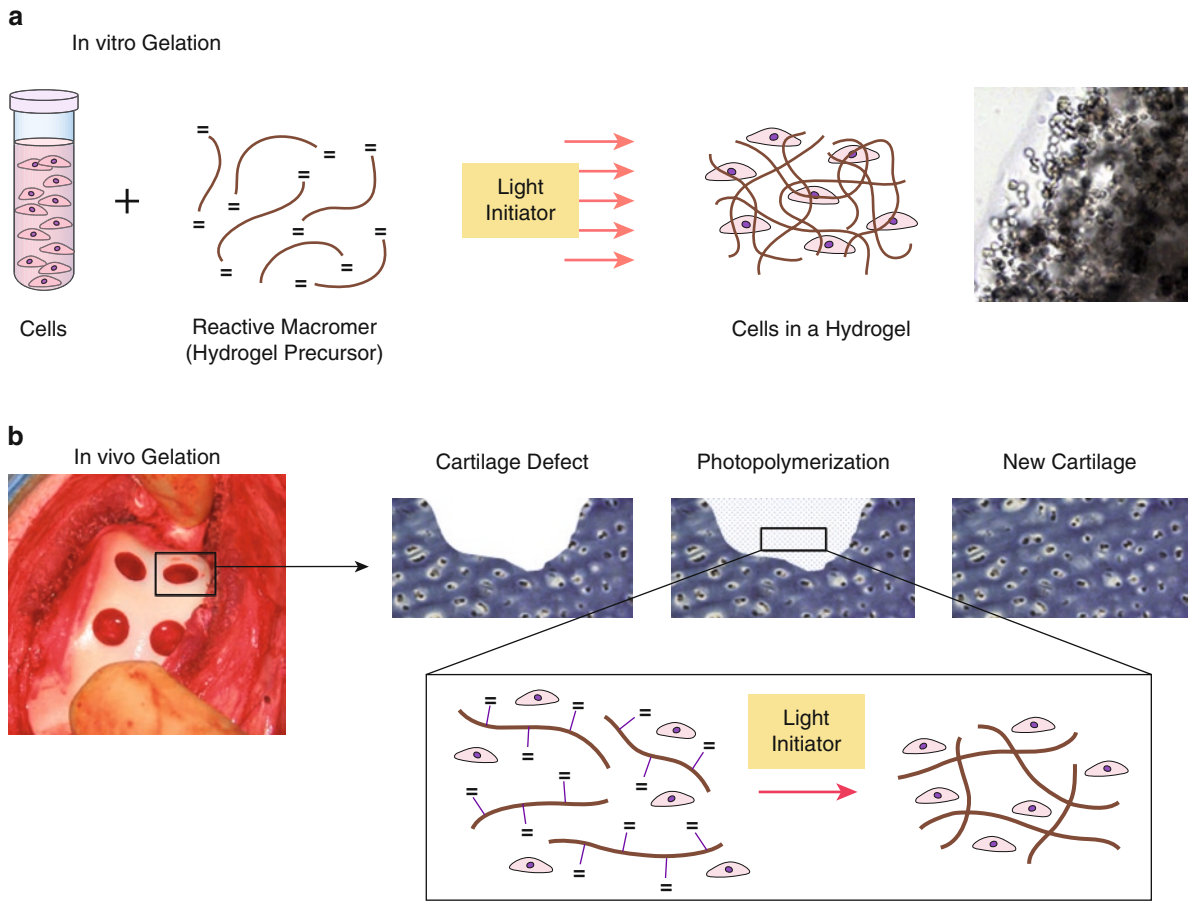

Fig. 23.5 Photopolymerization in cartilage tissue engineering. (**a**) In vitro process of suspending cells in a reactive macromer solution containing polymer and initiator followed by exposure to light to induce hydrogel gelation. (**b**) In vivo process wherein the cell suspension can be injected into a defect and gelled in situ using light

23.3.2 *Tailoring Hydrogels to Promote Cartilage Tissue Formation*

While a wide range of hydrogels have been developed, perhaps the most interesting are ones that are responsive to their local microenvironment or that exhibit temporal changes in properties. In gels, chondrocytes interact with their microenvironment and act as a point source for the production of ECM. Buschmann used agarose gels to illustrate the time-dependence of spatial accumulation of ECM, starting as small islands that eventually join with one another to form a contiguous ECM [24]. Buschmann also speculated that the high local concentration of PGs that form at the cell borders might act (through negative feedback mechanisms) to down-regulate further PG production, thereby limiting the rate of matrix accumulation. Others have quantified the temporal aspects of matrix accumulation around cells and diffusivity limitations by visualizing PG deposition using an autoradiographic technique [136] and via modeling with a finite element approach [145] and showed that diffusivity and binding of newly formed ECM may hinder large molecule transport. Theoretical analyses performed on the basis of mixture theory modeling of cartilage and hydrogels showed that molecular diffusion was impeded with increasing matrix density [101].

On the basis of this understanding, a number of investigators have examined how changes in hydrogel properties (e.g., crosslinking density) influence the synthesis and distribution of collagen and PGs by encapsulated chondrocytes [20]. This work generally

showed that higher crosslinking densities limit matrix distribution. To hasten matrix distribution, dynamic hydrogels have been developed that incorporate degradable linkages as have enzymatic treatment methods been applied to cell-seeded constructs during maturation. For example, investigators have applied agarase (which degrades agarose) to remove the remnants of this gel from the neo-tissue [118] while others have applied lipase to PEG-based hydrogels to degrade caprolactone crosslinks [138]. Others have engineered the hydrogel itself via the inclusion of hydrolytically degradable linkages in synthetic PEG gels; findings from this work show a markedly greater level of collagen deposition and distribution by chondrocytes seeded within [20]. Similarly, we have recently modified hydrogels based on the natural polysaccharide hyaluronic acid (HA) to incorporate a range of tunable hydrolytic linkages [142]. In these gels, greater matrix formation, and to a wider extent, was observed when a chondrogenic cell source was encapsulated in these hydrolytically degrading gels compared to slower enzymatically degrading hydrogels (see Fig. 23.6).

Still other hydrogel systems have been designed with MMP-cleavable linkages and backbones, such that remodeling of a synthetic matrix can occur by natural, cell-mediated mechanisms. For example, an MMP-sensitive PEG based gel developed by Lutolf [94] was used to encapsulate bovine chondrocytes [128]. This study showed a greater distribution of formed matrix in gels with MMP-sensitive linkages, as well as greater expression of aggrecan and type II collagen. From this body of work, it is clear that a cell encapsulating material must be appropriately designed to promote maximum levels of matrix formation, as well as eventually be removed to further ECM distribution throughout the construct.

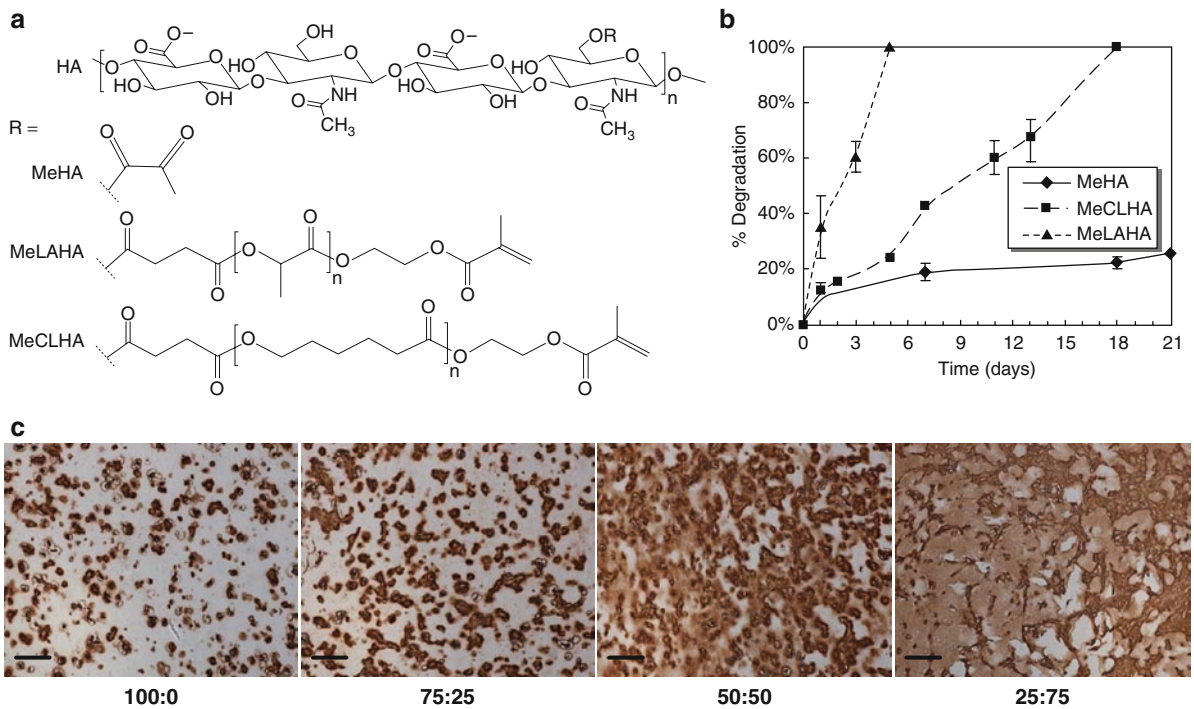

Fig. 23.6 Tuning tissue production with polymer degradation. (**a**) Chemical structure of methacrylated hyaluronic acid (MeHA) with various hydrolytically degradable groups (e.g., *LA* lactic acid; *CL* caprolactone) situated between the hyaluronic acid (HA) and the reactive group. (**b**) Degradation profiles for hydrogels formed from MeLAHA, MeCLHA, and MeHA macromers indicating control over degradation timescale. (**c**) Chondroitin sulfate staining of mesenchymal stem cells (MSCs) encapsulated in copolymer gels of MeHA:MeLAHA indicating enhanced distribution with greater fractions of the rapidly degrading MeLAHA macromer

23.3.3 Hyaluronic Acid: Biologic Relevance, Role in Cartilage, and Hydrogel Formation

One attractive molecule that we have used extensively for the formation of hydrogels in cartilage tissue engineering is HA. This linear polysaccharide is comprised of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine. During development, HA regulates a multiplicity of cellular functions, (e.g., gene expression, signaling, proliferation, motility, adhesion, metastasis, and morphogenesis) [84]. HA content is highest in undifferentiated cell aggregates during early embryogenesis, and then decreases with differentiation [158], where it has a crucial role in angiogenesis [139, 148]. It was recently shown that human embryonic stem cells can be maintained in an undifferentiated state in 3D HA networks [52]. In the musculoskeletal system, HA is down-regulated in the developing limb bud, allowing for cell aggregation and differentiation to proceed [90]. At the site of future joint formation, HA production is increased, allowing for separation of cartilage elements at the forming interzone through the process of cavitation [8]. HA interactions are MW dependent and mediated through cell surface receptors (e.g., CD44, ICAM-1, and RHAMM) [106], with CD44 particularly implicated in chondrocyte attachment [82].

In adult cartilage, HA is present in the form of long linear chains with MWs of up to 10 million kDa [153] and serves as an anchorage point for aggregated PGs via coupling to the aggrecan core protein via link protein [63]. HA–CD44 interactions are crucial for the formation of the chondrocyte pericellular matrix, an important region that mediates cell–matrix interactions [82]. Under normal conditions, HA in cartilage turns over rapidly (half-life of 1–3 weeks) [111] primarily because of hyaluronidases (HYALs) [154]. In mammalian somatic tissue, three HYAL isoforms (HYAL1, HYAL2, and HYAL3) are present, though only the first two are thought to be active. HA degradation can be controlled by inflammatory cytokines that increase expression and activity of HYALs in cartilage [46] and cell-generated reactive oxygen species [154]. While native HA is rapidly turned over in vivo, it can be modified to enhance stability and enable the formation of 3D structures. For example, HA is readily modified through both its carboxy groups with esterification [97] and hydroxyl groups that are crosslinked with divinyl sulfone [137] or via photopolymerization [21, 129, 149]. An esterified version of HA has been formed into nonwoven 3D scaffolds for use in cartilage TE [97, 112], though this formulation is hydrophobic and cannot be formed into hydrogel structures. For photopolymerization, HA can be modified with methacrylates [117, 149] (see Fig. 23.6). Formed into macromolecular networks, HA hydrogels show a remarkable resistance to catabolic breakdown [21]. HA can also be added into otherwise non-cell interactive gels (i.e., PEG) to promote fibroblast attachment and invasion [88]. Coating collagen sponges with HA can increase cartilage matrix gene expression by chondrocytes [3]. Notably, this study showed stimulatory effects for low HA levels, and inhibitory effects for high HA levels, suggesting that cell–HA interactions must be carefully tuned to ensure robust growth. Finally, by varying the molecular weight and concentration of modified HA formed into hydrogels, a wide range of properties can be obtained [21] that influence ECM production by encapsulated chondrocytes [34].

23.3.4 Cells Used in Cartilage Repair and Tissue Engineering

There are a range of cell types that are being explored for cartilage regeneration and the optimal cell source for cartilage tissue engineering is still being identified [32]. Examples of cells that have been explored for cartilage tissue engineering are outlined in Table 23.3. Chondrocytes, fibroblasts, stem cells, and genetically modified cells have all been explored for their potential

Table 23.3 Cell sources for use in cartilage tissue engineering

Type	Examples
Chondrocytes	Articular Auricular Costal Nasoseptal
Fibroblasts	
Stem cells	Bone-marrow derived Adipose-derived Muscle-derived Synovium-derived Periosteum-derived Embryonic

as a viable cell source for cartilage repair and vary in their source for implantation and capacity to elaborate a cartilage-like ECM. Chondrocytes have the advantage that they are the cell type found in native cartilage and have been extensively studied, yet their isolation can cause damage to the cartilage tissue and may be insufficient in number for implantation into large defects. Recent work has also focused on the use of stem cells, which have multi-lineage potential, can be isolated from numerous tissues, and can be expanded through several passages without significant loss of differentiation potential. Also, fibroblasts are easily obtained in high numbers and can be directed toward a chondrogenic phenotype [121]. Ultimately, the goal is to find a cell source that can be easily isolated, is capable of expansion, and can be cultured to express and synthesize cartilage-specific molecules for functional repair.

23.3.5 Mesenchymal Stem Cells in Cartilage Regeneration

While chondrocytes have been important in delineating a path for cartilage TE, it is likely that fully differentiated cells will not be used in future therapies because of their limited supply, potentially diseased source leading to inferior cells, and complications with tissue harvesting. One opportunity for overcoming such concerns is the use of adult MSCs [27] that are easily obtained from bone marrow aspirates via plastic adherence [49] or by selecting for surface markers (i.e., CD105 and STRO-1 [95, 147]). MSCs have also been isolated from adipose tissue, periosteum, bone chips, and cartilage [39, 123, 156] and retain a multi-lineage potential (i.e., ability to differentiate along numerous pathways, including fat, muscle, bone, and cartilage) [134, 135] when provided the proper cues. MSCs are expandable in culture [18], and may be grown in sufficient numbers to populate engineered scaffolds. Micrographs of both chondrocytes and MSCs are shown in Fig. 23.7. In general, MSC chondrogenic differentiation is initiated with growth factors from the transforming growth factor-β (TGFβ) superfamily, including TGF-β1 and -β3, and BMP-2 [75, 95] in the presence of ascorbate and dexamethasone. Original studies of MSC chondrogenesis were carried out in culture systems, such as micromass or pellet culture that induce a rounded shape and mimic events occurring in forming limb bud, with many of the same signaling pathways observed.

Toward tissue engineering, recent studies have demonstrated MSC chondrogenesis in scaffolds such as fibrin [169], agarose [12, 105], gelatin [12], and alginate hydrogels [12, 39, 95], as well as in polyester foams [99] and polyester/alginate amalgams [29]. Similar to chondrocyte-laden hydrogels, increasing

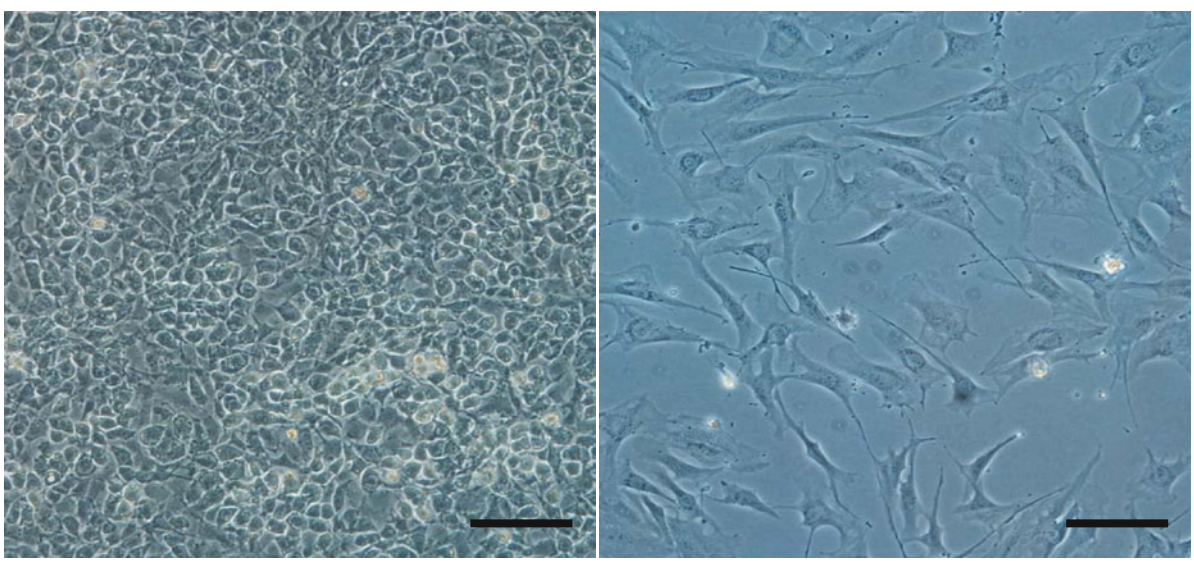

Fig. 23.7 Morphology of chondrocytes and MSCs in primary culture. Primary high-density culture of bovine chondrocytes (*left*) and expansion of bovine bone marrow derived MSCs (*right*) demonstrating differences in cellular morphology. Scale bar: 100 μm

MSC seeding density increases matrix formation rates (on a per cell basis) at low seeding densities (1–5 million cells/mL) in collagen microspheres [70], though culture at much higher densities has yet to be fully characterized. In one recent study, we showed that MSC encapsulation (see Fig. 23.8 for representative MSC chondrogenesis in hydrogels) in agarose hydrogels at densities of 10 and 30 million cells/mL resulted in increasing biochemical content, but did not alter the final tensile properties achieved [66]. This study also indicated that although there are similarities, the expressions of many genes are different between chondrocytes and MSCs differentiated in chondrogenic media, when both are cultured in hydrogels (see Fig. 23.9). Additionally, several studies have shown that the composition of the extracellular housing can itself influence MSC chondrogenesis. Bosnakovksy et al. induced MSC chondrogenesis in alginate and alginate supplemented with type II collagen, and showed that the presence of type II collagen enhanced the differentiation process [16]. We have demonstrated that bovine and human MSCs differentiate and form cartilaginous tissues with increasing compressive properties in agarose [105], self-assembling peptide gels [41], and in HA [33, 41].

As stated above, hydrogels based on HA may be a promising approach for cartilage tissue engineering. Despite the defined role of HA during limb development [90], little is known about the effect of HA on adult MSCs. MSCs cultured in HA hydrogels upregulate

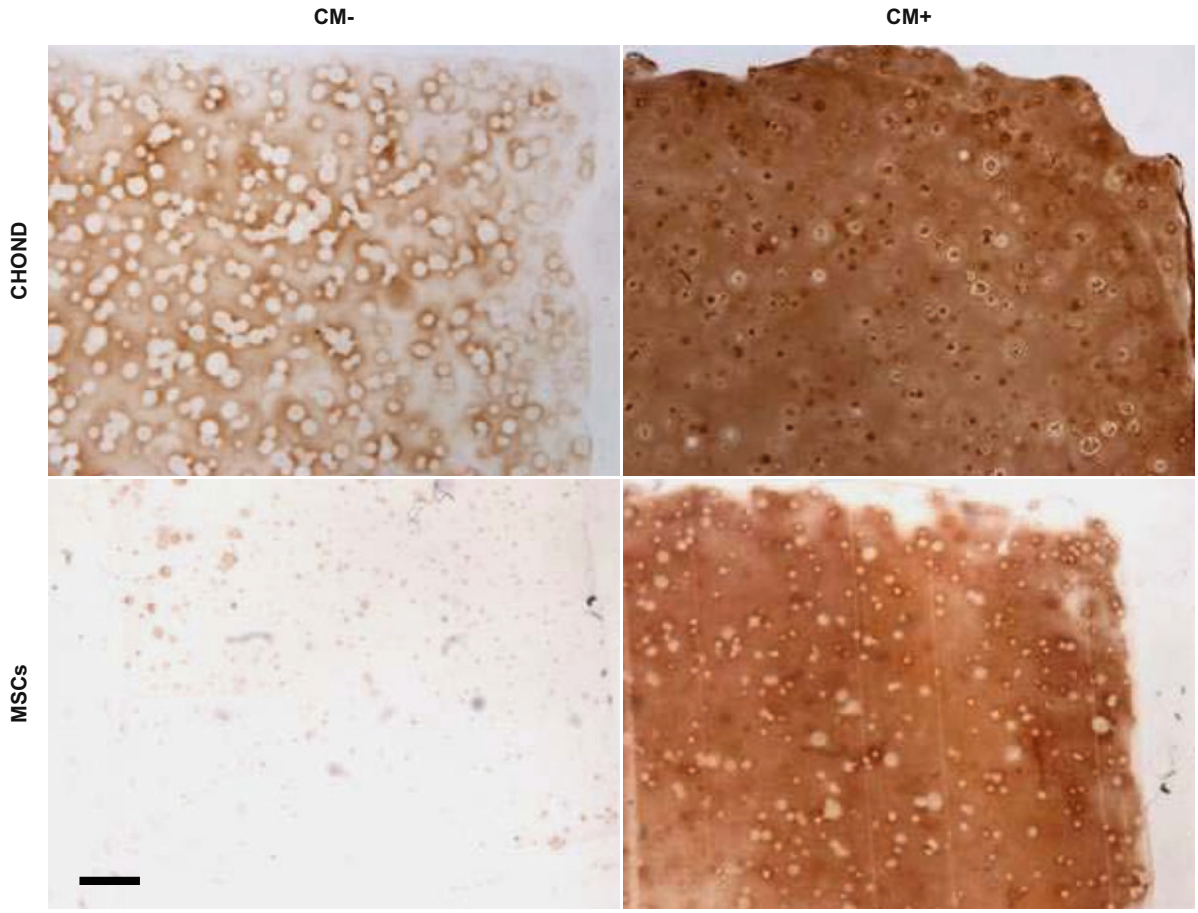

Fig. 23.8 Influence of inductive media on the formation of cartilage tissue by chondrocytes and MSCs in 3D hydrogel culture. Type II collagen staining of agarose hydrogels seeded with bovine MSCs or chondrocytes at 20 million cells/mL on day 56. When differentiated chondrocytes are maintained in a chemically defined medium with (*right*, CM+) or without (*left*, CM–) supplementation with 10 ng/mL TGF-β3, type II collagen staining is present throughout the gel, and enhanced deposition is observed with TGF-β3. For MSCs, type II collagen is only deposited in the presence of TGF-β3. Scale bar: 100 μm

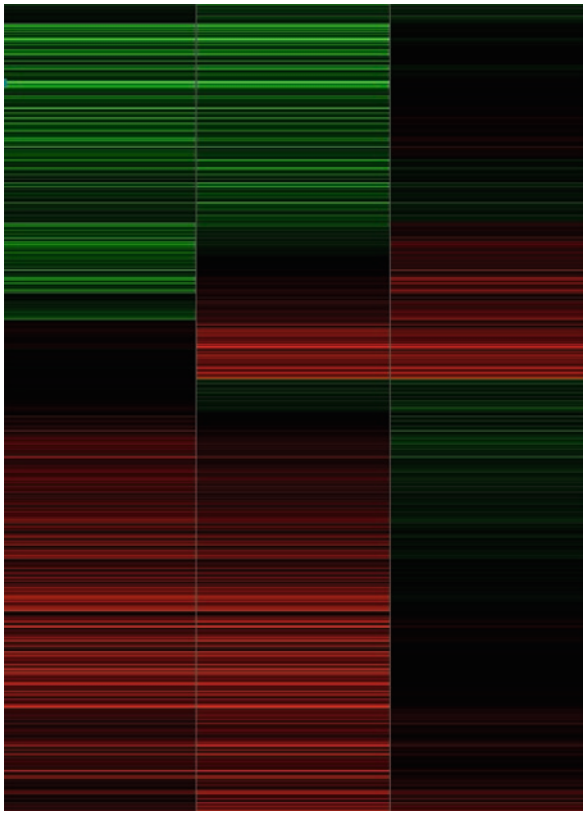

Fig. 23.9 Molecular differences between chondrocytes and MSCs. Heat maps from microarray analysis showing gene expression differences between undifferentiated MSCs (M0), MSCs exposed to chondrogenic medium containing TGF-β3 in 3D agarose culture for 28 days (M28), and donor matched chondrocytes cultured similarly (C28). Results indicate that even after prolonged exposure to chondrogenic medium, a large number of genes are differentially regulated (*up* = *red*, *down* = *green*) between the two cell types (column 3). These molecular level differences may underlie the reduced capacity of MSCs to form functional matrix compared to articular chondrocytes in 3D culture systems for tissue engineering

various chondrogenic genes compared to a relatively inert hydrogel based on PEG [33] and HA provides a supportive environment for ECM deposition and functional maturation with long-term culture [40] (summarized in Fig. 23.10). Also, Liu et al. investigated the quality of repair using HA-gelatin hydrogels seeded with MSCs in a rabbit defect model [93]. Defects with MSCs alone exhibited hyaline-like cartilage on the peripheral defect area and fibrous repair in the middle, whereas defects filled with a scaffold and MSCs resulted in elastic, firm, translucent cartilage with zonal architecture and good integration with the surrounding cartilage [93]. Collectively, these findings suggest that HA-based hydrogels are an effective environment for promoting MSC chondrogenesis and may have direct application to cartilage repair.

Although MSCs are a promising cell source, several questions regarding their efficacy relative to chondrocytes and the stability of their phenotype remain to be addressed. Using current best practices, it has been shown that MSCs can become chondrocyte-like cells, as evidenced by the ECM that is formed with chondro-induction, and the array of genes activated. For example, work by Kisiday et al. has shown that the molecular size of the large aggrecan molecules produced by differentiated MSCs is similar to that produced by juvenile chondrocytes, and greater than that produced by aged chondrocytes [80, 85]. However, our recent microarray data (see Fig. 23.9) show that MSCs remain clearly distinguishable from juvenile chondrocytes even after long periods of chondrogenic differentiation. Many chondrocyte signaling pathways have been investigated in MSCs, with some overlap and some differences noted. We believe that these continued differences might underlie the failure of MSCs to achieve properties that are achieved by chondrocytes cultured similarly. Identification of these complex biological differences and developing methods for their correction may further improve the capacity of MSCs. For example, we have recently developed high throughput screening (HTS) methods to identify novel molecular modulators of chondrogenesis [65]. Such protocols may be used to identify new factors in chemical libraries (such as the National institute of Health (NIH) Small Molecule Repository that contains >200,000 compounds of unknown function).

In addition to functional potential, several recent studies have raised questions on the issue of MSC hypertrophy and potential for ectopic bone formation. It has been reported that upon implantation, some MSC aggregates have progressed toward a bone-like phenotype [132]. A recent study by Mueller and Tuan showed that several critical alterations in the culture environment, including addition of T3 and phosphate, and reduction of dexamethasone and TGF-β3 are required for this transition to occur in vitro [115]. While noting these potential limitations in MSCs, we suggest that these are the most realistic and clinically relevant supply of cells. We posit that the engineered microenvironment provided by carefully engineered hydrogels will accelerate the differentiation process by virtue of cell–matrix interactions, and that coupling of advanced materials and instructive mechanical loading conditions will lead to optimized properties in MSC-based constructs.

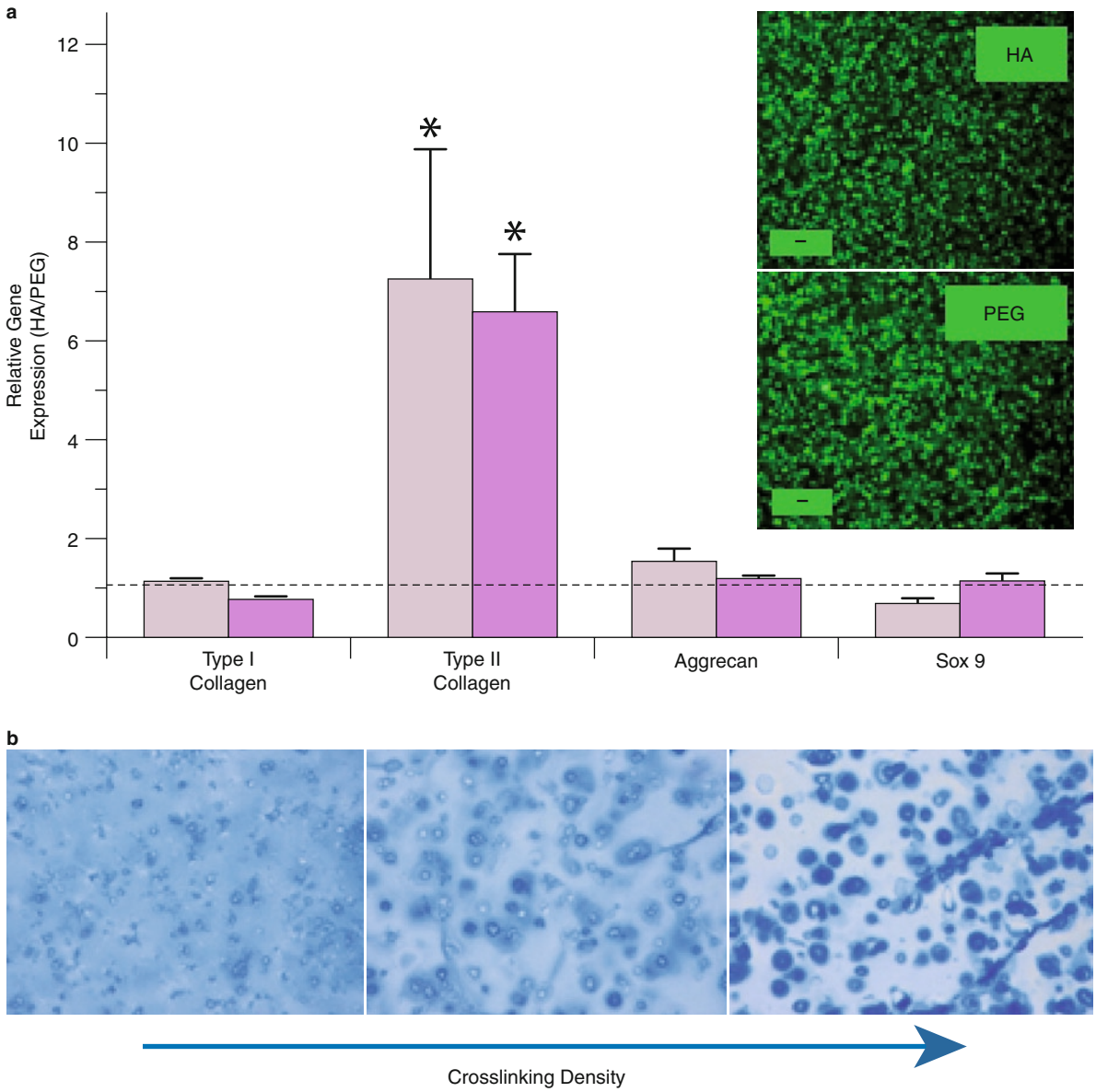

Fig. 23.10 Hydrogel chemistry and macromer density control MSC differentiation and matrix formation. (**a**) Expression of chondrogenic genes (Type II collagen, aggrecan, Sox 9) by MSCs encapsulated in HA gels normalized to PEG gels after 7 (*white*) and 14 (*black*) days (inset: live/dead staining of MSCs in both HA and PEG gels). These results illustrate an upregulation of chondrogenic genes in the HA gels. (**b**) Alterations in distribution of GAG produced by MSCs in HA hydrogels as a result of changes in crosslinking density on day 21 (1, 2, 5 wt.% gels from left to right)

23.3.6 Tissue Engineering of Articular Cartilage with Mechanical Preconditioning

Loading of cartilage generates mechanical signals [114] that modulate chondrocyte biosynthetic activities [59] depending on parameters such as magnitude, duty cycle, frequency, and duration [61]. A schematic of various loading modalities is shown in Fig. 23.11. These changes are not restricted to anabolic genes, as both static and dynamic loading modulate expression and activation of MMPs [42, 45]. This suggests that mechanical stimuli can control matrix remodeling. For tissue engineering, it has been shown that cell-based constructs subjected to deformational loading respond with biosynthetic

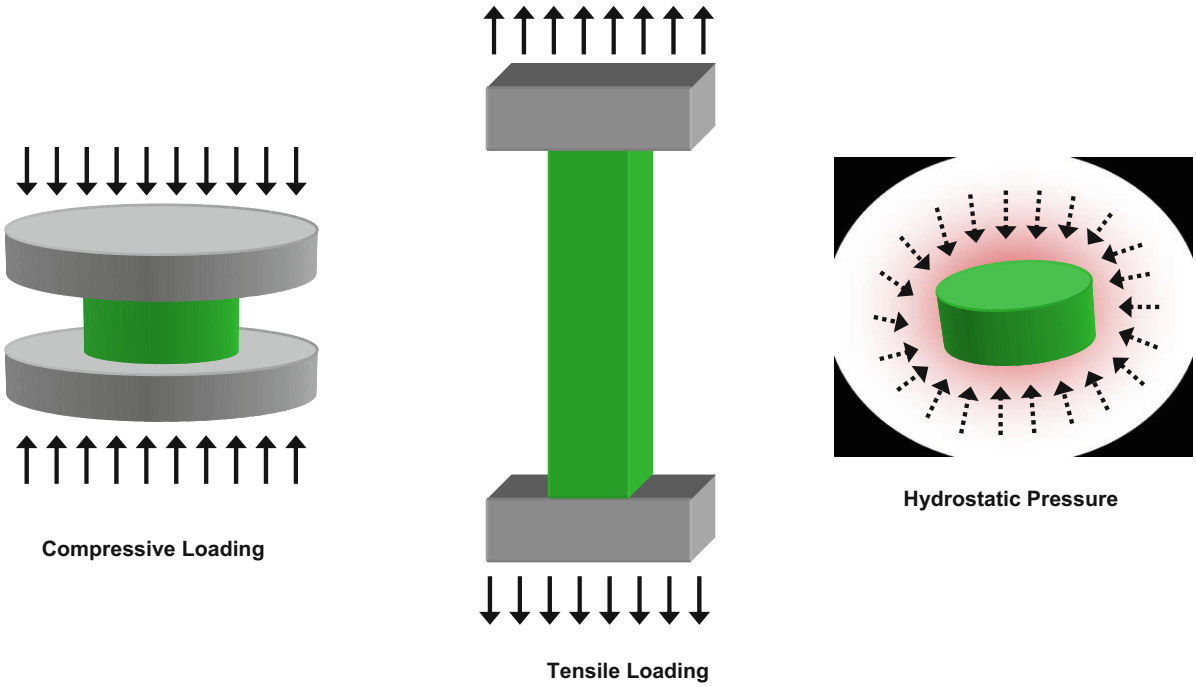

Fig. 23.11 Mechanical loading bioreactors for cartilage tissue engineering. Mechanical bioreactors have been developed to apply physiologic loading to engineered 3D cartilage constructs over the course of long term in vitro culture. Compression, tension, and hydrostatic pressurization each present different mechanical and physico-chemical signals to embedded cells. Compression and tension can further be coupled with shear and/or torsion, and hydrostatic pressurization (which acts equally in every direction and elicits no change in volume in incompressible materials) can be superimposed on each deformation-based loading modality. These complex bioreactor systems recreate at least a portion of the physiologic loading environment of cartilage and have been shown to promote anabolic changes in matrix production and improve mechanical properties in engineered constructs

changes similar to explants [23, 89]. An example bioreactor for compressive loading, as well as results indicating a change in matrix synthesis with loading of chondrocyte-seeded hydrogels, is shown in Fig. 23.12. In this "functional tissue engineering" approach, bioreactors are designed to recapitulate the in vivo mechanical environment in in vitro culture [60]. Beneficial effects of physical forces on engineered constructs have been demonstrated for hydrostatic pressure and perfusion [28] and for deformational loading [78, 103, 104]. Cyclical shear deformation increases cartilage properties on calcium phosphate scaffolds [160]. In short-term studies, chondrocytes in a porous polyurethane scaffold exposed to compression coupled with rotation increased ECM expression [58]. The mechanical environment also interacts synergistically with anabolic growth factors [56, 102], perhaps via enhanced solute transport [101]. An example, wherein type II collagen production and distribution are enhanced with both dynamic compression and the addition of TGF-β1, is shown in Fig. 23.13. With long-term culture in rotating wall bioreactors and with mechanical stimulation, constructs have achieved cartilage-like equilibrium properties, though collagen content and dynamic and tensile properties remain below native levels [104, 131].

23.3.7 Mechanical Sensitivity of Mesenchymal Progenitor Cells

While many studies have employed growth factors to induce chondrogenic differentiation of MSCs, the role of mechanical signals in this process is not fully characterized. Recent studies have shown that even fully differentiated tissues alter their phenotype with changes in the mechanical environment [96]. This appreciation of physical forces on cellular phenotype is clearly related to functional cartilage tissue engineering approaches. Indeed, some of the very signals that cause differentiation may be used to optimize MSC-based construct growth. To date, few studies have directly examined mechanically induced differentiation of MSCs in a tissue engineering environment. Both static

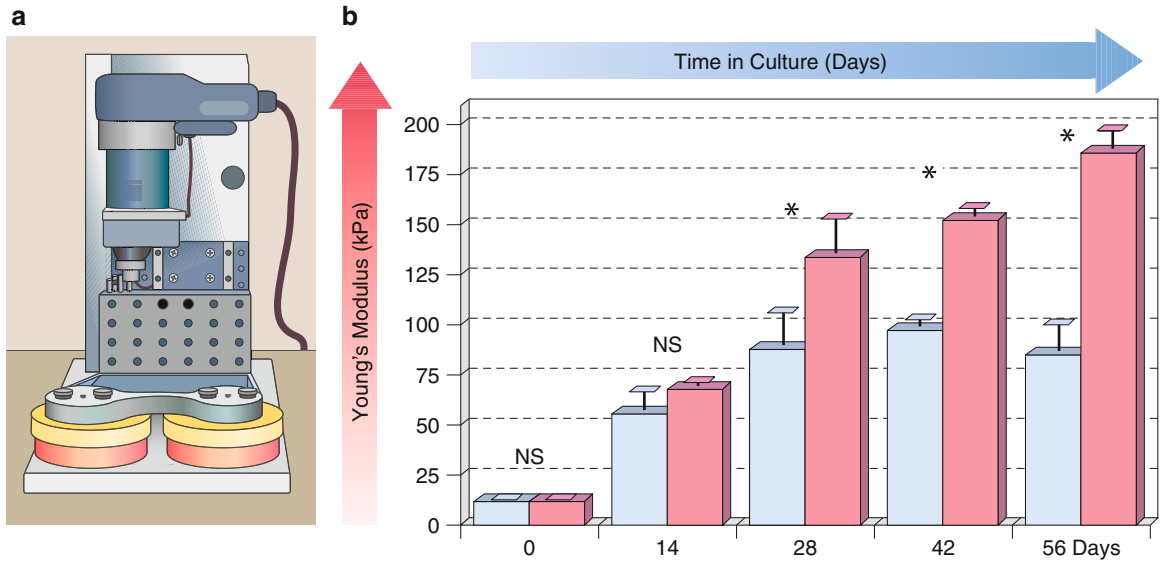

Fig. 23.12 Compressive loading of chondrocytes in hydrogels. (**a**) Image of compressive loading bioreactor for stimulation of cell seeded hydrogels, where the frequency, timing, and magnitude of deformation can be varied. (**b**) Increase in the compressive modulus of chondrocyte-seeded agarose hydrogels with culture in either free swelling (*blue*) or loaded (*pink*) conditions over a 2 month period. Asterisks indicate significant difference between loaded samples and free swelling controls. Adapted from Mauck et al. [104] with permission from Elsevier

and dynamic loading have been shown to increase the chondrogenesis of limb bud cells in collagen and agarose [36, 155], depending on the frequency and duration of loading. More recently, studies in HA sponges and agarose and PEG hydrogels [5, 67, 68, 100, 157] have demonstrated enhanced MSC chondrogenesis with dynamic loading, potentially through the smad signaling pathway [113]. Fluid pressure also enhances chondrogenesis in pellet and hydrogel cultures [6, 44, 110]. In one recent study, we demonstrated that aggrecan promoter activity in MSCs is modulated by the duration and frequency of mechanical compression in 3D hydrogels (see results in Fig. 23.14). While this area of research is still in its infancy, successful production of engineered cartilage with MSCs may ultimately require mechanical conditioning to induce, maintain, and optimize chondrogenic activity.

23.4 Clinical Application

23.4.1 Articular Cartilage Injury and Repair

While cartilage may function well throughout a lifetime, damage to this tissue is prominent and afflicts more than 21 million patients each year in the United States alone. This damage may arise from normal wear and aging, disease, or traumatic injuries. The natural healing response of cartilage to injury is unsatisfactory, largely because of its avascular nature and demanding physical environment. As a result, 9% of the U.S. population aged 30 and older has osteoarthritis (OA) of the hip or knee, costing an estimated $28.6 billion dollars [43] with >200,000 knee replacements currently being performed each year in the U.S. alone [47]. Unfortunately, there are very few viable options for patients with damaged articular cartilage, and many of the current treatments are invasive and their long-term efficacy is unclear. Current strategies for the repair of cartilage include the use of tissue adhesives [64], periosteal grafting [124], microfracture [73, 152], autologous osteochondral grafting [62], and autologous chondrocyte implantation (ACI) [17], with the latter three being the most common. Each of these methods has its own characteristic advantages and limitations. Microfracture involves the piercing of the subchondral bone to allow mesenchymal elements from the marrow to colonize the wound bed, which promotes an inferior fibrous healing response. Osteochondral allografting involves the transfer of bone-cartilage units from "healthy" regions to damaged regions and rapidly restores load bearing capacity and cartilage structure, yet there are issues with donor site morbidity,

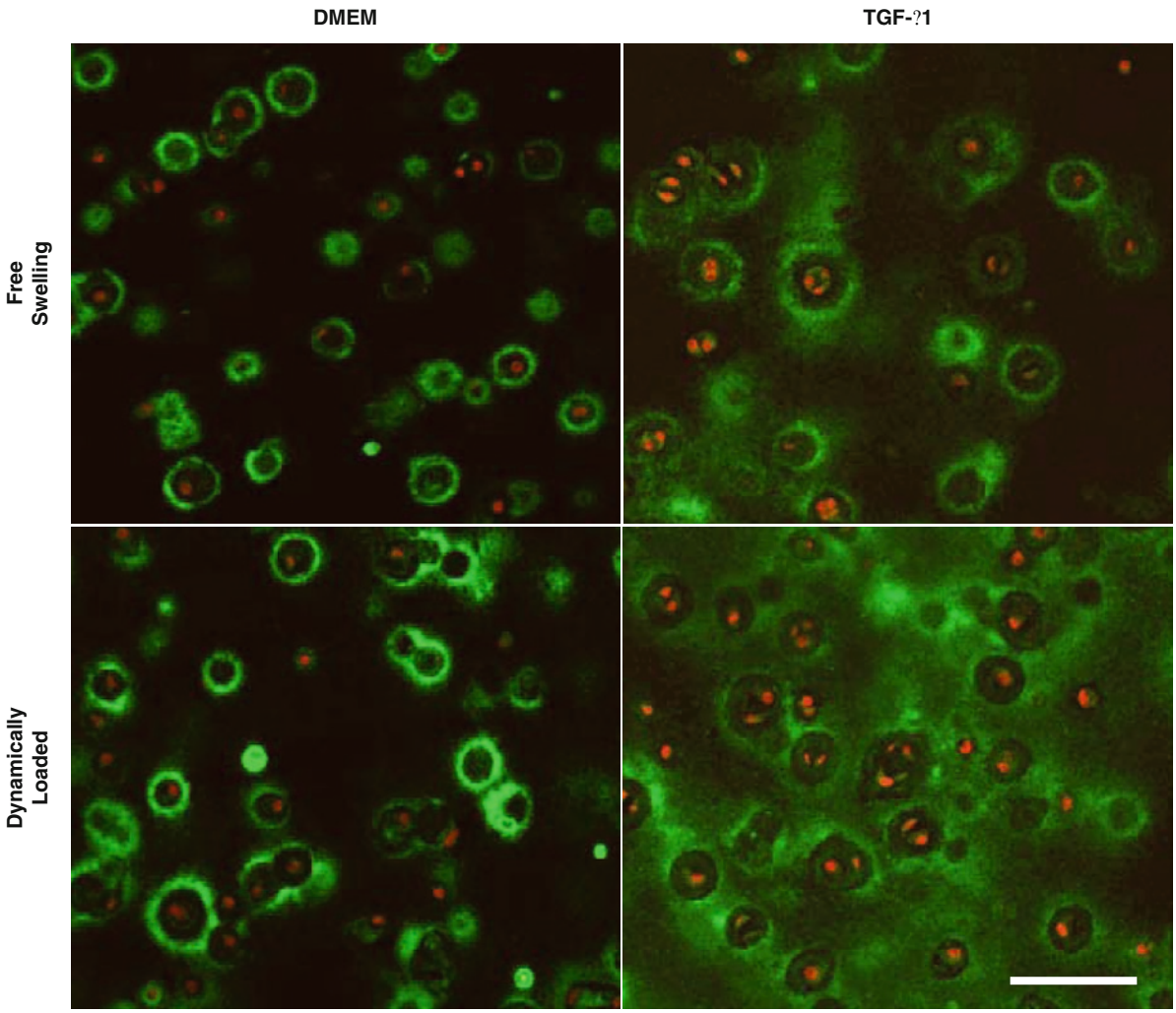

Fig. 23.13 Synergistic interaction between dynamic loading and growth factors. Type II collagen deposition on day 35 in chondrocyte-seeded constructs cultured under free swelling conditions or with daily dynamic loading in the presence or absence of TGF-β1. Growth factor addition increased type II collagen deposition in free swelling culture, and dynamic loading further increased the amount and distribution of this ECM component. Scale bar: 100 µm. Adapted from Mauck et al. [102] with permission from Mary Ann Liebert

lack of healthy donor tissue, and insufficient integration. Finally, ACI (implantation of high concentration of chondrocytes under a periosteal flap) has shown clinical promise in small defects in non- and low-load bearing sites. Yet, this method results in a mixture of fibrous and hyaline cartilage and is not definitively superior to microfracture in the long term [83]. Further, human chondrocytes expanded from OA cartilage possess a limited capacity to form repair tissue rich in collagen compared to prepubertal cells [1]. These drawbacks to current clinical practice motivate efforts toward engineering cartilage replacement tissues de novo.

23.4.2 Clinical Translation of Engineered Cartilage

Even in the eventuality that engineered cartilage constructs can match that of the native tissue, a considerable challenge remains in the anchoring of engineered layers to both the subchondral bone and the adjacent articular cartilage (in the case of focal defects). A number of recent studies have examined the inclusion of a bony layer to produce an osteochondral construct, similar to those used for autologous transplantation.

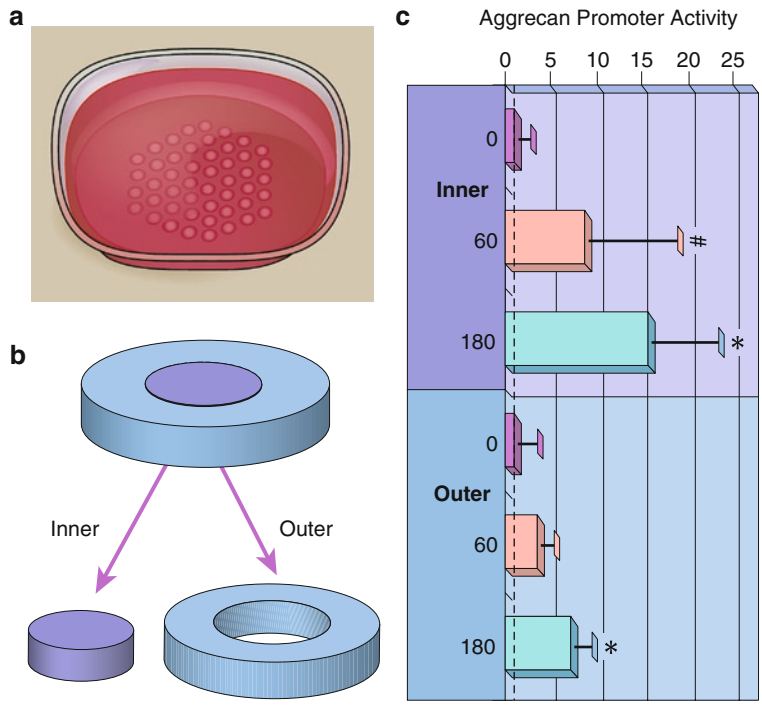

Fig. 23.14 Dynamic loading of MSCs in 3D hydrogels (schematic in (**a**) and (**b**)) enhances chondrogenesis. (**c**) Dynamic compressive loading of MSC-seeded constructs in serum free media without pro-chondrogenic factors increases aggrecan promoter activity with increasing duration (0, 60, or 180 min) of applied load. Asterisks indicate $p<0.05$ vs. free swelling (0 min) controls. Adapted from Mauck et al. [100] with permission from Springer

Such studies have utilized a diverse array of scaffolding materials and mechanisms for joining the disparate layers [4, 71, 143, 146], including biodegradable polymers and foams and allograft trabecular bone. For example, osteochondral constructs produced in vitro and composed of a cell-seeded agarose layer integrated with a trabecular bony layer showed increase in functional properties with time in culture [71]. Importantly, when mechanical deformation is applied to such constructs with different properties in different regions, a spectrum of mechanical signals will be generated, potentially influencing region-specific growth [92] (see Fig. 23.15). Of clinical note, several recent studies have used a tantalum based trabecular metal (TM) material for the "bone" region, combining high density cartilage monolayer or synovium layers directly to the TM [57, 98]. These studies have demonstrated the chondro-conductivity of TM, but have suffered from a lack of dimensional stability in the "cartilage" layer. Still more recently, studies have been carried out using MSC-seeded hydrogel layers overlying a TM "bone" layer for cartilage repair in rabbit defect model [13]. This study, while promising, failed to optimize the mechanical properties of the "cartilage" layer or its functional union with the TM prior to or after in vivo implantation. Though good bone in-growth into the TM layer was observed, defect filling was still limited at 12 weeks postimplantation, and cartilage properties were not evaluated. Regardless, this and similar approaches hold promise for bridging the gap between lab-grown cartilage constructs and in vivo application. In particular, these bio-hybrid constructs, which incorporate some components of materials currently in use in prostheses, may hasten clinical implementation of this new technology.

23.5 Expert Opinion

While there has been extensive work performed in the field of cartilage tissue engineering, several crucial questions have yet to be addressed in detail. Broaching these topics, and engineering solutions when necessary, constitutes the next stage of cartilage tissue engineering. When engineering implantable constructs for musculoskeletal tissue repair, the most critical questions to be considered are the threshold at which an in vitro generated implant should be assayed in vivo in a large animal model, and the criteria for success in long term in vivo studies. Indeed, this was the topic of a recent NIH sponsored Consensus Conference ("Tissue Engineering Evaluation Criteria for Musculoskeletal Tissue Repair,"

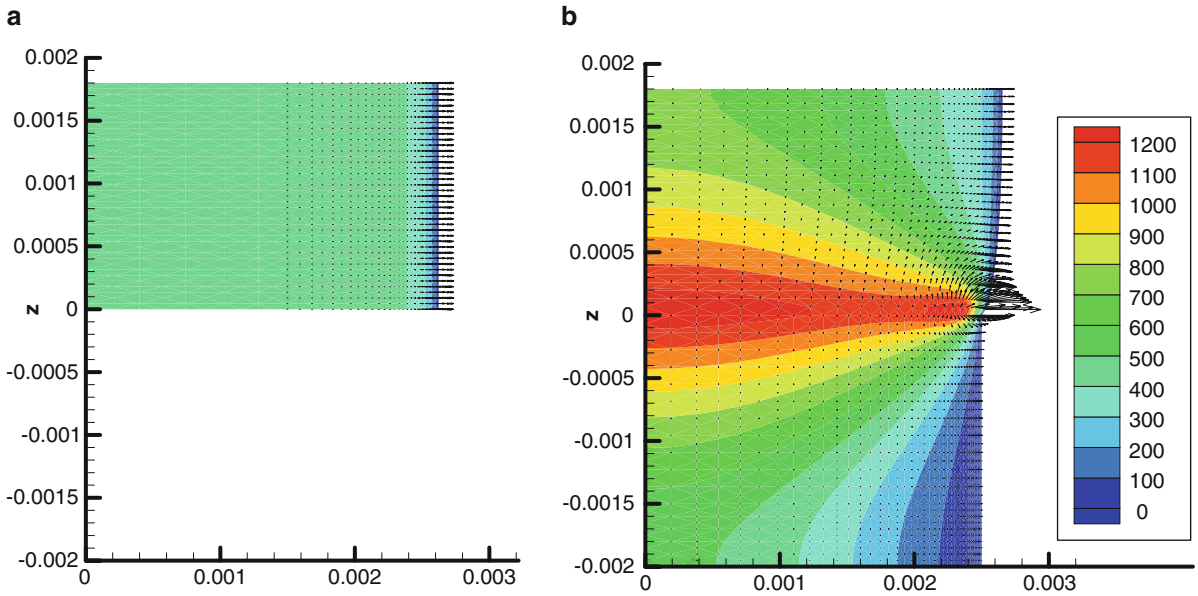

Fig. 23.15 Dynamic loading of osteochondral constructs. Finite element modeling (FEM) of fluid flow (*arrows*) and hydrostatic pressure (color map, Pa) in gel (**a**) and osteochondral (**b**) constructs in response to 10% deformation applied at 1 Hz. Inclusion of a rigid porous "bone" region alters the magnitude and location of physical signals generated within the "cartilage" region of the osteochondral construct. Adapted from Lima et al. [92] with permission from IOS Press

Hilton Head, SC, April 26–29, 2007). At that meeting, and in the field in general, a growing consensus has emerged that for an engineered construct to be acceptable for implantation into a load bearing site, several critical features must be met [50]. For all musculoskeletal tissues, these properties can be extracted from the native tissue in question and the kinematics and forces found at its site of action, and can be tuned to the developmental stages through which it transits during maturation and repair processes.

As summarized in Table 23.1, numerous properties can be measured to describe the unique mechanical properties of articular cartilage, and some subsets of these properties will be essential for articular cartilage and engineered constructs to function properly in vivo. Our study of this large body of literature identified several key benchmark qualities that must be addressed in a realistic and rational fashion in any engineered replacement (see Table 23.1 for mechanical values of articular cartilage with age). Specifically, it is well accepted that the compressive properties (both confined and unconfined) of cartilage are critical for its function – it is a load bearing tissue, and it operates by transmitting very large stresses across articulating surfaces. It is important to address these properties both at equilibrium and dynamically as they are both representative of in vivo loading conditions and vary considerably in magnitude (i.e., dynamic properties are much higher than equilibrium properties). Though less studied than compressive properties, cartilage tensile properties increase the fluid pressurization capacity of the tissue, and enhance the dynamic properties in unconfined compression and thus are important for native tissue function. Although molecular and biochemical features are important, and indeed underlie these mechanical properties, ultimately the functional properties are most critical. The goal is an engineered tissue that mimics the properties of the native tissues, and one that can recapitulate all of the critical features necessary to enable long-term function. Numerous attempts have been made to engineer articular cartilage, and a considerable effort has been devoted to understanding its properties as a function of age and the mechanical environment in which it operates; however, these efforts have yet to culminate in a fully functional cartilage substitute.

So, what criteria are most important for success, where does the field stand with respect to these criteria, and what route is the field taking to bridge this gap? In our engineering efforts, we use native juvenile

articular cartilage as a benchmark, as it may be posited that achieving these properties is sufficient for implantation of a load-bearing neocartilage construct as many animals bear load immediately or soon after birth. The specific metrics for engineered cartilage tissue produced in the field can be found in the many citations of this chapter, though the general finding is that to date these properties are insufficient with respect to biochemical content and biomechanical properties of the native tissue. Often, GAG content in constructs can be quite high (matching or exceeding native tissue levels) and several measures of mechanics come close to that of native tissue (for example, the equilibrium compressive modulus [25, 48]). However, many of the shortcomings in engineered tissue properties (specifically the dynamic and tensile moduli) can be attributed to an overall lack of collagen content and organization.

New technologies discussed above (i.e., dynamic hydrogels, temporal exposure to custom growth factor cocktails, and mechanical loading), may help overcome these limitations and improve properties to match those of juvenile articular cartilage. For example, our recent data show that transient exposure to transforming growth factor-beta3 (TGF-β3) can increase chondrocyte laden hydrogel compressive properties to match or exceed native tissue [25]. Likewise, dynamic loading can further increase compressive properties ~100% compared to those of free swelling culture [104]. Approaches that incorporate controlled gel degradation have been shown to improve collagen content of engineered cartilage constructs up to tenfold compared to that of nondegradable hydrogels [20]. Rational combination of these factors has the potential to dramatically improve overall engineered cartilage properties.

In addition to these "principal" components that define cartilage functionality, there are higher order considerations that are actively being investigated. As noted above, the adult tissue possesses significant inhomogeneity and anisotropy, as well as a vanishingly small coefficient of friction. While it is not clear whether duplication of these features is necessary prior to implantation, it does seem apparent from development and physiologic use that these could be critical considerations on which to focus future efforts. Indeed, there are several important recent publications on these topics. For example, layered hydrogels of differing composition have been used to form constructs with depth dependent properties, and varying mechanical response in these regions has been observed [119] (see for example, Fig. 23.16). Likewise, combination of chondrocytes from different regions (surface and deep zones) has been used as a means to

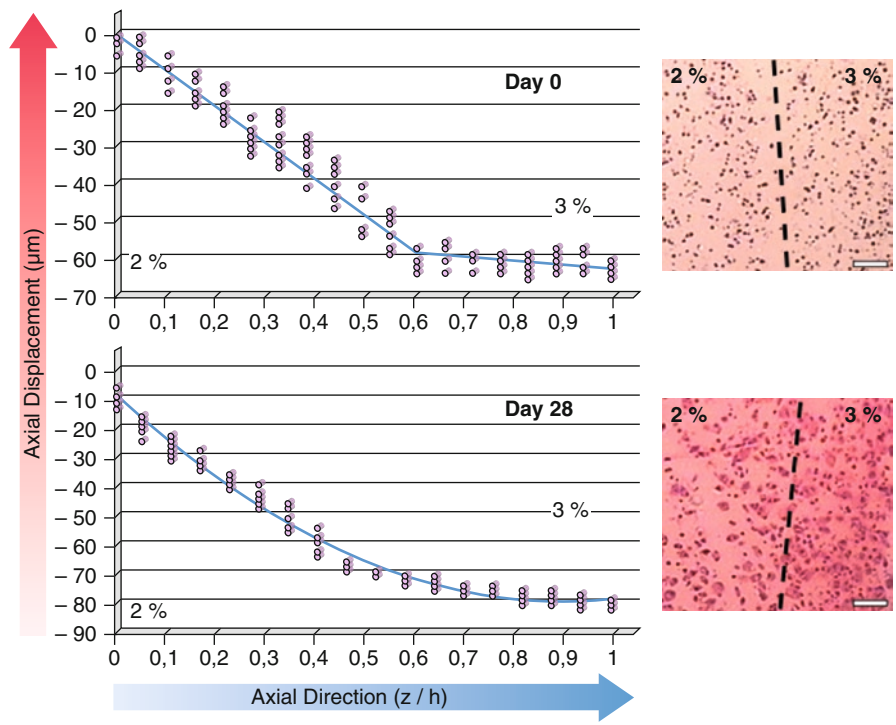

Fig. 23.16 Engineering depth-dependent inhomogeneity in 3D cartilage constructs. Local deformation gradient and histological analysis of GAG deposition in agarose hydrogels formed in layers of 2% and 3% w/v and seeded with bovine chondrocytes. On day 0, a clear transition in local deformation (and modulus) is observed across the layered interface. By day 28, matrix has differentially accumulated in the two layers. Deformation gradients (and local moduli) remain different through the depth of the construct, while the interface between layers becomes less distinct. Adapted from Ng et al. [120] with permission from Wiley

engineer both depth-dependent biochemical and histological properties [81]. Recent studies have also begun to characterize frictional response as part of the standard metric for evaluating success in engineered constructs [54]. Both hydrogel and scaffold-free methods have evaluated tensile properties directly [11, 66], though these studies have generally found this property lacking with respect to the native tissue. These new methods and measures will add to the understanding of the state of the engineered construct at the time of implantation, and on the basis of long-term outcome studies they may be used to predict in vivo success in future iterations.

23.6 Five-Year Perspective

In the last decade, considerable progress has been made in the formation of engineered cartilages that possess mechanical and architectural hallmarks of the native tissue. Coincident work has better defined the precise mechanical properties of the native tissue, and the contributions of these features to physiologic operation in a demanding joint environment. Current tissue engineering efforts build on this broad foundation, and over the next 5 years the expectation is that these technologies will begin to progress toward clinical implementation in humans. The next wave of advance in clinical usage of engineered cartilage will undoubtedly come from second generation ACI type approaches. These approaches, sometimes referred to as matrix-based autologous chondrocyte implantation (MACI), involve the combination of cells typically used with ACI with synthetic or biologic materials [172]. This advance helps situate and retain chondrocytes in the defect space, and may obviate the need for periosteal flaps during the procedure. This alone would decrease morbidity associated with these surgeries, and may improve clinical outcomes. Likewise, significant progress has been made in hydrogels that can be gelled in situ and retained within the defect space. A recent report from Elisseeff et al. detailed a new gel formulation that, when coupled with treatment of the cartilage surface, can be covalently bonded in place, potentially enhancing in situ retention in a load bearing space [163]. Still further, with the advent of sophisticated imaging modalities, investigations have begun to consider re-surfacing entire joint surfaces on the basis of patient-specific topographies. A recent report on the engineering of the entire patellar articular cartilage coupled to subchondral bone [71] provides a first glimpse at what could be a revolutionary concept in cartilage replacement and the treatment of degenerative OA of whole joints (Fig. 23.17). As with all new technologies, these advances are likely to move forward in a nonlinear manner. Regardless, the progress made in the last decade presages the potential of the coming 5 year period. As these new technologies are deployed in large animal models and in humans, careful evaluation of efficacy and cost must be considered.

23.7 Limitations/Critical View

There is no question that cartilage tissue engineering holds promise for the repair of an otherwise irreparable tissue. That being said, it is necessary to specifically enunciate the current limitations in clinical, corporate, and academic efforts to engineer cartilage tissues, and to set realistic benchmarks for evaluating in vitro and in vivo success. It is not clear that every benchmark must be met, and indeed new technologies must be evaluated through to clinical implementation in challenging large animal models at each stage of this process. If one key limitation were to be singled out for further discussion, the most striking failure of all cartilage tissue engineering efforts has been one of collagen content. As in the rest of the body, collagen is by far the most abundant molecule in cartilage, comprising up to 20% of its wet weight, and 75% of its dry weight (compared to ~95% of the dry weight in tissues like tendon and ligament). While most of the field focuses on PGs, which do unquestionably play a dominant role in the compressive characteristics of the tissue, collagen content in engineered cartilage remains extremely low relative to that in the native tissue. It is clear from developmental processes that collagen production as well as crosslinking is critical for the tissue to take on its mature functional properties, and yet the field has not been able to recreate cartilage tissue with collagen levels comparable to that in the native tissue. Even in recent reports, where compressive properties and PG contents meet or exceed native tissue values [25, 91], collagen content remains at best a quarter of native levels. The final critical feature in forming a

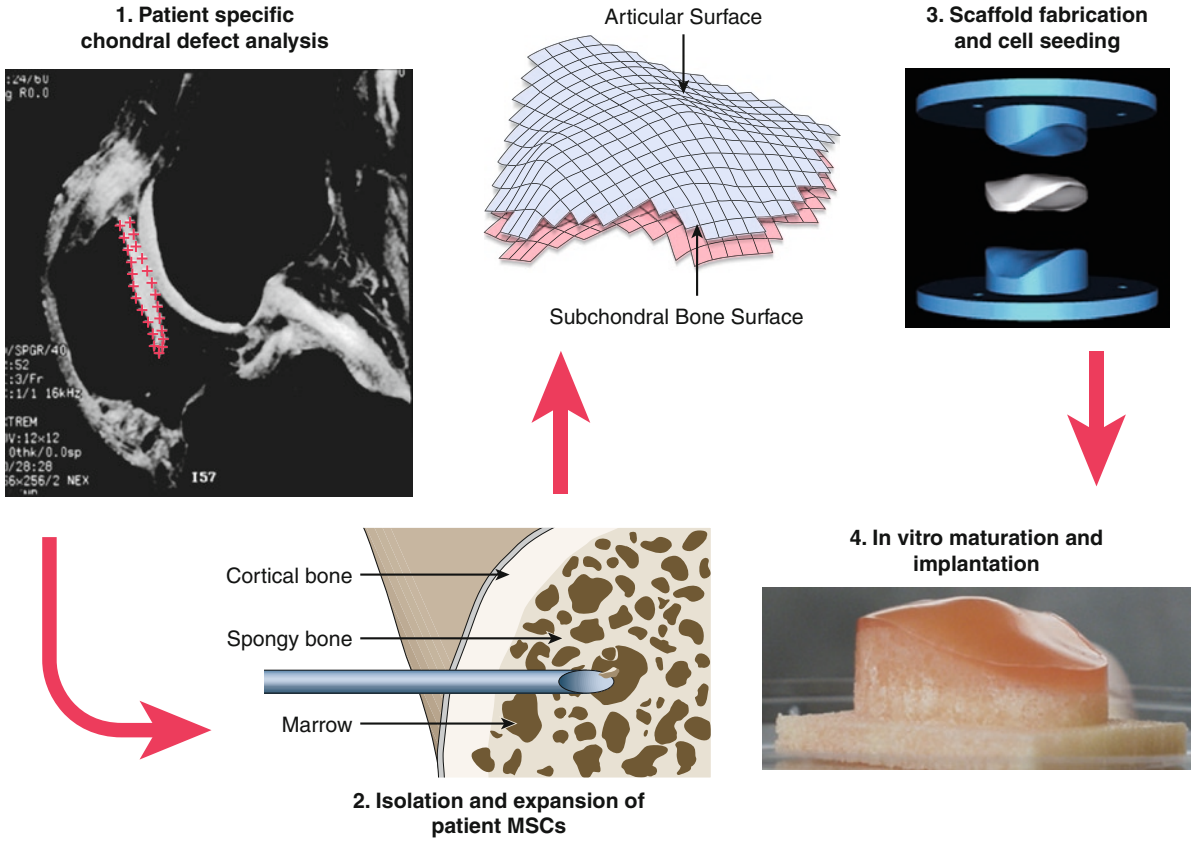

Fig. 23.17 Clinical implementation of joint and/or patient specific engineered cartilage formed from autologous MSCs. When presenting with a focal defect or joint-wide destruction of articulating surfaces, patient MSCs can be isolated via a simple bone marrow aspiration. A space filling chondral or osteochondral construct mold can then be generated from MRI images using computer-aided design (CAD) methods. Following a period of in vitro prematuration, the anatomically correct engineered cartilage surface can be re-implanted to restore structure and function to damaged or diseased articulating joints. Adapted from Hung et al. [71] with permission from Elsevier

successful cartilage replacement is in the recognition and optimization of a common cell source. It is clear from the literature that aged chondrocytes are not particularly useful in these efforts while MSCs and other progenitors show some promise. To deploy these cells, however, further work will be required toward optimizing their ability not just to take on a chondrocyte-like phenotype, but to actually form tissue that has the same functionality. Moreover, additional basic science work will be required to elucidate the mechanisms controlling phenotypic state in these cells, so as to better induce and preserve the cartilage-like state after implantation in the joint environment. Overcoming these limitations should be a primary focus of the field to ensure the potential for adequate maturation and clinical relevance of engineered cartilage.

23.8 Conclusion/Summary

The clinical need for engineered articular cartilage tissue is underscored by the increasing prevalence of degenerative joint diseases and the large numbers of researchers focused on this important problem. It has been noted that the number of total joint replacement procedures occurring over the next 20 years will undergo a rapid increase (up to 700,000 procedures/year in 2030 in the US alone) [47]. These numbers can be easily multiplied by a factor of 10 as emerging economies (such as India and China) and improving health care systems add to life expectancies and demand for improved quality of life world-wide. Indeed, large purveyors of prosthetic devices have begun to increase production in anticipation of this growing demand for

their products. Cartilage tissue engineering strategies stand poised to play a major role in this field as the technologies advance and efficacy is demonstrated relative to the current standard of care. Critical to this evolution is the continued development of new polymer systems for encapsulation, better understanding of the transitions cartilage undergoes as it achieves its mature state, realistic optimization strategies for the use of progenitor cell populations, and an increased focus on validated and challenging preclinical models that have the capacity to demonstrate efficacy over the long term. These challenges are significant, but their successful resolution will immeasurably improve quality of life for the aging population in the nation and the world.

Suggested Reading with Abstracts

Chung C, Burdick JA. Engineering cartilage tissue. Adv Drug Del Rev. 2008;60:243–62.

This is a current review article on cartilage tissue engineering that covers the various cell sources (chondrocytes, fibroblasts, stem cells), scaffolds (hydrogels, sponges, meshes), and stimulatory molecules (growth factors, bioreactors) that have been investigated for the regeneration of cartilage tissues.

Elisseeff J, Anseth K, Sims D, McIntosh W, Randolph M, Langer R. Transdermal photopolymerization for minimally invasive implantation. PNAS U S A. 1999;96:3104–7.

This is the first example where photopolymerizable hydrogels are used for the encapsulation of cells toward cartilage tissue engineering. In this study, solutions of macromers, cells, and photoinitiator were injected subcutaneously, polymerized through transdermal light exposure, and produced cartilage ECM with time. Photopolymerization has since become an important cell encapsulation process in many areas of tissue engineering.

Bryant SJ, Anseth KS. Controlling the spatial distribution of ECM components in degradable PEG hydrogels for tissue engineering cartilage. J Biomed Mat Res A. 2003; 64A:70–9.

This work investigated the influence of network degradation on cartilage tissue evolution using chondrocytes encapsulated in hydrogels. It was determined that control over degradation could lead to enhanced distribution of ECM molecules whose distribution is typically inhibited by small mesh sizes. Uniform ECM distribution is important toward mimicking the native tissue architecture and mechanical properties.

Li WJ, Tuli R, Okafor C, Derfoul A, Danielson KG, Hall DJ, et al. A three-dimensional nanofibrous scaffold for cartilage tissue engineering using human mesenchymal stem cells. Biomaterials. 2005;26:599–609.

These investigators utilized nanofibrous scaffolds that mimic the size-scale of the ECM from a biodegradable polymer seeded with mesenchymal stem cells toward the production of cartilage tissues. In the presence of chondrogenic media, the cells underwent chondro genesis and produced a neo-tissue that encompassed components of cartilage extracellular matrix.

Reddi AH. Morphogenesis and tissue engineering of bone and cartilage: inductive signals, stem cells, and biomimetic biomaterials. Tissue Eng. 2000;6:351–9.

This article reviews tissue morphogenesis in the context of tissue engineering and highlights the many signaling molecules involved. Importantly, the many microenvironmental signals involved in cartilage development and morphogenesis are covered as they may be applied to approaches in tissue regeneration.

Mauck RL, Yuan X, Tuan RS. Chondrogenic differentiation and functional maturation of bovine mesenchymal stem cells in long-term agarose culture. Osteoarthr Cartil. 2006;14(2): 179–89.

This article is one of the first addressing the functional capacity of progenitor cell populations with respect to fully differentiated and donor-matched chondrocytes. The authors find that stem cells can produce functional matrix, but do so to a lesser extent than fully differentiated chondrocytes. This remains an important hurdle to overcome for the efficacious use of progenitor cells in cartilage tissue engineering.

Buschmann MD, Gluzband YA, Grodzinsky AJ, Hunziker EB. Mechanical compression modulates matrix biosynthesis in chondrocyte/agarose culture. J Cell Sci. 1995;108:1497–508.

This seminal paper was one of the first to describe key mechanical concepts in the tissue engineering of cartilage with hydrogels. Specifically, this work showed the time dependent increase in mechanical properties of hydrogel constructs when seeded with chondrocytes. Moreover, and perhaps more importantly, this work demonstrated the feasibility of mechanical preconditioning to promote cartilage matrix formation in engineered constructs. The authors show that dynamic loading after various periods of free swelling in vitro

preculture could promote proteoglycan and collagen synthesis.

Freed LE, Langer R, Martin I, Pellis NR, Vunjak-Novakovic G. Tissue engineering of cartilage in space. Proc Natl Acad Sci U S A. 1997;94(25):13885–90.

This manuscript demonstrated the robust capacity of chondrocytes to form cartilage tissue on biodegradable scaffolds with long-term culture under microgravity conditions in a rotating wall bioreactor system. In many ways, this work continues to be the base to which all engineered constructs are compared, as the properties reported here come very close to native tissue levels. The use of the rotating wall bioreactor and discussion of nutritional constraints on engineered cartilage also set the stage for a large body of subsequent work and bioreactor designs.

Sah RL, Kim YJ, Doong JY, Grodzinsky AJ, Plaas AH, Sandy JD. Biosynthetic response of cartilage explants to dynamic compression. J Orthop Res. 1989;7(5):619–36.

This exhaustive study demonstrated how mechanical loading, within physiologic magnitudes and frequencies, modulates the anabolic behavior of articular cartilage explants. Along with a number of works of this kind, this area of focus helped to guide future efforts in mechanical preconditioning for articular cartilage tissue engineering.

Hung CT, Mauck RL, Wang CC, Lima EG, Ateshian GA. A paradigm for functional tissue engineering of articular cartilage via applied physiologic deformational loading. Ann Biomed Eng. 2004;32(1):35–49.

This manuscript reviews a body of literature focused on using mechanical preconditioning as a tool for expediting the growth of engineered cartilage constructs. The authors detail the considerations and hurdles that had to be overcome in using dynamic loading for this purpose. The manuscript may serve as a quick guide to those using similar approaches for cartilage tissue engineering with primary and progenitor cells.

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Pancreatic Tissues

24

Grace J. Lim and Anthony Atala

24.1 Introduction

The pancreas is a glandular organ with roles in both the digestive and endocrine systems of vertebrates. It is both an endocrine gland (secretes several important hormones, including insulin, glucagon, and somatostatin into the bloodstream) and an exocrine gland (secretes pancreatic juices containing digestive enzymes into the small intestine). The part of the pancreas that mediates endocrine functions is made of millions of cell clusters called islets of Langerhans. There are four main cell types in the islets as classified by their secretions: α cells secrete glucagon, β cells secrete insulin, δ cells secrete somatostatin, and PP cells secrete pancreatic polypeptide [10, 11]. Loss of insulin-producing β-cells leads to diabetes mellitus, which is a leading cause of morbidity in the world [4]. Diabetes currently affects at least 200 million people in the world, and this number is expected to double by the year 2030, according to the World Health Organization [63]. Insulin replacement therapy was developed a number of years ago and has become the standard treatment for diabetes, but it is apparent that injection of insulin does not completely compensate for the loss of the insulin producing β-cells of the pancreas.

Over the past two to three decades, two different transplant strategies have evolved to replace lost or insufficient β-cell function. Transplantation of the whole pancreas and transplantation of just the islets of Langerhans, which contain the β cells, have been studied. The ultimate purpose of transplantation is to prevent the secondary complications of diabetes through normal physiologic control of blood glucose, which cannot be completely mimicked by insulin injections. Transplantation of the whole pancreas has become a successful technology in some cases, as it maintains glucose homeostasis over the long-term, eliminates hypoglycemic unawareness, and improves quality of life by improving or stabilizing diabetic nephropathy, retinopathy, and neuropathy [5, 15, 21, 30, 32, 33]. However, waiting lists for pancreatic transplants are growing quickly, as the supply of human pancreatic tissue is limited. In addition, many patients do not have the opportunity to consider pancreas transplantation because the cost of the procedure is prohibitive (typical hospital charges exceed $100,000) Insurance may not cover this procedure [67, 83]. Unfortunately, some reports also suggest that solitary pancreas transplantation may increase patient mortality. Up to 20% of whole organ pancreatic transplants fail within the first year after transplantation because of complications such as thrombosis, infection, leakage, or rejection [33]. The frequency of complications associated with whole organ pancreatic transplantation creates an upper limit to the potential success and acceptability of the procedure at this time.

Transplantation of pancreatic islets is an attractive alternative to whole organ pancreatic transplantation, primarily because islet transplantation is potentially less likely to lead to serious clinical complications. Pancreatic islet transplantation has the potential to successfully treat diabetes because transplanted β cells can respond to both increasing and decreasing blood glucose levels [22, 69, 71, 76]. Intraportal transplantation of islets is technically less complicated than the laparotomy with multiple vascular and intestinal

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anastomoses required for transplantation of the whole pancreas. However, although potentially simpler and safer than whole organ transplantation, there has only been short term success of islet transplantation in Type 1 diabetes (T1DM) patients [2, 6, 7, 8, 16, 25, 26, 73]. In December 1995, 270 islet transplants were performed for T1DM, with an overall insulin-independence rate of 10% [36, 72]. In 2000, the University of Alberta in Edmonton, Canada reported that several patients with T1DM were insulin-independent following transplantation of freshly isolated islets from multiple donors [71]. Other islet transplant centers have confirmed the Edmonton results, and more recently, Hering has reported insulin-independence following islet transplantation from single donors [37, 74]. The major limitation of islet transplantation is that in order to treat diabetes, a large number of cells are required, and this number can be difficult to obtain. Multiple donors must often be used, and typically only patients who require small amounts of insulin achieve insulin-independence and maintain it long-term. These inefficiencies in islet transplantation can lead to an even higher cost than a whole organ transplant.

Obtaining insulin producing β-cells is a major challenge in cell therapy techniques for diabetes. Recent advancements in stem cell research suggest that pluripotent cells may be promising new cell sources for the treatment of various diseases. Importantly, stem cells have the potential to generate pancreatic β-cells and potentially cure diabetes mellitus [17, 23].

Another important factor that must be considered during pancreatic tissue or cell transplantation is the response of the host's immune system. A major obstacle associated with the transplantation of non-autologous cells or tissue is graft rejection [74, 79]. To overcome this problem, recipients are given immunosuppressive drugs for extensive periods of time. However, immunosuppression often has major side effects, including loss of resistance to infections, increased metastatic potential of malignant cells, hypertension, anemia, hyperglycemia, peptic ulcers, and nephrotoxicity [20, 82]. Immunosuppressive drugs also interact with other medicines and affect their metabolism. Various immunoisolation techniques for donor cells and tissues have been developed to overcome this problem. Cell encapsulation with biocompatible protective polymers is a promising and efficient method which will be clinically available during the next few years [51].

24.2 Aims of Pancreatic Tissue Engineering

Tissue engineering is an emerging, cutting edge field that aims to provide functional recovery of damaged tissues or organs and to build bioartificial tissue and organ replacements using various types of cells and biomaterials. Techniques developed in tissue engineering laboratories worldwide are now being applied to a number of organ systems, including the pancreas. Engineering pancreatic tissue involves understanding the limitations of currently available clinical approaches for treating diabetic patients and providing new methodologies to maintain long-term glucose homeostasis while preventing the secondary complications of diabetes. However, despite the great potential of tissue engineering and regenerative medicine techniques to successfully treat diabetes, there are still many problems that must be solved in order to obtain β-cells and transplant them in such a way that they are able to respond to blood glucose levels.

In this chapter, scientific and technological challenges encountered in the engineering of pancreatic tissues will be presented. In particular, sources of insulin producing cells, solutions to host immune rejection, and efficient and safe methods for delivering β-cells to the patient will be discussed.

24.3 State of the Art Technologies in Pancreatic Tissue Engineering

24.3.1 Sources of Insulin Producing Cells

The shortage of organ donors for regenerative medicine has stimulated research on stem cells as a potential resource for cell-based therapy. Stem cells have been used widely for regenerative medicine applications. The development of innovative methods to generate stem cells from different sources suggests that there may be new alternatives for cell-based therapies. Obtaining insulin producing β-cells or islets is an urgent clinical need and a major challenge for pancreatic tissue engineering. Recent advances in stem cell research suggests that these pluripotent cells may serve as new cell sources for a variety of therapeutic applications, and stem cell transplantation has the potential to

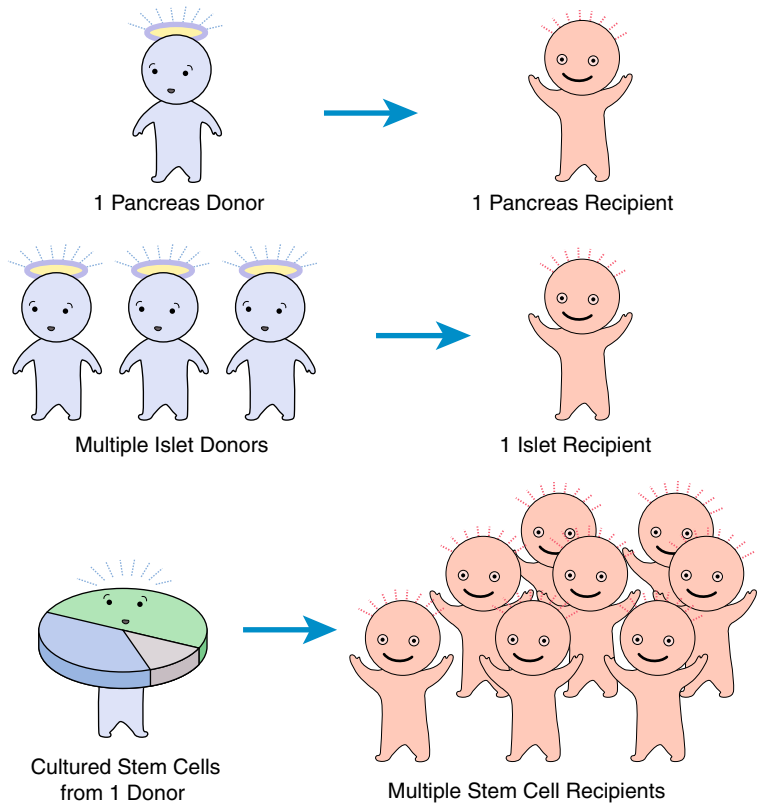

Fig. 24.1 Strategies for islet transplantation

overcome problems with both cell supply and host immunity [17]. Figure 24.1 shows a potential strategy for islet transplantation and the benefits of using stem cells to treat diabetes. In this section, various cell types, such as human embryonic stem cells (hES) and other multipotent or pluripotent cell types, will be discussed in terms of their potential for generating insulin-producing cells (IPCs) to treat diabetes.

24.3.1.1 Pancreatic β Cells Generated from Embryonic Stem Cells (ESC)

The isolation of pluripotent ES cell lines from the inner cell mass of early stage mouse embryos [35, 52] and, 17 years later, human embryos [79] offers the potential to generate any specialized cell type in large quantities. Early efforts established that ES cells could give rise to cells expressing endodermal lineage markers, including insulin expression, but yields were low and properties such as levels of hormone and regulation by glucose did not closely mirror those of mature pancreatic β-cells [22, 44, 48, 55, 77]. A major step forward came as investigators identified known developmental cues that could induce ES cells to replicate key aspects of the segregation of specific germ layers that occurs at gastrulation in the normal embryo. As the pancreas derives from the endoderm, an important breakthrough was to induce differentiation of ES cells to mesoendoderm and definitive endoderm. This was achieved by exposing mouse or human ES cells to activin A, a member of the TGF-β family closely related to the appropriate developmental signal, Nodal, or to Nodal itself, in the absence of overriding signals from serum or other growth factors [18, 49, 65].

A team led by Emmanuel Baetge at Novocell, Inc. continued to identify culture conditions that would drive further differentiation of human ES cell-derived definitive endoderm through subsequent stages on the desired path – posterior foregut, pancreatic endoderm, progenitors of endocrine pancreas, and hormone-producing endocrine cells [18, 47]. Insulin secretion was not responsive to glucose levels, but could be increased by other compounds known to act on β-cells in the fetal pancreas, which also responds poorly to glucose signaling. Two other groups, using somewhat

different "cocktails" of factors acting downstream from definitive endoderm, published confirmation that cells able to synthesize and secrete insulin and other pancreatic hormones can be derived from human ES cells. These teams observed at least modest insulin secretion to elevated serum glucose, perhaps because these groups allowed the cells to mature in culture for longer periods and maintained them under 3D culture conditions that promoted the formation of "islet-like" cell clusters [42, 43]. The data indicated that the human ES-derived cells had differentiated to a functional β-cell-like state. Experts in pancreatic islet physiology have noted that the ES-derived endocrine cells may still have a somewhat immature phenotype [69]. Nonetheless, the work stands as a remarkable proof of principle study for the potential clinical use of stem cells as a source of pancreatic β-cells. Human ESC can give rise to cells that secrete insulin in a glucose-responsive manner. This encourages expectations that cell therapy ultimately will cure many patients with diabetes.

However, significant further studies will be necessary before ES cells can be considered a safe, robust source for such treatment. For example, Kroon et al. [46] found that more than 15% of animals that received transplants created from ES cells developed teratomas or similar cell growths. It will be essential to ensure that there are no residual pluripotent ES cells present in populations implanted into patients [1].

24.3.1.2 Amniotic Fluid-Derived Stem Cells (AFSC) as a Source for Insulin Producing Pancreatic β Cells

Atala's research group [4, 19, 38] discovered that a novel class of pluripotent stem cells can be isolated from the amniotic fluid of murine and human females in the early second trimester of pregnancy. This group has shown that these AFSC can give rise to differentiated derivatives of all three embryonic germ layers (ectoderm, endoderm, and mesoderm) in culture and in vivo. Cells derived from AFSC have been found to express osteogenic, adipogenic, myogenic, neurogenic, vasculogenic, neurogenic, and hepatic phenotypes. Unlike previously described stem cell populations such as ESC, the undifferentiated AFSC can be expanded for many generations in culture with no detectable changes in karyotype, and they do not form tumors when injected into animals.

The experiments with murine AFSC demonstrated that the forced expression of the pancreatic duodenal homeobox-1 (PDX-1) transcription factor, driven by an adenovirus vector, predisposes the cells to efficiently produce functional pancreatic islet-like cells that are capable of producing glucagon and insulin both in culture and in vivo [53, 59, 81]. The engineered cells restore euglycemia in an animal model of diabetes [29, 38]. Recently, these observations were reproduced with human AFSC. Research is now focused on functional genomics approaches to define key molecular steps in β-cell neogenesis, especially those associated with the commitment of stem cells to the β-cell fate. A major goal is to identify genes that are transcriptionally active in progenitors of pancreatic islet cells. Currently, reporter gene systems are used to create new cell-based, high-throughput screening assays for the discovery of protein factors and/or new chemical entities (NCEs) capable of driving β cell regeneration in human diabetics.

These studies suggest that amniotic fluid stem cells are suitable for β-cell replacement and pancreatic tissue engineering. The manipulation of these novel stem cells seems to be particularly promising, as it overcomes many of the ethical issues related to ESC research.

24.3.1.3 Progenitor Cells

A major goal of research into islet cell replacement therapy is to find the most suitable progenitor cell type from which functional β-cells can be generated in large numbers. Many possibilities have been raised, including β-cells themselves, as well as embryonic and adult stem cells. Reprogramming of other cell types has also been suggested. For example, an attractive possibility would be to develop the ability to reprogram acinar exocrine cells from available pancreas tissue derived from cadaveric donors.

Human IPCs can be derived in vitro from precursors and notably from pancreatic duct cells. Some would argue that this is due to proliferation of contaminating cells in culture. However, at least one study has demonstrated the need to trigger recapitulation of the embryonic cell differentiation pathway [86]. It has been reported that pancreatic stem cells reside within the duct epithelium in both mice and humans, and that these cells can be used to generate islet-like cell clusters

that partially reverse insulin-dependent diabetes when transplanted into an animal model [86]. However the β-cells in these in vitro clusters display an immature phenotype. Furthermore, both the levels of produced insulin and the rates of proliferation of cells in these clusters appear low. Both of these factors may limit the therapeutic application of this technology. To be clinically relevant, any protocol for in vitro amplification or generation of cells from adult human pancreas must result in sufficient numbers of fully differentiated cells to allow many individuals to be treated with cells derived from a single donor. At present, this goal has not yet been achieved using human pancreas for cell derivation in vitro.

Another potential strategy for generation of islets would be to derive β cells from autologous nonpancreatic stem cells, such as those that can be obtained from bone marrow [45]. After reconstitution of lethally irradiated mice with bone marrow cells that had been genetically marked to reveal cells that upregulated expression of insulin, it was found that a small percentage of islet cells in the recipient mice were of donor origin, and did not appear to result from fusion to host cells [56]. The nature of the cells in bone marrow that participate directly in the generation of new islets is not clear. Of particular interest in this regard is a subset of cells isolated from mesenchymal stem cell (MSC) cultures, termed multipotent adult progenitor cells (MAPC) [42]. These cells can be expanded extensively in culture without apparent senescence, and they are capable of differentiating into a wide variety of cell types in culture as well as in vivo after injection into blastocysts. While they have great potential in tissue engineering and regenerative medicine applications, these cells are rare in the marrow and other tissues, and can be somewhat difficult to isolate and expand in culture. Thus, their practical utility for wide-scale therapy remains to be established.

24.3.1.4 Induced Pluripotent Stem Cell (iPS cells)

New technologies for reprogramming adult cells to an induced pluripotent state ("iPS cells"), similar to ES cells, may change the landscape of future β-cell therapy [85]. Theoretically, it should be a straightforward process to make iPS cells from readily accessible skin or blood cells from any individual, and to use these as a source of transplantable cells. This process would generate autologous cells, thus obviating the need for immunosuppression following implantation. However, at a practical level, it is not clear whether or not this degree of individualized medicine can be made cost effective for patient populations that could eventually number in the hundreds of thousands or millions. However, assessments of cell banking strategies have indicated that as few as ten pluripotent cell lines carefully chosen for homozygosity of the most common alleles at the major histocompatibility (MHC) [79] genetic loci would provide considerable benefit for transplantation matching. Obtaining such a bank of MHC-homozygous pluripotent cells would be logistically and ethically difficult if ES cell lines from embryos were used [29]. On the other hand, it would appear quite feasible to identify homozygous individuals from, for example, volunteers for bone marrow donation who are tested to determine their MHC genotype. A study of the British population indicated that approximately 10,000 individuals would suffice to find donors for the desired set of ten cell lines. This would allow beneficial matching of "off the shelf" donor cells to a majority of potential transplant recipients, and thereby significantly decrease the need for immunosuppression.

24.3.2 *Encapsulation: Strategies to Engineer Bioartificial Pancreatic Tissue*

In the past several decades, many attempts have been made to prevent the rejection of transplanted cells by the recipient's immune system by immunoisolation [74, 76]. Cell encapsulation is probably the most preferable system for cell transplantation and the formation of functional new tissues because the system enables the continuous delivery of various secreted factors emanating from the encapsulated transplanted cells to the host.

The development and application of various immunoisolation systems to transplant insulin producing islets and other cells and tissues offers the opportunity to revolutionize current therapy for many human diseases. There have been a few successful clinical applications of encapsulated pancreatic islets and many preclinical trials are underway [3, 27, 40]. Understanding the current status and limits of this

technology will enable the development of new strategies and successful cell therapy and transplantation.

24.3.2.1 Pancreatic Islet Cell Encapsulation as Immunoisolation

Cell encapsulation allows for cell transplantation while minimizing or totally overcoming the need for immunosuppressive drugs. The encapsulation technique is the process of isolating xenogenic or allogenic cells from the host's immune system by surrounding them with a biocompatible, semipermeable membrane prior to implantation within the host. In addition to protecting the cells from host immune attack, encapsulation can enhance or prolong transplanted cell function in vivo (Fig. 24.2) [12–14].

Membrane permeability is a function of both transport and thermodynamic properties, which are dependent upon the molecular characteristics of both the membrane and solute. The use of different membranes allows for variations in permeability, mass transfer, mechanical stability, buffering capability, biocompatibility, and other characteristics. A balance, however, has to be maintained among the physical properties of capsule membranes so as to support the entrapped cells' survival.

Several different types of systems employing selectively permeable membranes and matrix supports for pancreatic islets have been successfully tested in animals, including devices anastomosed to the vascular system as arteriovenous (AV) shunts, tubular membrane chambers, and spherical micro and macrocapsules. Results in diabetic animals indicate that these systems can function for periods of several months to one year without the use of any immunosuppression [66].

The impact of the natural environment of the cell on its function is a confounding issue adding a further degree of complexity. β cells reside naturally in the islets of Langerhans, microorgans consisting of a few thousand endocrine cells, of which some 75% are β cells. Their characteristic cellular architecture allows for specific intercellular communication that, in combination with other outside-in signals, is critical for well regulated insulin secretion.

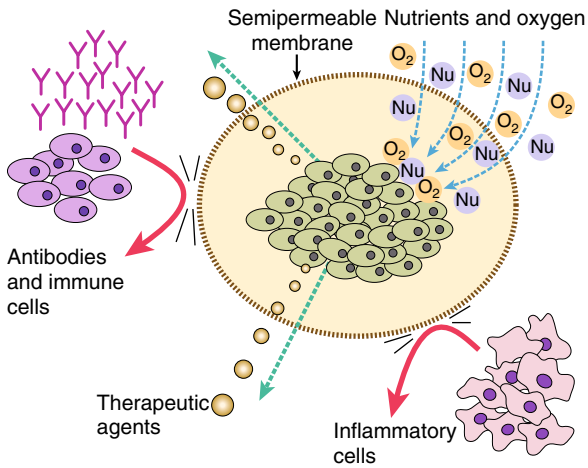

Fig. 24.2 Schematic diagram of cell encapsulation techniques. Nutrients and oxygen can diffuse across the membrane, whereas inflammatory cells, antibodies, and immune cells are excluded

24.3.2.2 Types of Islet Cell Encapsulation

Islets can be encapsulated within diffusion chambers or spherical micro- or macrocapsules and placed intraperitoneally, subcutaneously, or in other sites [40, 82]. Lim and Sun published the use of a method of encapsulation of islets of Langerhans using mild electrostatic crosslinking of sodium alginate and poly(L-lysine) to treat diabetes, and this is now the most commonly used cell encapsulation technique [39, 54]. A great number of other techniques for cell encapsulation using various polymeric materials have also been proposed.

The use of biologically compatible polymeric materials in the construction of these devices is critical to successful cell encapsulation. The cell encapsulation material plays important roles not only by providing immune protection by isolating encapsulated cells from host tissue but also by keeping the cells well distributed in the capsule and maintaining the phenotype of cells by providing a proper 3D environment, which subsequently enhances the production of therapeutic biologics from cells [50]. For example, alginate is a family of polyanionic copolymers derived from brown sea algae and comprises 1, 4-linked β-D-mannuronic (M) and a-L-guluronic (G) residues in varying proportions. Sodium alginate is soluble in aqueous solutions and forms stable gels at room temperature in the presence of noncytotoxic concentrations of certain divalent cations (i.e., Ba^{2+}, Ca^{2+}) when these ions interact with the guluronic acid groups. This enables the formation

of 3D shapes, with viable cells embedded in the gel [28, 31, 57].

One approach has been to place cells within hollow fibers made of synthetic polymers including polyacrylonitrile/polyvinylchloride (PAN/PVC), polyurethane, and polypropylene. This implanted fiber can be conveniently retrieved when the cells are no longer needed or if a problem occurs in vivo [60, 71]. Loss of cell viability due to a relatively small surface area, however, has limited the supply of nutrients and oxygen [51]. Table 24.1 lists the widely used materials for pancreatic β-cell encapsulation to treat diabetes.

24.3.2.3 Geometry of Capsules

Capsule geometry critically affects the cells within. The mass transport properties of a capsule membrane are critical as the influx rate of molecules, essential for cell survival, and the outflow rate of metabolic waste ultimately determine the viability of entrapped cells. Spherical capsule geometry can be made using the drop method and it is advantageous because of the high surface area to volume ratio. An islet (~150 μm in diameter) encapsulated in an alginate bead (600–800 μm in diameter) was shown to be less susceptible to oxygen mass transfer than islets in tubular or planar diffusion chambers [40, 54, 62]. Studies of long-term viability and functionality of encapsulated cells compared with capsule size revealed that the reduction in capsule size from 1 mm to 400 μm was effective in improving capsule quality, mechanical stability, diffusion properties, and in vitro activities of the encapsulated cells [10, 31, 76]. The capsule's mechanical stability was largely dependent on the volume ratio of the capsule over the membrane, and microcapsules are more durable and stable than macrocapsules. The in vitro cellular activities of both primary rat islets and murine hepatocytes were improved for cells encapsulated in the 400 μm capsules compared with those encapsulated in the 1 mm capsules. All experimental findings suggest that the smaller capsules present better properties for future clinical applications and at the same time widen the choice of implantation site. This strengthens the notion that slight changes in the capsular morphological parameters can largely influence graft function in vivo. However, there are two fundamental flaws which render microencapsulation unacceptable in the long run. First, millions of microbeads inserted into the host cannot reliably be retrieved if needed, and the accumulated presence of large quantities of nonfunctioning foreign biological tissue in the host is undesirable. Second, immunoprotective hydrogels currently used for microencapsulation have inherently low oxygen permeability. Oxygen is essential for pancreatic islet function. O_2 transport through the water channels of these hydrogels is only 3 mg O_2/100 g H_2O. Pancreatic islets require an oxygen consumption rate of 2.5–3 nmol/min per 100 islets in vitro and this rate increases in response to elevated glucose levels [24].

Additional problems of microencapsulating hydrogels include fragility, poor mechanical properties, bioincompatibility, fouling of pores, fibrosis, poor reproducibility, broad and ill-defined channel distributions, and variability between lots of alginate. All in all, none of the many kinds of hydrogels investigated to date has yielded convincing information for major human trials. Numerous secondary solutions have been tried to remedy the various shortcomings, but to date, there have been no fundamental advances in microimmunoisolatory materials. In view of these facts, we believe it is important to continue research on alternative technologies, such as macroencapsulation. Implantation of immunoisolated, living IPCs by macroencapsulation in semipermeable membranes has been attempted to correct the metabolic defects of T1DM.

Table 24.1 Materials used for pancreatic islet encapsulation for diabetes therapy

Cells	Materials
Pancreatic islets	Alginate-poly (L-lysine)-alginate Alginate-aminopropylsilicate-alginate Alginate-poly(L-ornithine) Alginate-cellulose sulfate-poly(methylene-*co*-guanidine) Agarose-poly(styrene sulfonic acid) Poly(*N*-isopropylacrylamide-*co*-acrylic acid) Aginate-poly(L-lysine)-poly(ethyleneimine)-Protamine-heparin Alginate-chitosan-polyethylene glycol
Bone marrow stem cells	Alginate-poly(L-lysine)-alginate
Embryonic cells	Polyethersulfone hollow fiber
Mesenchymal stem cells	Collagen-agarose

24.4 Clinical Application

Restoration of normal glucose metabolism has already been achieved in diabetic patients by the transplantation of isolated human islets. Unfortunately, the requirement for immunosuppressive drugs exposes these patients to a wide variety of problems including renal failure and osteoporosis. Ultimately, however, the goal of islet transplantation is to treat patients without the need for immunosuppressive regimens.

Cell encapsulation is one system for cell transplantation and represents an exciting biotechnological approach for both organ replacement and continuous delivery of drugs. This concept of cell-based therapy requires advances in cell encapsulation technology, and there have been successful efforts in applying this technology for the treatment of human diseases including diabetes, cancer, renal failure, and liver diseases. In some diabetic patients, this strategy has resulted in a long-term source of insulin without development of significant tolerance, eliminating the need for repeated insulin injection. A summary of these attempts to using pancreatic cells to treat diabetes is shown in Table 24.2.

24.5 Expert Opinion

The discovery of insulin and its use to treat diabetes mellitus represents one of the great chapters in the history of medicine. However, from the earliest days of insulin therapy, it was apparent that injection of the hormone did not completely compensate for the loss of the insulin-producing β-cells of the pancreas. Although life-saving to individuals with T1DM, and beneficial to many with severe Type 2 diabetes (T2DM), even state-of-the-art insulin therapy does not prevent long-term complications, notably cardiovascular disease and damage to the microvasculature and nerves, associated with elevated blood glucose levels. These individuals have benefitted from pancreas or pancreatic islet transplantation, in which the restoration of β-cells improves glucose homeostasis.

Regardless of the origin of newly generated insulin-secreting cells, there are common deliverables that must be in place for clinical use. Treatment of T1DM has improved with the availability of increasingly versatile insulin analogs, sharp needles, and home glucose monitoring. Individuals with T2DM have greatly improved glycemic control using contemporary oral hypoglycemic agents, sometimes in combination with insulin injection. However, most patients and their families state that they continue to live in hope of improved therapy that will ideally allow them to dispense with injections and provide a life-long cure. Any new therapy must be safe, effective, relatively inexpensive, and readily available to the rapidly growing population with diabetes. Finally, it must not depend on immunosuppression, yet it should allow new β-cells to thrive in the hostile environment of an individual with Type I diabetes. The race is on to provide a plentiful supply of

Table 24.2 CurrenVt pancreatic islet cell therapies

Company	Technology	Major disease focus
BioHybrid Technologies (Shrewsbury, MA)	Encapsulation system for allografts	Encapsulated pancreatic islet cell allografts; therapeutic protein delivery
Islet Sheet Medical (San Francisco, CA)	Encapsulated pancreatic islet cells	Retrievable bioartificial pancreas for diabetes
Ixion Biotechnology (Alachua, FL)	Pancreatic islet-producing human stem cells	Unencapsulated islet cell allografts for diabetes
Novocell (Irvine, CA)	Individually polymer-coated pancreatic islet cells	Encapsulated islet cell allografts for diabetes
Geron (Menlo Park, CA)	Human ES cells; dendritic cell vaccines	Cell-based treatments for cancer, diabetes, osteoarthritis
TEI Biosciences (Boston, MA)	Signaling molecules to induce stem cell differentiation	Tissue engineering using derived cell types
Progenitor Cell Therapy (Saddle Brook, NJ)	Cell therapy manufacturing services	GMP factory and distribution system to grow and deliver autologous therapies nationwide

fully functional cells to individuals with diabetes. The initial rush to publication of the first observations of newly derived or regenerating cells is over and we are now entering a more challenging and rigorous period of consolidation that must be combined with innovative and multidisciplinary research in basic cell and developmental biology.

24.6 Five-Year Perspective

One of the major challenges in the field of tissue engineering over the past two decades has been the requirement for large-scale engineered constructs comprising precisely organized cellular microenvironments. For vital organ assist and replacement devices, microfluidic-based systems such as the microcirculation, biliary, or renal filtration and resorption systems, and other functional elements containing multiple cell types must be generated to provide for viable engineered tissues and clinical benefit [9]. Over the last several years, microfabrication technology has emerged as a versatile and powerful approach for fabricating scaffolds for engineered tissues. These methods will be improved to provide ideal scaffolds for the engineering of 3D organ shapes with complicated vascular structures.

A recent approach to pancreatic engineering is in utilizing the natural ultra-structure of organs that can be obtained through decellularization processes. Scaffolds for complex organs such as liver, heart, and pancreas are difficult to engineer using artificial materials, but with decellularization, the natural structure of a donor organ can be preserved while removing the cellular components that cause immune reactions. The most important feature of this approach is the strong possibility that the original, natural vascular structure in the organ may be preserved even after the decellularization process.

Decellularization of an organ and recellularization via cell seeding technology provide new potential and promise in engineering functional whole organs and tissues. This approach as well as process is illustrated in Fig. 24.3. As an example, Atala's group used this method to obtain scaffolds for and subsequently construct a functional heart valve, blood vessel, liver, and pancreas. This approach may enable engineering of complex organs within 5 years.

24.7 Limitations/Critical View

Despite promising results of some cell encapsulation technology, there have been continuous questions about the use of this method. The major challenges are the maintenance of long term cell survival or cell viability in the capsules. Cell survival in capsules is limited by the supply of nutrients and oxygen. Nutrients typically include low molecular weight solutes such as glucose, macromolecules such as albumin, and transferrin for iron uptake. Growth factors may also be required. Although the transport limitations for macromolecules have not yet been quantified, it is likely that oxygen supply limitations are the most serious.

A class of microporous membranes that induce neovascularization can be placed in direct contact with the bloodstream at an arterial pO_2 (~100 mmHg). By contrast, extravascular devices implanted intraperitoneally or in subcutaneous tissue are exposed to the average pO_2 of the microvasculature (~40 mmHg). Implantation in soft tissue may also precipitate a foreign-body response, which can lead to the production of an avascular layer, typically ~100 μm thick. This fibrotic tissue increases the distance between blood vessels and the implant, and the fibroblasts in the avascular layer consume oxygen, further reducing the amount of oxygen that reaches the implant.

In the absence of a proper cell encapsulating material, adherent cells aggregate to form clusters. When the clusters grow too large, they typically develop a central necrotic core. Dying cells accumulate around the core and, upon lysing, release factors detrimental to the health of neighboring cells. The lysed cell fragments are also transported to the host environment, eliciting an antigenic response.

Cell sourcing is another challenge in cell encapsulation approaches for tissue engineering. Genetically engineered cells have a higher capacity for in vivo survival when used as cell sources for cell therapy, but problems associated with engineered cells include gene transfection efficiency and the risk of viral transmission, which necessitates multiple purification steps before the cells can be used. Stem cells have the potential to be used in cell-based therapeutics because they are a virtually unlimited donor source for transplantation, and they are flexible to a wide spectrum of genetic manipulations. However, extensive studies must be completed to determine the long-term safety and efficacy of encapsulated stem cells.

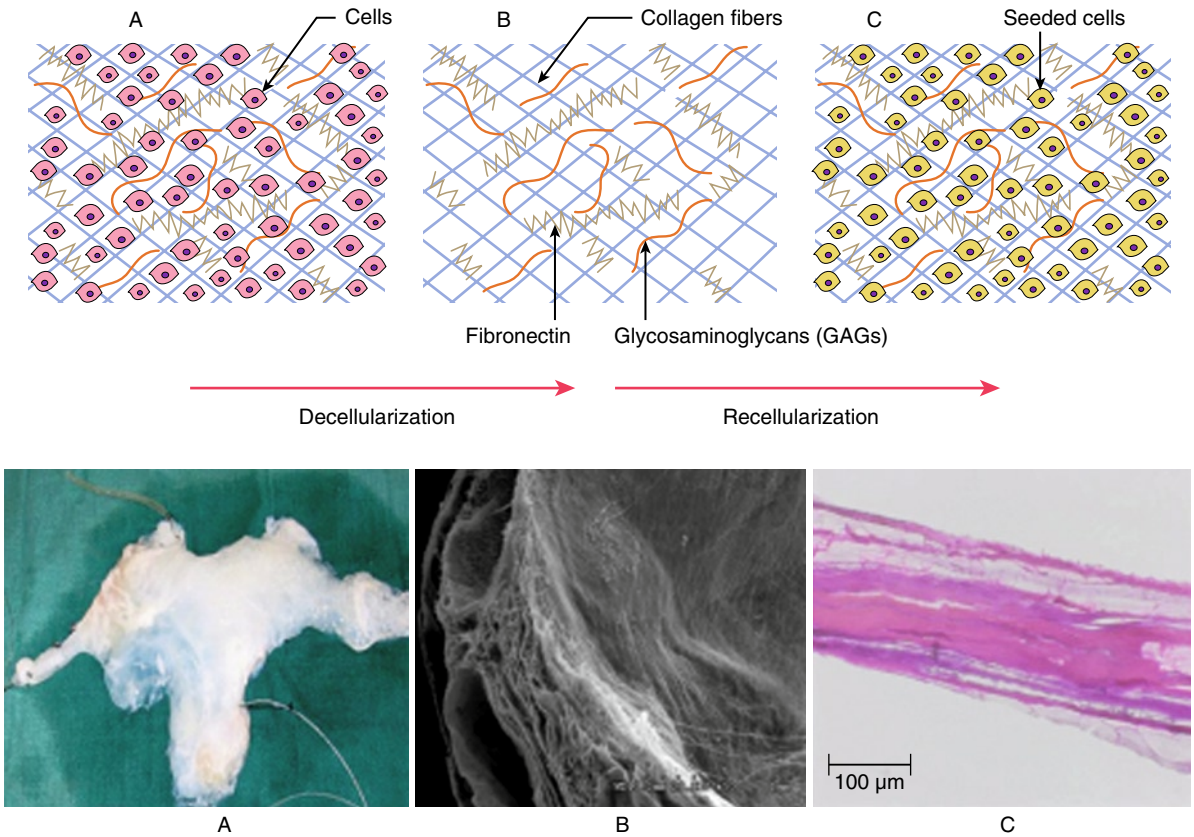

Fig. 24.3 Future approaches to engineering pancreatic tissue using decellularization methods. (**a**) Decellularized porcine pancreas, (**b**) scanning electron micrographs of decellularized porcine pancreas, (**c**) recellularized porcine scaffold with human pancreatic islets

Disturbing cell interactions will most probably have a profound negative effect on function. Driving in situ regeneration of, or replacement therapy using, isolated cells or a collective of cells deprived of their natural neighbors may not result in a clinically satisfactory solution and may even be unsafe if insulin secretion is severely disturbed. The greater challenge may then be the need to recreate islets, including normal endocrine cell architecture, microvasculature, and enervation [34].

24.8 Conclusion/Summary

A number of strategies for therapeutic implementation of pancreatic tissue engineering for diabetes have been explored, including the use of various types of stem cell sources, advanced fabrication methods, and encapsulation technology. However, there are several important hurdles to overcome to bring pancreatic engineering to the bedside. One example is the safety concerns surrounding the use of undifferentiated ES cells, which have been shown to give rise to teratomas. In addition, ideal scaffolding materials possessing appropriate physico-chemical properties to meet the requirements of a complex organ such as pancreas must be developed. Cell encapsulation is a core technology which enables long term delivery of biological products, such as insulin-producing, living pancreatic islet cells, in response to biological need [61, 62]. However, issues of long term viability, risk of immune development, retrieval of the unwanted cells, and other safety concerns should be addressed before this technique is used in further clinical applications.

Suggested Reading with Abstract

Atala A. Engineering tissues, organs and cells. J Tissue Eng Regen Med. 2007;1(2):83–96.

Patients suffering from diseased and injured organs may be treated with transplanted organs; however, there is a severe shortage of donor organs that is worsening yearly, given the aging population. In the field of regenerative medicine and tissue engineering, scientists apply the principles of cell transplantation, materials science, and bioengineering to construct biological substitutes that will restore and maintain normal function in diseased and injured tissues. Therapeutic cloning, where the nucleus from a donor cell is transferred into an enucleated oocyte in order to extract pluripotent ESC, offers a potentially limitless source of cells for tissue engineering applications. The stem cell field is also advancing rapidly, opening new options for therapy, including the use of amniotic and placental fetal stem cells. This review covers recent advances that have occurred in regenerative medicine and describes applications of these technologies using chemical compounds that may offer novel therapies for patients with end-stage organ failure.

Beck J, Angus R, Madsen B, et al. Islet encapsulation: strategies to enhance islet cell functions. Tissue Eng. 2007;13:3.

Diabetes is one of the most prevalent, costly, and debilitating diseases in the world. Although traditional insulin therapy has alleviated the short-term effects, long-term complications are ubiquitous and harmful. For these reasons, alternative treatment options are being developed. This review investigates one appealing area: cell replacement using encapsulated islets. Encapsulation materials, encapsulation methods, and cell sources are presented and discussed. In addition, the major factors that currently limit cell viability and functionality are reviewed, and strategies to overcome these limitations are examined. This review is designed to introduce the reader to cell replacement therapy and cell and tissue encapsulation, especially as they apply to diabetes.

Best M, Carroll M, Hanley NA, et al. Embryonic stem cells to beta-cells by understanding pancreas development. Mol Cell Endocrinol. 2008;288:86–94.

Insulin injections treat but do not cure T1DM. The success of islet transplantation suggests cell replacement therapies may offer a curative strategy. However, cadaver islets are of insufficient number for this to become a widespread treatment. To address this deficiency, the production of β cells from pluripotent stem cells offers an ambitious far-sighted opportunity. Recent progress in generating IPCs from ESC has shown promise, highlighting the potential of trying to mimic normal developmental pathways. Here, we provide an overview of the current methodology that has been used to differentiate stem cells toward a β-cell fate. Parallels are drawn with what is known about normal development, especially regarding the human pancreas.

Borenstein JT, Weinberg EJ, Orrick BK, et al. Microfabrication of three-dimensional engineered scaffolds. Tissue Eng. 2007;13(8):1837–44.

One of the principal challenges facing the field of tissue engineering over the past two decades has been the requirement for large-scale engineered constructs comprising precisely organized cellular microenvironments. For vital organ assist and replacement devices, microfluidic-based systems such as the microcirculation, biliary, or renal filtration and resorption systems, and other functional elements containing multiple cell types must be generated to provide for viable engineered tissues and clinical benefit. Over the last several years, microfabrication technology has emerged as a versatile and powerful approach for generating precisely engineered scaffolds for engineered tissues. Fabrication process tools such as photolithography, etching, molding, and lamination have been established for applications involving a range of biocompatible and biodegradable polymeric scaffolding materials. Computational fluid dynamic designs have been used to generate scaffold designs suitable for microvasculature and a number of organ-specific constructs; these designs have been translated into 3D scaffolding using microfabrication processes. Here, a brief overview of the fundamental microfabrication technologies used for tissue engineering will be presented, along with a summary of progress in a number of applications, including the liver and kidney.

De Coppi P, Bartsch G Jr, Siddiqui MM, et al. Isolation of amniotic stem cell lines with potential for therapy. Nat Biotechnol. 2007;25(1):100–6.

Stem cells capable of differentiating to multiple lineages may be valuable for therapy. We report the isolation of human and rodent AFSC that express embryonic and adult stem cell markers. Undifferentiated AFS

cells expand extensively without feeders, double in 36 h, and are not tumorigenic. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells are broadly multipotent. Clonal human lines verified by retroviral marking were induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal, and hepatic lineages. Examples of differentiated cells derived from human AFS cells and displaying specialized functions include neuronal lineage cells secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue-engineered bone.

Furth ME, Atala A. Stem cell sources to treat diabetes. J Cell Biochem. 2009;106(4):507–11.

We review progress towards the goal of utilizing stem cells as a source of engineered pancreatic β-cells for therapy of diabetes. Protocols for the in vitro differentiation of ESC based on normal developmental cues have generated β-like cells that produce high levels of insulin, albeit at low efficiency and without full responsiveness to extracellular levels of glucose. iPS cells also can yield IPCs following similar approaches. An important recent report shows that when transplanted into mice, human ES-derived cells with a phenotype corresponding to pancreatic endoderm matured to yield cells capable of maintaining near-normal regulation of blood sugar (Kroon et al. Nat Biotechnol. 2008;26:443–52). Major hurdles that must be overcome to enable the broad clinical translation of these advances include teratoma formation by ES and iPS cells, and the need for immunosuppressive drugs. Classes of stem cells that can be expanded extensively in culture but do not form teratomas, such as amniotic fluid-derived stem cells and hepatic stem cells, offer possible alternatives for the production of β-like cells, but further evidence is required to document this potential. Generation of autologous iPS cells should prevent transplant rejection, but may prove prohibitively expensive. Banking strategies to identify small numbers of stem cell lines homozygous for MHC loci have been proposed to enable beneficial genetic matching that would decrease the need for immunosuppression.

Gallo R, Gambelli F, Gava B, et al. Generation and expansion of multipotent mesenchymal progenitor cells from cultured human pancreatic islets. Cell Death and Differ. 2007; 14:1860–71.

Cellular models and culture conditions for in vitro expansion of IPCs represent a key element to develop cell therapy for diabetes. Initial evidence that human β-cells could be expanded after undergoing a reversible epithelial–mesenchymal transition has been recently negated by genetic lineage tracing studies in mice. Here, we report that culturing human pancreatic islets in the presence of serum resulted in the emergence of a population of nestin-positive cells. These proliferating cells were mainly C-peptide negative, although in the first week in culture, proliferating cells, insulin promoter factor-1 (Ipf-1) positive, were observed. Later passages of islet-derived cells were Ipf-1 negative and displayed a mesenchymal phenotype. These human pancreatic islet-derived mesenchymal (hPIDM) cells were expanded up to 1,014 cells and were able to differentiate toward adipocytes, osteocytes, and chondrocytes, similarly to mesenchymal stem/precursor cells. Interestingly, however, under serum-free conditions, hPIDM cells lost the mesenchymal phenotype, formed islet-like clusters (ILCs) and were able to produce and secrete insulin. These data suggest that, although these cells are likely to result from preexisting mesenchymal cells rather than β-cells, hPIDM cells represent a valuable model for further developments toward future replacement therapy in diabetes.

Halban PA. Cellular sources of new pancreatic β cells and therapeutic implications for regenerative medicine. Nat Cell Biol. 2004;6:1021–25.

Replacing missing insulin-producing β cells to treat diabetes is a major challenge for regenerative medicine. A better understanding of β-cell embryogenesis and regeneration in adult life is needed to devise means to derive these specialized cells in sufficiently large numbers from stem or precursor cells. It is also critical to ensure that any surrogate or regenerated β cells have perfectly regulated insulin production, which is essential for physiological glucose homeostasis.

Koblas T, Pektorova L, Zacharovova K, et al. Differentiation of CD133-positive pancreatic cells into insulin-producing islet-like cell clusters. Transplant Proc 2008;40:415–18.

Adult pancreatic stem and progenitor cells could represent an alternative source of insulin-producing tissue for diabetes treatment. In order to identify these cells, we have focused on the human pancreatic cells expressing

cell surface molecule CD133, a marker of adult stem cells. We found that population of human CD133-positive pancreatic cells contains endocrine progenitors expressing neurogenin-3 and cells expressing human telomerase, ABCG2, Oct-3/4, Nanog, and Rex-1, markers of pluripotent stem cells. These cells were able to differentiate into IPCs in vitro and secreted C-peptide in a glucose-dependent manner. On the basis of our results, we suppose that the CD133 molecule represents another cell surface marker suitable for identification and isolation of pancreatic endocrine progenitors.

Liu M, Han ZC. Mesenchymal stem cells: biology and clinical potential in type 1 diabetes therapy. J Cell Mol Med. 2008;12(4):1155–68.

MSCs can be derived from adult bone marrow, fat, and several fetal tissues. In vitro, MSCs have the capacity to differentiate into multiple mesodermal and nonmesodermal cell lineages. Besides, MSCs possess immunosuppressive effects by modulating the immune function of the major cell populations involved in alloantigen recognition and elimination. The intriguing biology of MSCs makes them strong candidates for cell-based therapy against various human diseases. T1DM is caused by a cellmediated autoimmune destruction of pancreatic cells. While insulin replacement remains the cornerstone treatment for T1DM, the transplantation of pancreatic islets of Langerhans provides a cure for this disorder. And yet, islet transplantation is limited by the lack of donor pancreas. Generation of IPCs from MSCs represents an attractive alternative. On the one hand, MSCs from pancreas, bone marrow, adipose tissue, umbilical cord blood, and cord tissue have the potential to differentiate into IPCs by genetic modification and/or defined culture conditions in vitro. On the other hand, MSCs are able to serve as a cellular vehicle for the expression of human insulin gene. Moreover, protein transduction technology could offer a novel approach for generating IPCs from stem cells including MSCs. In this review, we first summarize the current knowledge on the biological characterization of MSCs. Next, we consider MSCs as surrogate – cell source for islet transplantation, and present some basic requirements for these replacement cells. Finally, MSCs-mediated therapeutic neovascularization in T1DM is discussed.

Wang AY, Ehrhardt A, Xu H, et al. Adenovirus transduction is required for the correction of diabetes using Pdx-1 or Neurogenin-3 in the liver. Mol Ther. 2007;15(2):255–63.

The regeneration of IPCs in vivo has emerged as a promising method for treating Type I diabetes. Pdx-1, NeuroD, and Neurogenin-3 (Ngn3) are pancreatic transcription factors important for the development of IPCs in the liver. Other groups have demonstrated that adenoviral-mediated transgene expression of these transcription factors in the liver can reverse hyperglycemia in diabetic mice. We delivered Pdx-1 and Ngn3 to the livers of diabetic mice using adeno-associated virus (AAV) serotype 8, a vector that has been shown to result in nontoxic, persistent, high level expression of the transgene. We were unable to correct hyperglycemia in mice with streptozotocininduced diabetes using AAV vectors expressing Pdx-1 and Ngn3. However, when we codelivered these transcription factor expression cassettes in nonviral vectors with an irrelevant adenoviral vector, we were able to correct hyperglycemia in diabetic animals. Further studies demonstrated that an antigen-dependent immune response elicited by the adenoviral capsid together with the expression of a pancreatic transcription factor was required for restoration of serum insulin levels by the liver. Our results suggest that a host response to adenovirus in combination with expression of a pro-endocrine pancreas transcription factor is sufficient to induce insulin production in the livers of diabetic mice.

Xu YX, Chen L, Wang R, et al. Mesenchymal stem cell therapy for diabetes through paracrine mechanisms. Med Hypotheses. 2008;71(3):390–93.

T1DM is a chronic disorder characterized by the destruction of pancreatic islet β-cells through autoimmune assault. Insulin replacement is the current main therapeutic approach. In recent years, several studies have showed that MSCs transplantation can improve the metabolic profiles of diabetic animal models. However, the exact mechanisms of reversing hyperglycemia remain to be elusive. Trans-differentiation of MSCs into IPCs has ever been regarded as the main mechanism. But other reports have contradicted these findings and it is difficult to explain the timing and extent of improvement by only the effect through trans-differentiation. Researchers have found that MSCs naturally produce a variety of cytokines and growth factors, promoting the survival of surrounding cells, called as paracrine mechanisms. Paracrine effects have been proved to play an important role in tissue regeneration and repair in recent researches. Therefore, we speculate that MSCs transplantation into diabetic animals may

prevent apoptosis of injured pancreatic β-cells and enhance regeneration of endogenous progenitor cells through paracrine actions such as angiogenic, cytoprotective, antiinflammatory, mitogenic, and anti-apoptotic effects. This hypothesis, if proved to be valid, may represent an important breakthrough in developing effective molecular or genetic therapeutics for diabetes.

Zhou Q, Brown J, Kanarek A, et al. In vivo reprogramming of adult pancreatic exocrine cells to b-cells. Nature. 2008; 455(7213):627–32.

One goal of regenerative medicine is to instructively convert adult cells into other cell types for tissue repair and regeneration. Although isolated examples of adult cell reprogramming are known, there is no general understanding of how to turn one cell type into another in a controlled manner. Here, using a strategy of reexpressing key developmental regulators in vivo, we identify a specific combination of three transcription factors (Ngn3 (also known as Neurog3) Pdx1 and Mafa) that reprograms differentiated pancreatic exocrine cells in adult mice into cells that closely resemble β-cells. The induced β-cells are indistinguishable from endogenous islet β-cells in size, shape, and ultrastructure. They express genes essential for β-cell function and can ameliorate hyperglycaemia by remodeling local vasculature and secreting insulin. This study provides an example of cellular reprogramming using defined factors in an adult organ and suggests a general paradigm for directing cell reprogramming without reversion to a pluripotent stem cell state.

Zalzman M, Gupta S, Giri RK, et al. Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci USA. 2003;100(12):7253–8.

β-Cell replacement is considered to be the most promising approach for treatment of T1DM. Its application on a large scale is hindered by a shortage of cells for transplantation. Activation of insulin expression, storage, and regulated secretion in stem-progenitor cells offers novel ways to overcome this shortage. We explored whether fetal human progenitor liver cells (FH) could be induced to differentiate into IPCs after expression of the Pdx1 gene, which is a key regulator of pancreatic development and insulin expression in β-cells. FH cells possess a considerable replication capacity, and this was further extended by introduction of the gene for the catalytic subunit of human telomerase. Immortalized FH cells expressing Pdx1 activated multiple β-cell genes, produced and stored considerable amounts of insulin, and released insulin in a regulated manner in response to glucose. When transplanted into hyperglycemic immunodeficient mice, the cells restored and maintained euglycemia for prolonged periods. Quantitation of human C-peptide in the mouse serum confirmed that the glycemia was normalized by the transplanted human cells. This approach offers the potential of a novel source of cells for transplantation into patients with T1DM.

Zaret KS. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nat Rev. 2008;9:329.

The liver and pancreas arise from a common multipotent population of endoderm cells and share many aspects of their early development. Yet each tissue originates from multiple spatial domains of the endoderm, under the influence of different genes and inductive cues, and obtains different regenerative capacities. Emerging genetic evidence is illuminating the ability of newly specified hepatic and pancreatic progenitors to reverse their course and develop into gut progenitors. Understanding how tissue programming can be reversed and how intrinsic regenerative capacities are determined should facilitate the discovery of the basis of cellular plasticity and aid in the targeted programming and growth of stem cells.

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Tendons: Engineering of Functional Tissues

25

D.I. Zeugolis, J.C.Y. Chan, and A. Pandit

25.1 Introduction

Tendon and ligament injuries represent some of the most common musculoskeletal disorders that clinicians address daily, ranging from as mundane as a mild ankle sprain to the crippling effects of Achilles tendon rupture or flexor tendon injury in the hand. Indeed, from over 33 million musculoskeletal injuries per year in United States alone; almost 50% of them are tendon and ligament related with approximately 95,000 new cases per year [69, 71, 74, 163, 304, 316]. Surgical repairs do not fully restore function due to fibrous adhesions or failure arising from the mechanical demands placed on imperfect integrative healing at tendon-tendon or tendon-bone interfaces [58, 512]. As the human population ages and the life expectancy increases, tendon injuries will continue to rise putting a further physical and financial strain on healthcare system. Therefore, to develop strategies for functional tendon regeneration is of paramount importance. Tissue engineering aims to mimic the native tissue properties with the ultimate goal being the fabrication of implants that would closely imitate native extracellular matrix assemblies and restore function. The behaviour of the tissue during development and ageing, and knowledge of the extracellular matrix components of the tissue to be replaced is essential for engineering functional constructs.

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25.2 Cellular Composition of Tendons

The extracellular matrix elements of tendons are produced by tenoblasts and tenocytes, which are elongated fibroblasts and fibrocytes that are organised in a complex hierarchical structure [95, 108, 211, 258]. Tendon cells accounts up to 90–95% of all the cellular components in the tendon. The remaining 5–10% consists of chondrocytes at bone insertions in the osteotendinous junctions, synovial cells from tendon sheath and vascular endothelial and smooth muscle cells from the vascular plexus [253, 259, 462]. Tendon cells appear to be arranged in a unicellular row in the space between adjacent tendon fibres; their cell bodies occupy the middle of these spaces and send smooth, flattened lamella outwards between the tendon fibres and small bundles of thin collagen fibrils [183, 488]. Tenoblasts have different shapes and sizes; some are round, some are spindle-shaped, and others spherical or quadrangular. Their size varies with length ranging from 20 to 70 μm and width from 8 to 20 μm. The newborn tendon has a very high cell to matrix ratio that decreases during development, maturation and ageing. Tenoblasts become tenocytes as they mature and become very elongated with width ranging from 80 to 300 μm. Tenocytes, although are metabolically active cells, are not as active as tenoblasts and may have different functions in vivo [85, 242, 253]. It has been demonstrated, for example, that tenoblasts, in contrast to tenocytes, are responsible for matrix remodelling in healthy tendons [108].

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25.3 Structural Elements of Tendons

Collagen and elastin fibres, proteoglycans and minerals are the major load-bearing elements in the extracellular matrix of tendon. Collagen type I constitutes 20–30% of the total body protein and in tendon ranges from 80 to 95% of its dry weight [47, 49, 56, 143, 217, 221, 353, 404, 408, 453, 581]. Moreover, collagen fibres function in biological structures to maintain tissue shape, transmit and absorb loads, prevent premature mechanical failure, partition cells and tissues into functional units, and act as a scaffold that supports tissue architecture [471]. Despite the pivotal role of type I collagen in tendons, the role and distribution of other less abundant collagen types (such as type III) [273, 419] or biological macromolecules (such as proteoglycans and glycosaminoglycans) [85, 175, 395, 454, 479] are of great importance when it comes to fabrication of biomaterials and replacement of the collagenous device by new functional tissue.

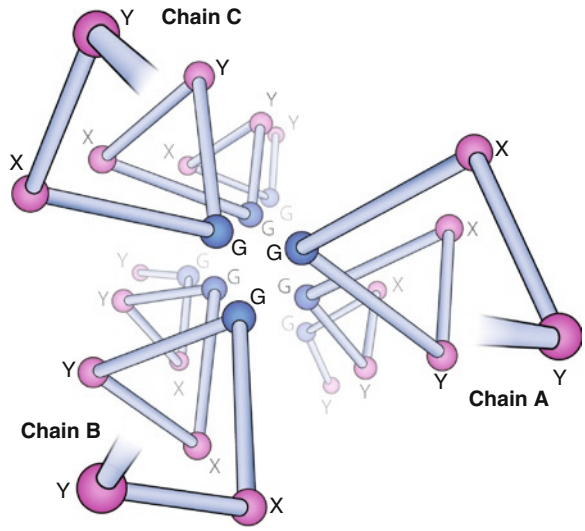

Fig. 25.1 Cross-section of collagen triple-helix; each propeptide form a left handed helix and assemble with the other two chains in a right handed super-helix. Glycine occupies the centre of the helix, whilst the X and Y residues are at the surface with their side chains extended outward of helical configuration. Note: the balls correspond to the α carbons of the amino-acyl residues, whilst the sticks to peptide bonds. Adopted from [522]

25.3.1 Collagen Type I

The term collagen is often used to describe a family of protein molecules that all share the same characteristic triple helical configuration as a major structural motif. To date, forty vertebrate collagen genes have been described, the products of which combine to form 28 distinct homo- and/or hetero– trimeric molecules [247, 273, 389, 522, 526]. Their common feature being the presence of at least one tri-peptide helical sequence of repeated $(Gly–X–Y)_{100–400}$, where X is often proline and Y sometimes hydroxyproline [35, 39, 273, 521, 522].

Type I collagen is hetero-polymer containing two α1(I) chains and one α2(I) chain. Each α-chain is a left-handed helix and the three chains are staggered by one residue relative to each other and are super-coiled around a central axis and form a right-handed super-helix (Fig. 25.1) [115, 220, 331, 355, 440, 522, 553]. The stability factor of the helix is attributed to intramolecular hydrogen bonds between glycines in adjacent chains. The hydroxyl groups of hydroxyproline residues are also involved in hydrogen bonding and are important for stabilising the triple helix structure. Two hydrogen bonds formed per triplet: one between the NH-group of a glycyl residue with the COO-group of the residue in the second position of the triplet in the adjacent chain and one via the water molecule participating in the formation of additional hydrogen bonds with the help of the hydroxyl group of hydroxyproline in the third position (Fig. 25.2) [39, 360, 398, 447]. Every third amino acid is in the centre of the helix and, for steric reasons, only glycine, with a side chain limited to a single hydrogen atom, can occupy this position without altering the triple-helical conformation. The presence of another amino acid in the glycine position or the presence of imperfections in this repetitive structure seriously alters the stability or the conformation of the helix. Proline and hydroxyproline stabilise the collagen molecule and due to their alicyclic nature, they stiffen the α-chain by preventing rotation around the C–N bond [188]. The presence of hydroxyproline in the molecule contributes significantly to the stability of the collagen triple helix; in the absence of hydroxyproline the collagen molecule is rapidly degraded [45, 249, 439].

The assembly of extracellular matrix gives rise to the distinct structural and functional properties of tissues. Collagen fibril assembly and growth are finely regulated during connective tissue development. The pathway of collagen synthesis from gene transcription to secretion

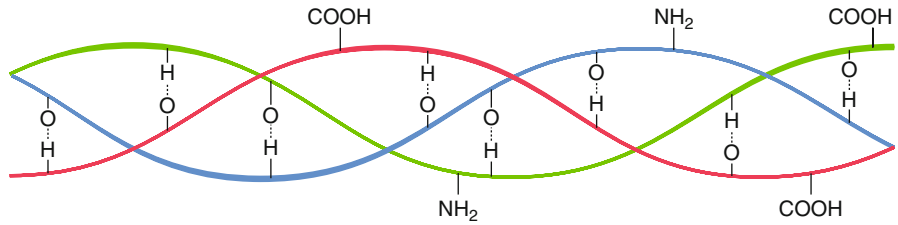

Fig. 25.2 Hydrogen bonds within the triple helix. Adopted from [398]

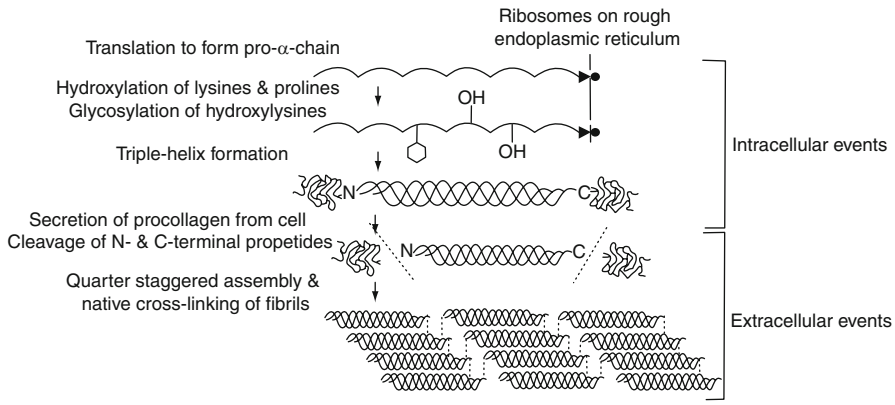

Fig. 25.3 Collagen biosynthesis in vivo

and aggregation of collagen monomers into functional fibrils in tissues is a complex multi-step process that requires the coordination of a very large number of biochemical events, both temporally and spatially (Fig. 25.3). Briefly, fibril-forming collagens are synthesised in precursor form, procollagens, with N- and C-terminal propeptide extensions. The C-propeptides direct chain association during intracellular assembly of the procollagen molecule form its three constituent polypeptide chains. Following or during secretion into the extra-cellular matrix, propeptides are cleaved by specific procollagen proteinases, thereby triggering fibril formation [49, 52, 76, 160, 188, 222, 231, 301, 409, 419, 522]. These cleavage reactions leave short, non-triple-helical domains, called telopeptides, at each end of the collagen molecule. These telopeptide domains play an essential role in the assembly and maturation of the collagen matrix by inter-molecular cross-link formation [188, 190, 222, 419, 554, 564] and they are also responsible for the inflammatory processes [440]. Losses of diameter uniformity, induction of anti-parallel packing and changes in the fibril assembly pathway have been observed depending on the extent of loss of the N- and C-telopeptides. Even with preservation of the telopeptides using protease inhibitors during extraction and purification of type I collagen, alternative pathways for fibril assembly have been observed [222, 223]. Individual collagen molecules then aggregate spontaneously in quarter-staggered arrangement to form elongated fibres (termed fibrils). After fibril formation, the lysyl-oxidase cross-linking occurs in a head-to-tail fashion and gives exceptional strength to collagen fibres [35, 39–41, 76, 122, 203, 283, 351, 352, 500, 570]. First hydroxylysines or lysines are oxidatively deaminated by the enzyme lysyl oxidase to form hydroxyallysine or allysine respectively. The resulting aldehydic side chains can then react with another hydroxylysine residue to form either dehydro-di-hydroxylysino-norleucine (dehydro-DHLNL) or dehydro-hydroxylysino-norleucine (dehydro-HLNL). Formation of hydroxylisino-5-ketonorleucine via an Amadori rearrangement of dehydro-DHLNL can give further stabilisation. The keto-imine cross-link and hydroxylysino-ketonorleucine polymerise the molecules in a head to tail fashion and this provides strength to the fibre. However, these divalent cross-links are only intermediates and with time are converted to multivalent cross-links capable of linking several molecules. For such a reaction to occur the molecules would have to be in register. It has been therefore proposed that cross-linking takes place in two stages; firstly longitudinal cross-linking of the end-overlapped molecules, and secondly by interaction of

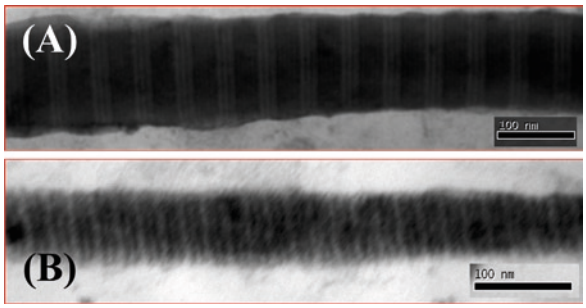

Fig. 25.4 Quarter staggered arrangement of collagen as revealed by Transmission Electron Microscopy. (**a**) Native rat tail tendon fibre; (**b**) Self-assembled collagen fibre from pepsin soluble rat tail tendon extracted collagen

these cross-links between two micro-fibrils in register. Individual fibrils can be greater than 500 μm in length, 500 nm in diameter and contain more than 10^7 molecules. The collagen fibrils possess a high degree of axial alignment, which results in high tensile strength and they exhibit a characteristic D banding, which results from alternating overlap and gap zones, produced by the specific packing arrangement of the 300 nm long and 1.5 nm wide collagen molecules on the surface of the fibril. This produces an average periodicity of 67 nm in the native hydrated state (Fig. 25.4) [48, 144, 193, 222, 385, 394, 438], although dehydration and shrinkage during conventional sample preparation for electron microscopy, results, in lower values of around 55–65 nm [96, 273].

25.3.2 Other Collagenous Structures of Tendons

The regulation of the molecular assembly involves the interaction of type I collagen with other, quantitatively minor, collagen types [48, 50, 320]. Collagen fibrils from tendon are composed of collagen type I and some type III and V in small quantities [76, 154, 268, 580, 581]. Collagen types III is an homo-polymer composed of three identical α1(III) chains, whilst collagen type V can be found as either an homo-polymer [three α1(V) chains] or as an hetero-polymer [2α1(V)α2(V) or α1(V)α2(V)α3(V)]. These collagen types (including collagen type II and XI) belong to the family of fibrous collagens that form quarter-staggered fibrils and are characterised by an uninterrupted helical domain of approximately 1,000 amino acids per chain and 300 nm in length; their COOH-terminal propeptides are highly homologous between themselves and their modes of aggregation appear to be very similar [35, 38–40, 230, 231, 273, 419, 522]. The presence of these collagen types have shown to influence the diameter and consequently the mechanical properties of the tissue.

Tissues rich in type I collagen have limited extensibility, in contrast to tissues with a high content of type II and III collagens, which are more extensible and tend to undergo greater deformation [150, 246, 474]. Certain pathophysiologies have been attributed to the absence of less abundant collagen types; the fragile skin of patients with Ehlers–Danlos syndrome results from the lack of type III collagen [411]. Fibril-associated collagens with interrupted triple helix (collagen types XII and XIV) [257, 463] are only indirectly associated with the surface of type I collagen banded fibrils in tendon, ligament and skin [267, 268], however they act to decrease the interactions between collagen fibrils thereby allowing them to slide past each other when an external force is applied [376].

25.3.3 Non-Collagenous Constituents of Tendons

Apart from collagen, connective tissues contain elastin and proteoglycans as predominant components. Elastin content in tissues varies depending on tissue function [183]. The elasticity of many tissues such as lung, dermis and large blood vessels depends on the presence of a high content of elastic fibres. In tendons, it constitutes approximately 1–2% of the dry mass [258]. These structures are composed of two distinct morphological elements: a more abundant amorphous core of which elastin is the major constituent; and highly-organised micro-fibrillar structures of about 10–12 nm diameter, which are located around the periphery of the amorphous component and consist primarily of fibrillin 1 and/or 2 [36, 95, 135, 191, 199, 444, 488]. One of the precursors to the elastin component of elastic fibres has been designated as tropoelastin. Tropoelastin undergoes a number of post-translational modifications before it is assembled into the elastic fibres. Each elastin molecule is composed of alternating hydrophobic and lysine-rich cross-linking domains that are critical for extracellular assembly and elastic function [274].

Lysine side chains oxidation (by the enzyme lysyl oxidase) allows formation of covalent cross-links with neighbouring molecules [33, 37, 441]. It is believed that the elastic properties are derived from the tendency of the hydrophobic regions to take on a random coil configuration following stretch. Over-extension is prevented by the cross-links and results in almost complete recovery of the wavy configuration of the collagen fibres after muscle contraction and tendon stretch [34, 37, 73, 397]. The microfibrils, which are mainly fibrillins [444] together with the elastin micro-fibril interface located proteins (EMILINS) [59, 364] act as a template for the organisation of the elastic tissue (elastogenesis) and are distributed in tissues where resilience and elastic recoil are prominent [36, 110]. EMILINS are found at the interface between amorphous elastin and microfibrils, and it is believed that they may regulate elastogenesis and vascular cell maintenance by stabilising molecular interactions between elastic fibre components and by endowing elastic fibre with specific cell adhesion properties [135, 577]. In pathophysiologies, such as Ehlers–Danlos syndrome or chronic uraemia, the number and volume of the tendon elastic fibres has been shown to increase [251, 252], although in skin and lungs both elastin and collagen type I reduction has been observed, in addition to disorganisation due to lysyl oxidase decrease [336, 465].

Proteoglycans consist of a family of glycoproteins with a core protein structure and a variable number of covalently attached carbohydrate moieties, glycosaminoglycans [279]. These molecules, together with other glycoproteins and the glycosaminoglycan hyaluronan, form the gel-like structure in which the collagen fibrils are embedded [147]. Glycoproteins are believed to have some function in stabilising the tissue, maintaining the structural stability of collagen fibrils and even playing a primary role in its antigenicity [13, 417]. There are two major classes of proteoglycans present interstitially in connective tissues; the first is represented by large molecules (>l,000 KDa) having the capacity to form aggregates with hyaluronan [206, 365] and constitute about 10% of the total proteoglycans in tendons [537], whilst the second class is characterised by low molecular mass (<l00 KDa) and is not capable of interacting with hyaluronate [207]. In tendons, specific interactions between proteoglycans and fibrillar collagens determine the organisation of the extracellular matrix assembly at the supramolecular level [204, 454, 456, 457, 460]. Proteoglycans make up less than 1% of the dry weight of most tensile tendons [538] and electron microscopy studies have localised them on the surface but not inside collagen fibrils [460]. Although they form a minor proportion of the extracellular matrix, proteoglycans and their constituent glycosaminoglycans influence many physiological processes, including collagen fibrillogenesis, cell-cell interactions, growth factor binding and cell regulation [197, 431, 442, 455]. Decorin, biglycan, fibromodulin and lumican are members of the family of small leucine rich proteoglycans and they are known to bind to the surface of collagen fibrils [204, 240, 241, 529, 551]. Both decorin and fibromodulin demonstrate similar affinity for collagen type I [204, 492]. Although biglycan has a structure very similar to decorin and fibromodulin, it does not bind to fibril-forming collagens [65]. These macromolecules are present in embryonic tendon [51] and are believed to regulate collagen fibril assembly and growth by preventing the aberrant lateral fusion of collagen fibrils in connective tissues such as tendon [153, 384]. Chondroitin sulphate and hyaluronate dominate in foetal tissue, whereas dermatan sulphate is the main glycosaminoglycan in mature tendon [460]. Regularly arranged mucopolysaccharides on collagen fibrils, revealed by small-angle X-ray diffraction [296], have been shown to contribute to the stabilisation of intact collagen fibrils through mechanical restraint, electrostatic interactions, and/or by means of water-binding capacity of the sulphated glycosaminoglycans [122].

Several phosphorylated and acidic non-collagenous proteins have been implicated in the mineralisation of vertebrate tissues, depending on their concentration and whether they are immobilised or free in solution [57, 99]. Studies on tendons suggest that such proteins regulate mineral deposition at the gap region of the collagen fibrils [513], which could result in stiffening the collagen triple helix and increasing the elastic spring constant. An increase in the elastic spring constant has been shown to increase the elastic energy storage in developing avian tendons [472].

25.4 Tendon Hierarchical Structure

Healthy tendons are brilliant white in colour and fibro-elastic in texture, demonstrating great resistance to applied mechanical loads. Tendons occur in many

physical forms ranging from wide and flat tendons to cylindrical, fan-shaped, and ribbon-shaped tendons [258]. Tendons are described as dense, regular connective tissues consisting primarily of type I collagen arranged in hierarchical order [316, 512]. Tropocollagen molecules form fibrils that would give rise to fibres and fascicles (Fig. 25.5). Parallel arrays of fascicles are bundled together to form epitenon, which forms a sheath around tendons, is composed of fine, loose connective tissue, allows the fascicles to slide past one another and contains the vascular, lymphatic and nerve supply [322, 512]. The epitenon extends into the tendon to form endotenon between tertiary bundles [261]. A loose areolar connective tissue, called paratenon, surrounds the epitenon superficially. Paratenon consists of type I and III collagen fibrils, elastic fibres and an inner lining of synovial cells [294]. Synovial sheaths have a lubricating function in sites of increased mechanical stress, such as the hands. The inner synovial sheath produces synovial fluid for lubrication of tendons [253], whilst the outer fibrous sheath forms fibrous aggregates that keep tendons close to the bone and prevent bowstringing of tendons by acting as fulcrums (pulley system) [138]. Without their normal function, the tendons will travel the shortest distance between adjacent remaining pulleys, causing bowstringing, which is described as a complication of release of trigger fingers [265]. The extracellular elements of the human tendon have been arbitrarily classified on the basis of diameter as small (100 Å), medium (600 Å), and large (1,750 Å) [142]. The diameter of a tropocollagen molecule is around 1.5 nm, that of a collagen fibril is around 80–100 nm and that of a collagen fibre is around 1–4 μm [427]; Achilles tendon fibre ranges from 26 to 30 μm [246], secondary bundles range

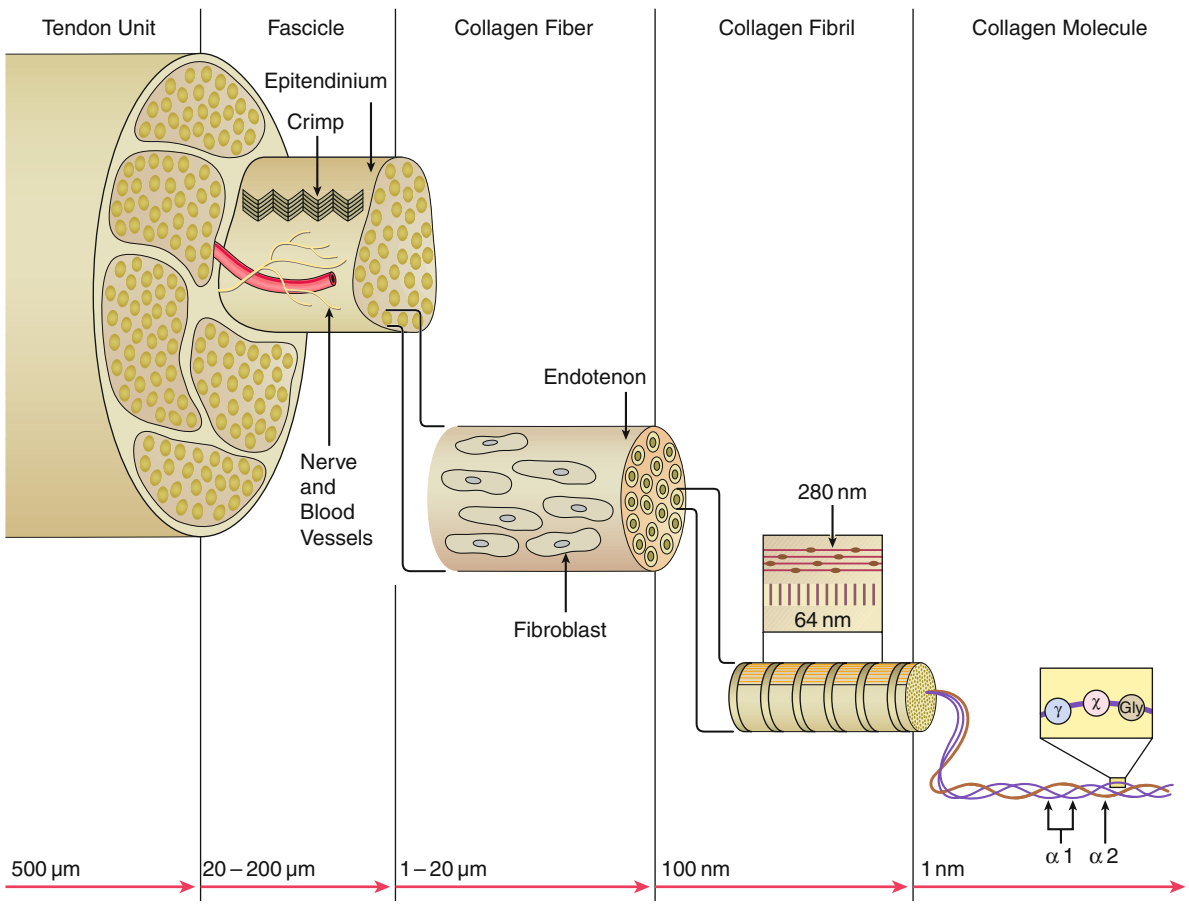

Fig. 25.5 Tendon hierarchical structure. Adopted from [473]

from 150 to 1,000 μm, whilst the diameter of the tertiary bundles varies from 1,000 to 3,000 μm. The diameter of both types of bundles is directly related to the macroscopic size of the tendon; small tendons like the flexors and extensors of the fingers and toes are the smaller in diameter, whilst big tendons, such as the Achilles are the thickest [258].

The collagen fibres in skin are structured in a three-dimensional wavy array, while in tendons, they are packed parallel, nearly straight, and aligned in the direction of applied loads. The specific architecture of the collagen network in tissues is believed to influence its function [299]. For example, the randomly orientated fibres of skin permit considerable extension of the tissue until the fibres themselves are loaded, whereas the fibres of tendons are aligned parallel and therefore loaded instantly, permitting maximum energy transfer [40]. The collagen fibril has been described as essentially a long, thin, single crystal [42, 525]. The positive intrinsic birefringence of collagen indicates a quasi-crystalline alignment parallel to the fibre and molecule axis of the amino acid residues of the polypeptide chains [347, 562]. X-ray diffraction studies indicate that the collagen molecules are arranged on a three-dimensional crystalline lattice [61, 232–234, 384] that, in vivo, can be highly polarisable and often assembles into large, ordered noncentrosymmetric structures [75, 560]. Its unique triple-helical structure and the high levels of crystallinity make collagenous structures exceptionally efficient in generating second harmonic generation signals of incident light [116, 117, 248, 560]. An excellent example is rat tail tendon, a tissue rich in type I collagen with extremely high level of crystallinity and structural alignment [116, 502] (Fig. 25.6).

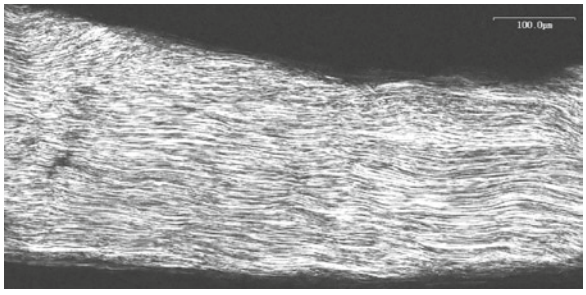

Fig. 25.6 Second harmonic generation micrograph of native rat tail tendon

25.5 Function of Native Tendon

Tendons play two major physiological functions; they function as structural components and play a mechanical role in linking and transmitting forces generated by muscle to bone, in order to mobilise and/or stabilise the joints that they cross. Indeed, tendons are specialised dense connective tissues, with viscoelastic and plastic properties, both essential in transmitting muscle-contraction-induced tensile strains into movements, while simultaneously maintaining the structural integrity of the tendon [200, 246, 299]. Elastic energy storage in tendons of many animals is an important mechanism that saves substantial quantities of muscular energy during movement [8, 68, 472]. Elastic recoil converts most of the stored energy back to kinetic energy [473]. Elastic energy storage involves direct stretching of the flexible regions within the collagen molecule; the ends of the molecule and its cross-links are probably not as flexible as the triple helix devoid of proline and hydroxyproline [468, 473].

The collagen network is considered to be the main load-bearing structure of connective tissues that are exposed to repeated tensile forces [54, 76, 121, 221, 386]. As such, collagen fibres perform many mechanical functions in the body, most of which require a tough, durable material [139, 188, 221, 422, 453]. The primary mechanical strength of individual collagen molecules is dependent upon the extra-cellular formation of the triple helix molecule that self-assembles into a collagen fibril. Given that individual collagen fibrils are discontinuous in extracellular matrices [515], to achieve mechanical integrity stabilisation through intra- and inter- molecular cross-links between the adjacent helical molecules has been postulated. It has also been proposed that non-collagenous macromolecules play an important role in forming such connections [130, 514]. The unique viscoelastic properties of elastin and the interactions of proteoglycans with the collagen fibre have been shown to allow the tissue to withstand compressive and tensile forces [226, 425]. The interaction between collagen and its water of hydration is of special importance as well since the mechanical properties of connective tissues are dependent upon this interaction [145, 509].

The age of the animal, the length and the diameter of the collagen fibres, the sampling position, the collagen content, the presence of non-collagenous components and the strain rate have been shown to play

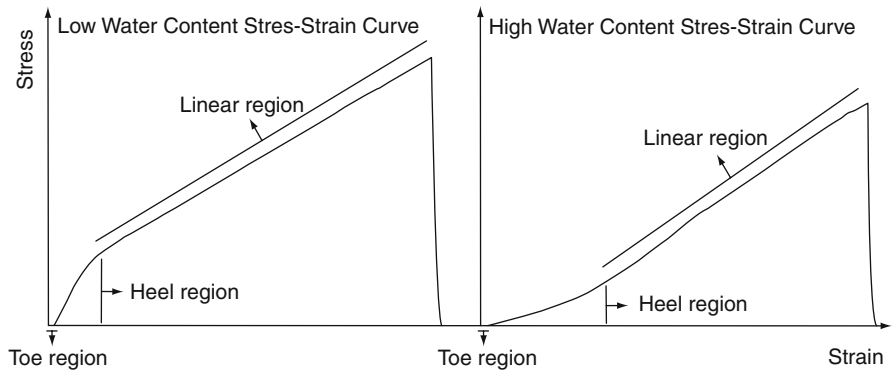

Fig. 25.7 Stress-strain curves for dry and wet native rat tail tendon

important roles in the study of the collagenous tissue [22, 118, 136, 165, 226, 406, 407, 418, 425]. The deformation mechanism of tendons can be studied by observing the stress-induced changes on the stress-strain curves. S-shape [27, 210, 262, 408, 429, 545] and j-shape [173, 184, 262, 408, 530] curves are observed in the dry and wet state respectively, similar to those of crystalline polymers that yield and undergo plastic flow (Fig. 25.7). During loading of collagen molecules, fibrils, and fibril bundles deform and finally fail by a process termed defibrillation. Molecular level studies on tendon indicate that up to a strain of 2% (toe region), stretching of the triple helix is the predominant mechanism of deformation [164, 406, 415, 470] and corresponds to the gradual removal of a macroscopic crimp in the collagen fibrils and this is visible in the light microscope [118, 162, 165, 176, 246, 298, 332, 395, 420, 490, 558]. Elastin is largely responsible for the mechanical events represented by the first region of the curve [380]. Elastin exhibits properties typical of amorphous polymers; it is a soft, rubbery solid when wet, but a brittle glassy solid when dry [33]. The crimp has been shown to act as a buffer or a "shock absorber" within the tendon, permitting small longitudinal elongation of individual fibrils without damage to the tissue [38, 246, 429]. In real life, the tendons on the outside of the bend are stretched until the wave pattern disappears, and thus, provide a highly efficient "safety measure" for tendons to resist sudden, possibly hazardous tensile strains subjected to them [38, 429].

At strains typically beyond 2%, the low modulus of the toe region gives rise to a non-linear stress-strain curve (heel region), which has been attributed to the reorientation and un-crimping of the collagen fibrils, as well as the initiation of stretching of the triple helix, the non-helical ends and the cross-links [415, 469, 470]. Once this wave pattern is straightened out, the tendon behaves like a "stiff spring" until a stress is reached at which certain chemical bonds or perhaps ground substance between the collagen fibres or other subunits, breaks. This might allow sub-fibres to move past one another [429]. X-ray studies have demonstrated lateral molecular packing of collagen molecules within fibrils, occurring as a result of the straightening of kinks. The straightening of the kinks allows an elongation of the fibrils and a resulting reduction in entropy, which provides the force acting against the elongation. The entropic forces increase as the number of kinks decreases leading to the typical upwards curvature of the stress-strain curve. When collagen is stretched beyond the heel region, most kinks are straightened out and no further extension is possible by the entropic mechanism described above. Increases in the D-period and in the gap region and relative slippage of laterally adjoining molecules along the fibre axis are also observed [262, 264, 406, 448]. The yielding mechanism involves some form of flow that occurs within the fibre, possibly inter-fibrillar slippage, which plays an important role in the tensile deformation of aligned connective tissue such as tendon [26, 282].

25.6 Changes in Tendon During Maturation and Ageing

During tendon development, maturation and ageing, alterations in composition, structure and biomechanics occur. Changes in collagen and elastin are associated with intermolecular cross-linking and side-chain modifications [36], which would increase the rigidity of the

tissue by making the fibres brittle [510, 517, 530] and ultimately bringing about deleterious effects to the optimal functioning of the locomotive system [40]. Understanding of the molecular and biophysical mechanisms involved would enable us to address the adverse effects of ageing and enhance the chances for a successful implantation.

Soft connective tissues attain an increasing tensile strength and strain at maximum load [124–126, 200, 348, 435, 534] as well as thermal stability [14, 122] with age that depends on a changed pattern of cross-linking of collagen [36, 315, 536]. The cross-link profiles are to a certain extent tissue-specific and consequently different tissues change with age by a different mechanism depending on the extent of lysyl hydroxylation [40]. The majority of tendons and ligaments contain both the stable keto-imine and the aldimine immature cross-links [315]. The ratio depends upon the particular function of the tissue, in particular whether there is tension on the tissue. Ligaments therefore, contain mainly keto-imine cross-links, whilst tendons have higher aldimine content [11, 33, 40].

The properties of the tissue can also be affected by the different collagen types that are present. At the early stages of development, for example, removal of type XIV collagen from the fibril surface has been documented to influence assembly and growth of fibrils [576]. It has also been shown that during ageing, type III content is decreased, whilst type I collagen content is increased [131, 214, 345, 496]. Rat tail tendon, a tissue that has been shown to comprise of mainly type I collagen (>95%) [356, 476] and readily soluble under mild acidic conditions [89], if extracted from animals of 6-month old, could contain up to 43% type III collagen [132]. In a similar manner, type III collagen content in bovine, human or porcine skin has been shown to range from 5 to 40% [87, 90, 92, 120, 149, 309, 494, 516, 568]. It has been reported previously that type I and III collagens are differentially lost during purification and precipitation procedures [196].

The mean diameter of collagen fibrils increased with age and was also associated with the content of non-collagenous proteins. Proteoglycans associated with the surface of collagen fibres in rat tail tendon was demonstrated to change throughout life. Dermatan sulphate was the main glycosaminoglycan in the mature tendon, whereas chondroitin sulphate and hyaluronic acid were preponderant in foetal tissue [85, 454, 459, 460].

25.7 Vascular Supply of Tendons

During development, tendons are highly cellular and metabolically active [399], whilst mature tendons are poorly vascularised [5, 451]. The vascular supply of tendons has been shown to depend on the number of arteries that supply the tendon [93, 381, 451], the shape of the tendon [421], even on the locality of tendons in regards to the sheath [60, 94]. Tendons receive their blood supply via the intrinsic system at the musculotendinous and osteotendinous junctions [5], whereas the paratenon or the synovial sheath contributes to the extrinsic system [81, 371, 462]. Tendons nutrition and vascular supply is more reliant on synovial fluid diffusion than vascular perfusion [157]. Blood vessels from the musculotendinous and osteotendinous junctions do not extend to the full length of the tendon [81]. Branches from the synovial sheaths [371], called vincula (mesotenon), contribute to tendon nutrition, reach the visceral synovial layer and form vascular plexus [254, 339]. Vessels from the vascular plexus supplies the tendon and penetrates the epitenon and into endotenon septae. In tendons without synovial sheath, the paratenon provides the extrinsic component. Blood vessels penetrates the paratenon and then, similarly into epitenon to the endotenon septae [426]. Vessels are generally arranged longitudinally within the tendon, passing around the collagen fibre bundles in the endotenon, a sheet of loose connective tissue contiguous with the external layer or epitenon [60, 399]. In the hand tendons, the very few found straight vessels tend to be curved allowing them to straighten out during tendon movement [60, 495].

25.8 Tendon Healing

Tendon injury occurs due to acute trauma or inflammation of either the tendon itself or the surrounding tissues. A variety of conditions ranging from inflammation to complete tendon rupture may be brought about. Injury to these structures can cause significant joint instability, resulting in injury to other tissues and the development of degenerative joint diseases. Tendon healing takes place in three steps. Firstly, inflammatory cells migrate to the injury site, phagocytise necrotic tissue and clot (inflammation phase). Following that, fibroblasts proliferate at the injury site and synthesise and deposit collagen and

other components of the extracellular matrix (scarring phase). Finally, newly produced collagen fibres align along the longitudinal axis of the tendon (remodelling phase) [23, 178, 179, 181]. Two mechanisms of tendon healing have been proposed. The extrinsic mechanism of tendon healing theory proposes that fibroblasts and inflammatory cells from tendon sheath and surrounding soft tissue invade and proliferate to lay down a new collagen matrix, whilst the intrinsic mechanism of tendon repair occurs through the resident tenocytes [43, 179, 338]. Provided that there is adequate blood supply and nutrition, damaged tendons are capable of intrinsic healing; synovial and extracellular fluids provide nutrition and contribute to tendon healing [324–326]. It is likely that both mechanisms occur, although the involvement of external cells depends on the site of injury and vascular perfusion [157]. The extrinsic mechanism of healing however leads to increased amounts of randomly organised collagen deposition at the site of injury. Scar formation and adhesions formed between the tendon and its surroundings are the most common complication after tendon repair. Such complications compromise the material properties of the reparative tendon tissue [344, 378], interfere with the tendon motion and gliding and ultimately result in compromised tendon function [43, 146, 412, 466]. Moreover, injuries that lacerate the tendon also disrupt the nutritional systems that feed the tendon and sustain the repair effort [43]. It is therefore of paramount importance to modulate the healing process by enhancing the intrinsic pathway and suppressing the extrinsic pathway to restore tendon function [498, 499]. Early physiotherapy and motion has been shown to enhance vascularity and facilitate collagen realignment and faster tendon healing, resulting in better gliding and higher tensile strength [178, 278, 480]. Early motion has been shown to be beneficial even for tendon autografts [272, 467]. Unfortunately, exogenous cells predominate over endogenous tenocytes, allowing the surrounding tissues to attach to the repair site, resulting in adhesion formation, which possess the major clinical challenge. Despite remodelling, the biochemical and mechanical properties of healed tendon will never match those of intact tendon [462].

25.9 Non-Invasive Strategies

Non-invasive pharmacological strategies towards the prevention of ligament and tendon injuries associated with inflammatory joint diseases have been introduced and advocate the use of slow acting antirheumatic drugs (SAARDs) or non-steroidal anti-inflammatory drugs (NSAIDs) to restrain inflammation and preserve tissue integrity. In fact, it has been shown that such substances increase the mechanical properties of various connective tissues and may alter the healing strength by decreasing collagen content [158, 532, 533, 535]. SAARDs, in combination with steroids, are used at the early stages of the disease; there is only a small window of opportunity (2 years) in which to get the disease into remission before irreversible damage is done to the joints. NSAIDs also work within a few days, but patients' response varies widely and their side effects, particularly on the stomach and kidney, have limited their use [15, 16, 128]. Moreover, such agents have an inhibitory effect on proteoglycan synthesis and cell proliferation [430]. It has also been argued whether they contribute at tendon regeneration at all [341]. Nutraceutical supplements including creatine and ephedrine alkaloids (e.g. ephedra) have been associated with side effects and lack rigorous quality assurance to warrant their use [507]. Recently, it was suggested that upregulation of collagen synthesis in vitro by a combination of nutraceuticals glucosamine and chondroitin sulphate may accelerate tissue repair and diminish the probability of osteoarthritic development [316].

Growth factors have drawn increasing interest in the field of tendon injury and repair, but still their in vivo potential has not yet proven successful [308] and there is no FDA approved treatment currently available. Growth factors are cell-secreted proteins that regulate cellular functions, including the normal processes of development and tissue repair. The initial stages of repair include the formation of weak tissue that is not capable to support tensile loads. Although immobilisation will enhance healing, it will inevitably result in formation of adhesions that will compromise gliding. Incorporation of growth factors has been shown to be crucial at the early stages of the repair and it is likely to result in superior repair and therefore a functional tissue. Vascular endothelial growth factor, insulin-like growth factor, platelet-derived growth factor, basic fibroblast growth factor and transforming growth factor-β have been shown to increase the mechanical properties of the tissue, to enhance intrinsic reparative processes after tendon injury, to enhance tendon healing and in general to modulate cell proliferation and to stimulate matrix synthesis [180, 194, 225, 244, 284, 354, 369, 400, 445, 504, 542, 543, 567, 571]. Despite

the overall success of growth factors to augment tendon repairs, their use is not sufficient to heal tendon injury in cases of early tendinopathy [458].

Mesenchymal stem cells (MSCs) are progenitor cells found in adult tissues and they are characterised by their extensive proliferative ability and the potential to differentiate along various lineages in response to appropriate stimuli. They are also capable to give rise to diverse tissues, including bone, cartilage, adipose, tendon and muscle. Their additional ready availability has established them as an attractive candidate progenitor cell type for tissue engineering applications [79, 101, 177, 413]. Moreover, MSCs can be transduced with retroviral and other vectors and are, thus, potential candidates to deliver somatic gene therapies for local or systemic pathologies [78]. A combination of mesenchymal adult stem cells, biological cues through growth factors and topographical cues through bioresorbable scaffolds architecture could provide a valuable tool for the treatment of difficult tendon injuries [227]. The rationale behind the use of MSCs in tendon repair lies on the limited number of cells that reside in the endotenon tissue between the collagen fascicles and adjacent to the vasculature, which might explain the limited repair process. Therefore, the use of MSCs in far greater numbers would have the potential of regenerating or repairing tendons and ligaments [428, 491]. In fact, MSCs and tenocytes have comparable proliferation rates, function, and viability and adipose-derived MSCs have been shown to proliferate fast in vitro and can be used to successfully repopulate acellularized tendon in vivo [290, 291]. Animal models and human clinical trials have demonstrated significantly improved repair biomechanics, structural, biological and biochemical characteristics and an overall efficiency in tendon repair [28, 69, 79, 198, 256, 363, 416, 432, 541, 575]. Despite early success in the field, such technologies are still not FDA approved and whether they will regenerate a functional tissue in vivo is still to be seen.

25.10 Clinical Need and Requirements

There are over 15 million tendon and ligament related injuries in USA alone with approximately 95,000 new cases per year [69, 71, 74, 163, 304, 316]. Tendon injuries may be induced due to trauma; resection related to an infiltrating tumour; or atrophy or shortening due to delayed presentation after tendon laceration [1, 239, 275, 323, 382, 508]. There is currently no efficacious therapy for enhancing the rate and/or ability of these tissues to heal [431]. As the human population ages and the life expectancy increases, tendon injuries will continue to rise putting a further physical and financial strain on the healthcare system. Surgical repairs do not fully restore function due to fibrous adhesions or failure arising from the mechanical demands placed on imperfect integrative healing at tendon-tendon or tendon-bone interfaces [58, 512]. Therefore, there is a compelling need to develop strategies for functional tendon regeneration.

Although successful replacement of skin, cartilage, urinary bladder and bone has been reported [25, 276, 277, 373, 401, 424, 518], there is no clinical acceptable tendon substitute available yet. The main challenges in tendon engineering include fabrication of complex designs that will mimic the native architecture of different tendons. The construct should possess adequate mechanical strength to withhold constant loads during healing. It is also important to interact with the surrounding tissues, stimulate healing and ultimately functional neotissue formation. The scaffold should also be immunologically acceptable and can be vascularised and/or permits nutrients to diffuse easily. In the case of an acellular tendon substitute, the appropriate type of host cells must be stimulated to proliferate, infiltrate the construct and lay new structural matrix to ensure remodelling as the material degrades. The outer surface morphology of the engineered tendon must be smooth to eliminate friction with the surrounding tissues at the site of implantation. Repair of damaged tendons requires the formation of crimped neo-tendon that has similar mechanical properties to those of normal tendon [187]. In many soft connective tissues, the mechanical properties are influenced by fibre alignment. The ability to efficiently generate aligned mats would be a valuable tool in studying the biomechanics of normal, developing, and healing native tissues and artificial tissues [511]. The construct also needs to be isolated from the surrounding tissues to prevent ingrowth of adhesions and allow gliding. Smooth and efficient tendon gliding, in addition to excursion are essential to ensure the reconstruction of a biomechanically stable construct. The tendon substitute should consist of the tendon substance/bundle and if possible, a tendon sheath equivalent to ensure free, frictionless movements. Matching tendon size and shape will prevent catching of the tendon within the fibro-osseous canal. In

addition, sufficient strength to allow end-to-end tendon suturing, weaving to the native tendon or insertion directly onto bone is critical. The force to failure for the interface between native and substitute tendon should be equivalent to that of primary tendon repair, to allow early motion and stimulation of intrinsic healing mechanisms. Early joint mobilisation is important to reduce adhesions (extrinsic healing), increase tensile strength and prevent subsequent postoperative joint stiffness. Hence, if the mechanical strength decreases well below that of a direct suture repair after implantation, postoperative therapy must be modified accordingly to prevent rupture, while maximising intrinsic tendon healing and avoidance of adhesions. The construct should also enhance remodelling once implanted, as an exceptionally mobile structure and must withstand constant mechanical loads during healing. Since the vascularity of tendons is relatively poor, the material degradation products must be biocompatible and if possible, to follow the body's natural mechanisms, as the removal process would be slower when compared to other tissues such as muscle or bone. Ultimately, the construct should be a living substitute that will "read" the requirements of the implantation. For example, if the tendon substitute is designed for a child, it should proportionally increase in length, diameter and strength as the child grows to avoid contracture that will lead to further surgery. In contrast, if the tendon scaffold is intended for developed adults, it is crucial that it does not lengthen or shorten inappropriately. The neotissue should allow active range of movement, without compromising the opposing movements or the overall limb function.

25.11 Approaches Towards Tendon Repair: Autografts, Allografts and Xenografts

Tendon injury remains a common cause of morbidity throughout the world. Although non-invasive approaches have been investigated, they have been found wanted. Efforts to treat tendon injuries have primarily focused on improving surgical techniques to provide a more robust repair and essentially a better quality of life. Tendon autografts and allografts constitute the gold standard when it comes to tendon repair. The choice of an ideal graft is based on a number of factors including: (1) graft availability and accessibility; (2) should result in little donor-site morbidity; (3) should allow immediate rigid fixation; (4) should undergo rapid healing; (5) must reproduce the structural and mechanical properties of the native tissue [186]. The utilisation of tendon autografts has been recommended during: (1) a continuing or an established disease processes (e.g. rheumatoid arthritis); (2) poor tendon quality to achieve a satisfactory repair; (3) tendon shortening (permanent retraction) or atrophy due to delayed presentation; (4) tendon loss due to trauma or disease (e.g. cancer resection); and (5) scarring or injury of the tendon bed [280]. The use of autografts has been advocated repeatedly due to superior functional results. Tendon autograft has been shown to be safe, effective and acceptable choice for posterior cruciate ligament reconstruction [88, 565]. Anterior cruciate ligament reconstruction, using the middle third of the quadriceps tendon without a patellar bone block and absorbable tibial and femoral cross-pin fixation, demonstrated very good clinical results [18]. The choice of autografts depends on the site and size of tendon to be reconstructed and on the availability of donor tendon. Donor sites are selected carefully to minimise morbidity and loss of function. Palmaris longus and plantaris longus tendon autografts are commonly employed for tendon reconstruction in the digit, hand and wrist, as they are similar in size and proven to be reliable. Digital flexor tendon reconstruction is commonly carried out in two stages [235]. The first stage involves introduction of a temporary silicon rod to prepare a new tendon tunnel and the reconstruction of the pulley system. This is then followed by rod removal and repair with autograft [156, 393, 481, 487]. Extensor tendon ruptures in hands of patients suffering from rheumatoid arthritis can be treated with tendon transfers or tendon grafts [107]. Results are reasonable for tendon grafts provided that the duration between rupture and surgery is short and muscle contractures are not severe. In patients with extensor mechanism ruptures secondary to rheumatoid arthritis, early surgery using autogenous palmaris longus tendon as interposition grafts for extensor tendons reconstruction at the wrists level results in good functional results [55, 107]. However, the quantity and quality of autografts needed may be insufficient in severe injury. In ruptured patella tendon reconstruction, a semitendinosus-gracilis graft has been used [195, 245, 303]. Porcine small-intestinal submucosa construct was characterised by earlier neovascularisation, increased transforming growth factor-β levels, increased

collagen deposition and greater intrinsic repair strength over to both autograft and suture material indicating a great potential for flexor tendon graft substitution [369]. Patella and Achilles tendons allografts have been used in knee, tendon and anterior cruciate ligament reconstructions with relatively limited success [20, 119, 446, 578]. In contrast, recent clinical study demonstrated that double-loop hamstring tendon autograft and Achilles tendon allograft for posterior cruciate ligament were equivalent [6]. Similarly proportional results were obtained from hamstring autograft or tibialis anterior allograft for the reconstruction of shoulder anterior capsule [7]. Moreover, another study demonstrated that patients treated with allograft patients experienced less pain, better functionality and fewer activity limitations during the follow-up period [410]. Furthermore, the use of allografts has been supported due to economical reasons [109]. However, in general, direct comparison between autografts and allografts demonstrates the superiority of the autografts. Indeed, anterior cruciate ligament reconstruction with bone–patellar tendon–bone autograft was favoured over the bone–patellar tendon–bone counterpart allograft [289]. In fact, bone–patellar tendon–bone autograft for anterior cruciate ligament reconstruction is the gold standard and remains the benchmark against any other method [452]. Similarly, allograft remodelling was delayed and of inferior stability and mechanical function over the correspondent autograft in anterior cruciate ligament reconstruction in sheep model [450]. Cell-seeded patellar tendon allografts supported cell proliferation creating viable tissue-engineered grafts potentially useful for anterior cruciate ligament reconstruction [82]. Xenografts have also been employed in tendon and ligament reconstruction. Their use has been promoted because they do not compromise patient's tissues and have identical structure as the tissue to be replaced. Early drawbacks [330, 362, 437, 523] have now been addressed [30, 250] and recent data demonstrate normal tissue ingrowth in several animal models [185, 358, 486, 557]. Tendon autografts and allografts are currently the most commonly used substitutes to reconstruct injured ligaments and tendons. However, allografts still face the risk of potential transmission of infectious diseases [24, 111, 151, 243] and autografts present the disadvantages of creating a secondary morbid site during harvesting. Development of tissue engineering strategies could provide a valuable alternative to transplantation crisis.

25.12 Approaches Towards Tendon Repair: Synthetic and Natural Biomaterials

Tissue engineering began with the use of materials designed to interact with the body and encourage tissue regeneration, replacement, or restoration of function. The scaffold material(s) and the cell-scaffold interactions are the major contestants in any tissue-engineered assembly. A porous scaffold is generally employed upon which cells will attach, proliferate and maintain their differentiated function. It is essential the scaffold material to provide mechanical stability/integrity and a template for the three-dimensional organisation for the developing tissue [53, 103, 213, 215, 236, 269, 305, 461, 555]. Micro- and nano- fabrication technologies of polymers have tremendous potential in the field of tissue engineering since they can achieve topographical, spatial, chemical and immunological control over cells and therefore create more functional tissue engineering constructs [86, 219, 236, 271, 305, 483]. For example, aligned fibres or channels on the surface of fibres have been shown to provide topographical cues that enhance directional growth of the neotissue [32, 105, 112, 501]. The principles of tissue engineering have been applied to virtually every single organ-system in the body [72, 300]. Depending on the application, the properties of the device may vary, but it should (a) be composed of a biocompatible material, which does not have the potential to elicit an immunological response or clinically detectable primary or secondary foreign body reaction; (b) have mechanical properties to match those of the tissues at the site of implantation; (c) be easily processed to form end-products of different textures; (d) closely imitate the native topography/architecture of extracellular matrix assemblies; and (e) offer opportunities for functionalisation to either enhance cell attachment and growth or delivery of therapeutic molecules. Currently, there are various polymers available that stimulate repair or regeneration of tissues in vivo [236, 237, 318, 370]. For tendon replacement, it has been postulated that synthetic devices will replace or reinforce biological materials and would act as scaffolds upon which fibrous tissue could be induced to proliferate and form ligamentous and tendinous tissues. Moreover, tissue-engineered tendon scaffolds have the potential to significantly improve the treatment of tendon and ligament injuries, especially those associated

with degenerative (e.g. rheumatoid arthritis) [55, 107, 367, 372] and congenital [382, 540] conditions, where autograft or allograft tissue might not be available in sufficient quantity for reconstruction. Tendon substitutes may also reduce the need for composite flaps (with donor tendons) for reconstruction [1, 239, 275], and therefore reduce morbidity. Finally, a series of appropriate experiments in conjugation with biological assessment [137] and animal models [80] is essential for the optimisation of the construct properties and final clinical application of tissue-engineered tendons and ligaments.

Materials tested include synthetic constructs such as polylactic acid [166], carbon-polylactic acid polymer composite [19, 335], polytetrafluoroethylene [63, 312], polyethylene terephthalate [12, 313], poly(glycolic acid) and Dacron composite [91, 402, 436], nylon [194, 531], polypropylene [228, 388], polyglycolide/polyglyconate and polydioxanone [335, 539] and polyglactin [434]. Panacryl has also been used and results demonstrate that it has more durable mechanical features and may well be suited to long-term tissue apposition, such as tendon repair or arthroplasty [159, 478]. Polytetrafluoroethylene and Dacron vascular grafts continue to be utilised in order to bypass a segment of diseased artery [91, 312, 402]. Although dacron prosthesis has been shown to have acceptable mechanical properties [405] and be capable of inducing and supporting tissue encapsulation and ingrowth, it was demonstrated that it does not allow the functional orientation of neotissue [21]. Dacron prosthesis has also failed to protect patellar tendon graft during neovascularisation [17]. Polyglycolic acid fibres, mimicking human extensor tendon complex, were seeded with human foetal extensor tenocytes. Constructs, that were mechanical stimulated, promoted collagen alignment, more mature collagen fibril structure with D-band periodicity and stronger mechanical properties, suggesting that that mechanical loading might be an optimal niche for engineering functional extensor tendon [544]. Similarly, polyglycolic acid fibres seeded with tenocytes tendon generated a tendon tissue during in vitro culture. However, the engineered tissue was thinner and significantly weaker than the natural tendon [77]. Nylon replacement of Achilles tendon did not lead to successful production of a new functional substitute [161]. Polypropylene braid device has demonstrated superior mechanical properties, however histological evaluation revealed a disordered fibrous capsule [350]. Carbon fibres and carbon fibres augmented with soft tissue have also been employed in tendon engineering. The results have demonstrated that although difficult, it is possible to obtain acceptable mechanical and structural properties; extrinsic and intrinsic fibroblastic activity; and ultimately a functional neotissue formation [152, 266, 292, 396, 506, 520]. However, association of carbon with skin diseases could limit its use in biomaterials field [297, 464, 477]. In general, synthetic polymers used for tissue engineering applications are easily formed into required shapes, have good mechanical strength and their biodegradation can be regulated by changing their molecular weight and/or chemical composition [493]. However, synthetic materials have been associated with foreign body reaction [70, 205, 255, 343], they lack cell-recognition signals and their hydrophobic property hinders smooth cell seeding [102, 103, 489]. To this end, several synthetic scaffolds (e.g. poly-ε-caprolactone, poly-D, L-lactide-co-glycolide, polyglycolic acid) have been coated with biological active molecules, such as collagen, gelatin, hyaluronic acid, growth factors and RGD peptides sequences, to modulate cell attachment, to decrease adhesions, to accelerate tendon healing and to improve the quality of repair tissue [127, 133, 134, 194, 201, 202, 238, 311, 329, 342, 377, 414, 443, 449, 497, 527, 566, 572]. Moreover, poly-lactide-co-glycolide scaffolds loaded with allogenic bone marrow stromal cells have the potential to regenerate and repair gap defect of Achilles tendon in the rabbit model and to effectively restore both structure and function [387]. Despite commendable efforts in the field, no artificial materials have been able to meet the requirement for functional tissue remodelling [31]. Moreover, there are still concerns; scaffolds that will remain for longer time periods in the body may impair tissue regeneration. Non-degradable synthetic materials may become harmful due to mechanical impingement or infection and require a second operation for removal [357], whilst the degradation products of biodegradable synthetic materials could be deleterious to the surrounding tissue and may cause suppression of cell growth [3, 209, 216]. Natural polymers possess several inherent advantages such as ability to present receptor-binding ligands to cells; susceptibility to cell initiated proteolytic degradation; and natural remodelling [29, 281, 337, 370, 519]. For these reasons, natural polymers (e.g. chitosan, silk and collagen) have been used extensively for tissue engineering applications.

Chitosan and chitosan-hyaluronic acid scaffolds have been employed for tendon and ligament reconstruction. In vitro studies demonstrated acceptable biodegradability and biocompatibility, whilst in vivo experimentation showed that these scaffolds enhanced collagen type I production and improved the mechanical strength in the regenerated tissues [32, 171, 172, 333, 334]. Fibrin is a biopolymer involved in the natural blood clotting process. In the presence of thrombin, spontaneous fibrillogenesis takes place that give rise to linear fibrils that can undergo lateral association to form fibres with diameter range from 10 to 200 nm. Due to its excellent biocompatibility, biodegradability, injectability, the presence of several extracellular matrix proteins, such as fibronectin, that enhance cell adhesion and proliferation, cross-linking ability that offers control over mechanical properties and degradation rate in the body and its association with bioactive molecules as a carrier vehicle, fibrin has been investigated for various tissue engineering applications [4, 370, 563]. It has been demonstrated that fibrin glue promotes smooth gliding surface and restricts adhesion after repair of flexor tendon [169]. However, treatment of fresh Achilles tendon ruptures with fibrin glue, although allowed postoperatively the same level of activity for leisure sports as preoperatively; competitive athletes should expect loss of performance. Similarly, use of fibrin on healing of rat supraspinatus tendon defects promoted collagen organisation over time; however biomechanical analysis although was improved over time, did not reach the properties of normal tendon [505]. On the other hand, biomechanical and histological analysis on healing ruptures in the Achilles tendon of rabbits revealed comparable characteristics among repair using sutures or fibrin glue [327, 503]. Moreover, ruptured Achilles tendons of patients treated with fibrin demonstrated significant better functional and cosmetic results over patients treated with sutures [218, 423]. The use of silk as suture material for tendon injuries has long been recognised [224, 270]. Silk is an example of natural material with incredible elasticity, strength and robustness unmatched by many synthetic materials [67]. In addition to its superior mechanical properties, silk is benefited by excellent biocompatible and biodegradability and therefore has found applications in several tissue engineering applications [528]. Silk sutures immobilised with RGD peptide sequence exhibited increased cell attachment and adhesion, whilst increased collagen type I and decorin transcription levels were observed [260]. Silk-based scaffold has also been shown to support attachment, expansion and differentiation of adult human progenitor bone marrow stromal cells and to express collagen types I and III and tenascin-C all characteristics of the ligament [10]. Moreover, silk-based scaffolds offer opportunities for sustained drug release [546, 547]. Early work however indicated that silk replacement of the excised Achilles tendon did not lead to successful production of a new substitute [161]. Moreover, possible allergic reactions and misconception about its biodegradability in vivo have restricted its use in tissue engineering [9]. Given that tendons are primarily composed of type I collagen, collagen-based biomaterials have extensively used for tendon and ligament reconstruction.

25.13 Approaches Towards Tendon Repair: Collagen-based Biomaterials

Collagen-based biomaterials of different physical forms (fibres, films, sponges and hydro-gels) are widely used for soft and hard tissue repair [106, 167, 307, 374, 375, 390, 419, 587]. The attractiveness of collagen as a biomaterial rests largely on the view that it is a natural material and is therefore received by the body as a normal constituent rather than a foreign matter [167]. Although potential allergic reactions to collagen as a foreign protein can be induced [328, 552], advancements in purification methods and analytical assays have essentially assured minimal immunogenicity [319]. Tissues rich in fibrous collagen such as skin and tendon are generally used to extract collagen. Dilute acidic solutions are used to break intermolecular cross-links of the aldimine type, whilst proteolytic enzymes, such as pepsin, are used to cleave the more stable cross-links of the keto-imine type. Limited pepsin digestion will cleave only the non-triple-helical C- and N-telopeptides, leaving the triple-helical molecule intact [167, 182, 314, 328, 581], unless it is partially unfolded, molten or broken [66]. Collagen possesses many desirable features making in an excellent choice as a biomaterial, among which are its high tensile strength, controllable cross-linking, inexpensive and easy to prepare in large reproducible quantities, high biodegradability and low

antigenicity [165, 167, 273, 398, 522]. Furthermore, its association with growth factors [340, 392, 485, 561, 574], proteoglycans and/or glycosaminoglycans [123, 403, 454] and drug/therapeutic molecules loading capabilities have enhanced its use in tissue engineering applications [170, 307, 317, 368, 569]. Its ability to promote cellular attachment and growth and consequently tissue healing and regeneration is well established [2, 64, 148, 281, 482, 556].

Collagen, under appropriate conditions of temperature, pH, ionic strength, collagen concentration and composition and presence of other connective tissue macromolecules will spontaneously self-assemble to form microscopic fibrils, fibril bundles and macroscopic fibres that exhibit D periodic banding patterns virtually indistinguishable from native fibres when examined by electron microscopy [62, 96, 129, 155, 191–193, 226, 310, 361, 408, 476, 548, 559, 579, 581–583, 586]. It is believed that type I collagen contains all the necessary structural information for its self-assembly into fibres in vitro, except maybe for some tissue-specific factors [38, 189, 293, 310, 346]. The ability of fibrous collagens to form striated fibrils is believed to involve specific charge-charge and hydrophobic interactions. Indeed, collagen fibril formation is an endothermic process involving hydrophobic and electrostatic interactions between adjacent molecules, with accompanying release of associated structured water. The fibril formation process is promoted by agents that increase the disorder of the bulk water, such as solution temperature increments or the addition of ions that "break" water structure, whilst fibril formation is retarded by factors that increase the order of the bulk water, such as decrease of the solution temperature or addition of ions that "make" water structure [129, 366]. Although the mechanisms for fibril formation in vitro and in vivo may be different, the final products have similar banding patterns [468]. The kinetics of the in vitro collagen fibril assembly can be divided into three stages: a lag phase during which the solution does not increase its turbidity; a growth period characterised by a sigmoid increase of the solution turbidity due to the appearance of collagen fibrils; and a plateau phase in which turbidity again remains constant. It has been shown that the first-formed aggregates are dimeric, with the most prevalent dimer having a maximal stagger (D = 67 nm) between constituent molecules [52, 62, 155, 301, 475, 548, 559]. The molecule contains three structural domains, the amino- and the carboxyl-terminal extra helical regions (the telopeptides) and the major triple helical rod-like domain. Comparison of the behaviour of protease-modified collagen and native collagen suggests that both the N-terminal and the distal region of the C-terminal telopeptides are important in the initial nucleation, while the C-terminal proximal moiety plays a relevant role in the growth phase [208]. Furthermore, it has been shown that the propeptides limit association of collagen molecules [286] in solution and may therefore play an important role in modulating collagen fibrillogenesis until they are proteolytically removed [44].

The principle of self-assembly, with excellent engineering craftsmanship, was used to fabricate extruded collagen fibres that closely imitate extracellular matrix assemblies [187, 262, 433, 549, 579]. Briefly, a collagen solution is extruded at constant rate through a thin laboratory tube into a series of neutral pH phosphate buffers maintained at 37°C. The collagen solution, upon contact with the phosphate-based formation buffer, instantly self-assembles to give rise to insoluble fibres (Fig. 25.8). The surface of the fibres is characterised by crevices and ridges running parallel to the fibre axis (Fig. 25.9a), whilst a compact fibrous-like interfibre space is apparent after detailed scanning electron microscopy study (Fig. 25.9b). Such nano-textured external characteristics have been shown to facilitate directional cell migration that it is essential for neotissue formation [112]. In general, fibres with wet diameter ranging from 50 to 650 μm have been fabricated [264, 408, 584, 585] and ultrastructural analysis demonstrated that they are composed of aligned collagen fibrils with diameter distributions similar to native collagen fibres [408, 581, 584]. Furthermore, recent optical analysis using second harmonic generation demonstrated that these fibres possess high level of crystallinity and structural alignment that is characterised by strong second harmonic generation signals in the presence of intense laser light [579]. Although the intensity of the signals was lower in comparison to native tissues as has been observed previously [117, 484], supplementary ultrastructural analysis suggested that the intensity difference between the in vitro and in vivo assemblies was attributed to the low and high respectively supramolecular configuration order.

Similar to native fibres, self-assembled fibres exhibited stress-strain curves dependant on the water content and/or cross-linking extent [585]; an s-shape curve was obtained in dry state, whilst a j-shape curve was

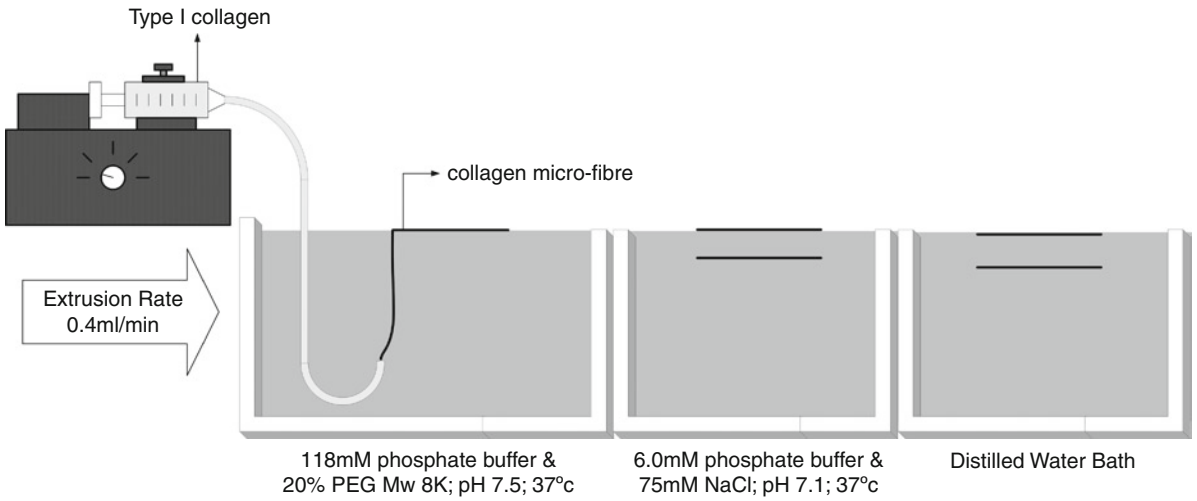

Fig. 25.8 Process diagram of the fabrication of extruded collagen fibres

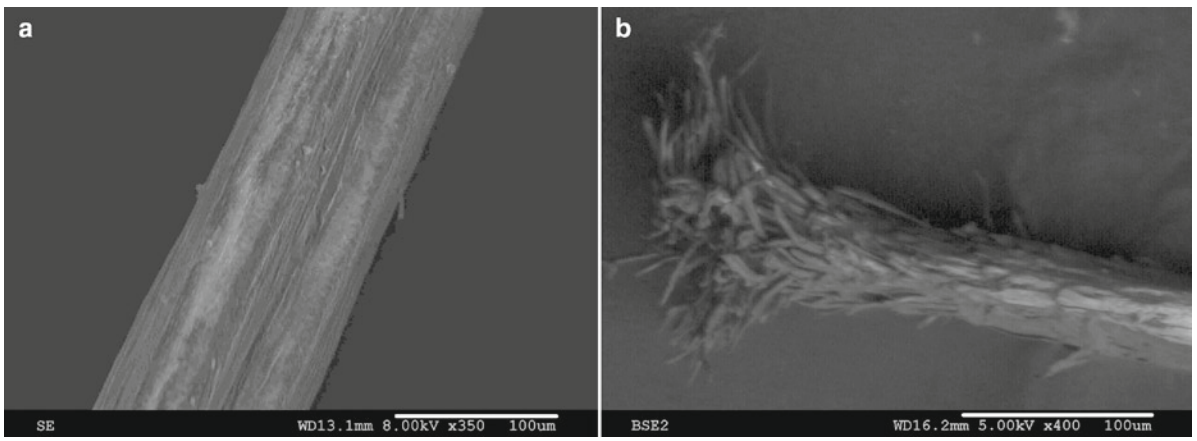

Fig. 25.9 Scanning electron micrograph of pepsin soluble bovine Achilles tendon derived fibres. (**a**) The surface morphology is characterised by undulations that enhance cell attachment and directional growth. (**b**) A fibrous inter-fibre space similar to native tendons is apparent

found under fully hydration conditions [584, 585]. An intermediate stress-strain curve has also been reported and is characterised by an s-shape curve up to the knee point, followed by a j-shape curve that persists until failure [585]. The effect of moisture content on stress-strain curve shape is due to the plasticising action of water that allows more freedom of movement to fibres and fibrils; thus allowing greater re-orientation of fibres and fibrils during stretching. In hydrated collagenous tissue the occurrence of a j-shaped curve is often associated with collagen fibres becoming more aligned along the strain axis during stretching [530]. The cross-linking density has been shown to constitute an important modulating factor because it affects the water uptake of the fibres [585]. Others have shown that water content plays an important role in determining the mechanical properties of collagen fibres [22] and cross-linking density influences the tensile deformation behaviour [306]; therefore, the higher the density of cross-links, the less water can be bound [100]. Typical s-shape curves (dry fibres/low swelling) have been reported for crystalline polymers that yield and undergo plastic flow [262, 408, 545]. The yielding mechanism involves some form of flow that occurs within the fibre, possibly inter-fibrillar slippage, which plays an important role in the tensile deformation of

aligned connective tissue such as tendon [282]. Similar curves have been documented for tendon [429], ligaments [210], extruded collagen fibres [84, 184, 406] and nano-fibrous meshes [201, 202, 295]; whilst analogous j-shape stress-strain curves (wet fibres/high swelling) have been reported for re-hydrated reformed collagen fibres [262, 408], pericardium tissue [173] and rat tail tendon [184]. The curves are consisted of a small toe region, a region of sharply increasing stress up to a knee point where the gradient of the curve reduced, followed by a long region of constant gradient which persisted until failure. A diameter dependent variation of the stress-strain curves has been documented; thin fibres demonstrate high stress/low strain graphs and short toe region, whilst thick fibres yield low stress to strain ratio graphs and a long toe region [582, 584, 585]. The slope of the stress-strain curve increases with strain; this is a characteristic of connective tissues [22, 164, 545] and has been attributed to the progressive orientation of the collagen fibres during straining [26, 415]. At low strains, the resistance to deformation is low, since the fibres themselves are not being stretched but are being aligned along the axis of applied tension. In native tissues, the region of low strain (toe region) corresponds to the gradual removal of the macroscopic crimp of the collagen fibrils that allows small longitudinal elongation of individual fibrils without damage to the tissue [38, 246, 429]. Although macroscopic crimp has extensively been studied for native tissues [118, 162, 165, 176, 246, 298, 332, 395, 420, 490, 558], its presence on extruded collagen fibres, although possible, is still unclear.

Tendons function as structural components and play an important mechanical role in linking and transmitting forces generated by muscle to bone, in order to mobilise and/or stabilise the joints that they cross. It is therefore essential that a tendon construct be able to withstand great mechanical loads. Natural cross-linking, the formation of covalent inter- and intramolecular bonds between proteins, is utilised by nature to create new entities with properties completely different from the original monomeric form [34]. The primary function of native cross-linking is to impart desired mechanical characteristics and proteolytic resistance on the collagen fibres in connective tissue [40, 76, 167, 359]. Lysyl oxidase is secreted from fibrogenic cells as a 50-kDa pro-enzyme that is proteolytically processed to the mature enzyme in the extracellular space. Inhibition of lysyl oxidase action toward collagen molecules results in the accumulation and ultimate proteolytic degradation of soluble collagen monomers, thus preventing the formation of insoluble collagen fibres [524]. The participation of this enzyme is therefore critical to the development and repair of connective tissues [391]. However, the lysyl oxidase mediated cross-linking does not occur in vitro and consequently collagen construct lack sufficient strength and may disintegrate upon handling or collapse under the pressure from surrounding tissue upon implantation. Incorporation of polymers [582, 583], polysaccharides [583] and biological macromolecules [406] has been used through the years to enhance the mechanical properties of the produced fibres. Although the mechanical properties were increased, the stability brought about is not sufficient for a successful tendon reconstruction. To this end, a number of cross-linking approaches (chemical, physical and biological) have been investigated through the years to control mechanical and thermal properties, biological stability, the residence time in the body and to some extent the immunogenicity and antigenicity of the device [97, 104, 114, 167, 168, 286, 349, 386, 398, 422, 550, 573, 585]. For extruded collagen fibres, a variety of cross-linking approaches has been evaluated [84, 112, 113, 140, 141, 184, 264, 285–288, 408, 545, 585] and overall, it is clear that these fibres provide a mechanically stable construct upon which tendon fibroblasts can attach, migrate and proliferate and therefore may be used to bridge gaps in ruptured, lacerated or surgically transected tendons. Indeed, when these fibres were used for tendon prosthesis, they rapidly resorbed and allowed the formation of aligned connective tissue similar to the one from the autogenous tendon graft and almost identical to the normal tendon [263, 549]. Similarly, when the scaffold was used for anterior cruciate ligament replacement, it was completely remodelled into the host tissue by 12 weeks [84], whilst in other report it was shown that at 20 weeks post-implantation the construct was completely degraded and was replaced by organised, crimped neoligament tissue [141]. Moreover collagen fibres avoid problems associated with long-term foreign body implantation or granuloma that could interfere with the smooth gliding of tendons repairs [84, 187, 262, 264, 406, 545].

Biomaterials design has evolved from basic constructs that match structural and mechanical properties, to biofunctional materials that aim to incorporate instructive signals into scaffolds and to modulate

cellular functions such as proliferation, differentiation and morphogenesis [86, 302]. It has been demonstrated that transglutaminase, the solely biological cross-linking approach, can be used to stabilise collagen scaffolds [98, 100, 174, 379, 383], albeit limited [585, 587]. Tissue transglutaminase belongs to a family of enzymes that catalyse several post-translational modifications of proteins by forming inter- and intra- molecular bonds; the process results in the formation of stable covalently cross-linked proteins in the extracellular matrix in a Ca^{+2} dependent manner [46, 83, 212, 229, 321]. We have been able to use the resultant covalent γ-glutamyl-ε-lysine isopeptide bond to incorporate peptides into the molecular structure [587], which indicates the functionalisation potential of transglutaminase in the biomaterials field that can be used to deliver therapeutic molecules at the site of the injury.

25.14 Conclusions

It is believed that the imitation of nature through tissue engineering will be able to address the patient need for functional tissue reconstruction. However, to imitate nature successful, we need to understand, firstly, the basic biology of the tissues of interest; only then we will be able to develop strategies for functional engineering of living tissue substitutes that will enable tissue repair and remodelling. Advancements in biochemistry, biology and clinical practice have provided invaluable insights in tendon healing and repair and therefore we now have the appropriate information for the fabrication of the ultimate constructs that will enable the successful reconstruction of functional tendon. Synthetic and naturally occurring polymers are an important element in new strategies for producing engineered tissue. Although synthetic polymers minimise batch-to-batch variations and can be tailored made to match specific applications, natural occurring polymers minimise chances of cytotoxicity, are characterised by high biocompatibility and offer opportunities for functionalisation. We believe that polymer chemistry and novel fabrication techniques will likely lead to the development of more effective tissue substitutes that will actively interact with the tissue to repair it. Incorporation and delivery of bioactive and therapeutic molecules, without loss of activity, at the site of the injury will enhance cell attachment and proliferation; guide cellular activity; and ultimately produce a new extracellular matrix that will support a functional neotissue growth.

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Tissue Engineering in Oral and Maxillofacial Surgery (OMFS)

26

Wolfgang C. Puelacher

26.1 Introduction

The technologies of biomedical tissue engineering represent a rapidly growing field in regenerative medicine and basic science [53, 102].

Tissue engineering has been defined as the application of the principles and methods of engineering and life sciences towards the fundamental understanding of structure and functional relationship in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve function (National Science Foundation, Lake Tahoe, CA, 1987).

In 1988, Vacanti [101] described four essential observations as the prerequisites for successful transplantation and engraftment:

- Every tissue undergoes constant remodeling due to attrition and changes of constituent cells.
- Isolated cells tend to reform appropriate tissue structure if proper conditions are provided [32].
- Isolated cells can remodel only to a limited degree when placed as a suspension in the middle of a mature tissue without intrinsic organization and with no template to guide restructuring and reorganization [27].
- Transplanted cells will survive only if they are implanted within a few hundred microns from the nearest capillary [17, 26].

Taking into account the postulated prerequisites, cartilaginous tissue may be seen as an ideal tissue to generate using tissue engineering: cartilage basically consists of one cell type, the chondrocyte, which shows a limited regenerative capacity clinically [11, 31]. Chondrocytes can be easily isolated in great numbers, show high viability, are readily multiplied in vitro, and most importantly, there is no significant vascularization of cartilage. The chondrocytes are nourished by diffusion and exhibit a low oxygen requirement compared to other tissues. All these properties make them an ideal candidate for transplantation and survival by diffusion until successful engraftment takes place.

26.2 Aim of the Discipline

26.2.1 *From Resection to Reconstruction to Regeneration*

In oral and maxillofacial surgery (OMFS), the autologous model gold standard in reconstructing missing tissue like cartilage and bone can be achieved by the transplantation of the patient's own tissue specimen originating from different topographic locations, whereby the transplant consists of the naturally occurring variety and types of cell and intercellular three-dimensional (3D) matrix structure with or without vascular supply. Patients requiring maxillofacial reconstruction with autografts still experience significant morbidity from donor site procedures [109].

Cell-based tissue reconstruction therapies offer new horizons in repairing lost structures. As a new option, tissue engineering offers a biologically oriented approach for the healing of diseased functional units in OMFS. The transfer of cells, harvested and augmented in number ex vivo, represents a promising alternative to the classical treatment options.

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Table 26.1 Tissue repair strategies

Biomaterials	Transplantation of tissue	Extracorporeal tissue engineering
Bioactive systems (BMP, PRP) tissue enhancement	Transplantation of cells	Implantation of cell housing constructs

Alternatively, various biomaterials alone or in combination with bioactive factors – including bone morphogenetic proteins (BMPs), platelet-rich plasma (PRP), or matrix proteins are promising alternatives for structural tissue repair [95, 99].

Tissue engineering combines the biological properties of living cells and physiochemical properties of individually designed materials in order to enable the foundation of artificial tissues.

To reach the ultimate goal of "restitutio ad integrum," 3D scaffolds, bioactive factors, and cells are used to produce an implantable tissue forming device for regenerative and functional repair. In tissue engineering, two main strategies of cell-based tissue repair are promoted: cell transplantation and cell enhancement technologies [62, 100] (Table 26.1).

26.3 State of the Art

26.3.1 Cell-Based Tissue Repair Strategies

26.3.1.1 General Principles

Cellular transplantation respectively cell-housing-construct implantation shows different important impacts on clinical outcome including harvesting procedure, culture technique, implantation procedure, methods of cell isolation, cell multiplication, cell screening, and immunological aspects [34].

The different donor sites show varieties of cell populations and extracellular matrix (ECM) composition. Various surgical techniques allow the harvesting of individual cell containing tissues. Topographical anatomical locations in autologous, homologous, and heterologous donors may offer different tissue types for cell harvesting. The different extraction of cells and the cell culture techniques influence the resulting cellular products [48]. The cells may be used as a therapeutic agent or may be seeded onto anatomically shaped scaffolds prior to implantation.

For example, gaining precursor cells for maxillofacial bone tissue engineering may involve the harvesting of periosteum pieces, bone marrow aspiration techniques, cancellous or cortical bone biopsies from os ileum, tibia, rib, mandible, maxilla, or calvaria.

Special attention is given to gaining selective stem cells (SCs). Different approaches have been used to allow the mesenchymal SCs to be cultured and expanded in numbers without differentiation and keeping their potential for osteogenic, chondrogenic, or adipogenic differentiation. The age-related decrease in bone marrow components and subsequently, partial loss of precursor cells may present a limitation in gaining sufficient number of adult SCs (osteoprogenitor cells count approximately 0.001% of the nucleated cells in the adult marrow). Although standard conditions have been established for cell culture, the creation of sufficient number of osteogenic or chondrogenic SCs may represent a major challenge for the engineering of structural tissue in the near future [106]. Under clinical conditions, culture media should contain autologous serum respectively artificially chemically defined media, when cell-based ex vivo tissue engineering strategies are used [42].

Besides the use of cytokines, mechanical stimulation, and guided tissue regeneration (GTR), there are genetic engineering strategies in cell-based tissue engineering, which include local or systemic delivery approaches in vivo, respectively transfection techniques ex vivo. Cells derived from an autologous source can be rendered immunogenic, when genetically modified.

The ex vivo tissue engineering approach includes the reimplantation of genetically altered cells attached to a carrier. This technique may be considered as a more defined method of cell-based engineering than vector applications to the host site, although controlled clinical trials have not been performed up to now (Table 26.2).

26.3.1.2 Polymer Cell System

To provide the appropriate environment for cell survival, differentiation, and expression of their specific function, defined spatial relationships have to be established [101].

Table 26.2 Cell-based tissue repair strategies

Cellular transplantation
Implantation of cell housing constructs
Stem cells
Precursor cells
Determined cells
Extracorporeal tissue generation
Cell enhancement
Stimulation and augmentation of cells
Cytokines (morphogens – mitogens)
Mechanochemical stimulation (distraction)
Guided tissue regeneration (membranes)
Vector application (genetic engineering)

Biodegradable natural occurring or synthetic polymers as templates [20] to which harvested cells adhere, provide structural support and induce intrinsic organization. The polymers act as a 3D scaffolding, which can be engineered to allow the implantation of the transplanted cells within only a few cell layers from the next capillaries and allow nutrition and gas exchange by diffusion until successful engraftment is achieved [6]. Although many of the issues associated with cell transplantation are the same, many individual problems associated with each cell type have to be solved [32] (Fig. 26.1).

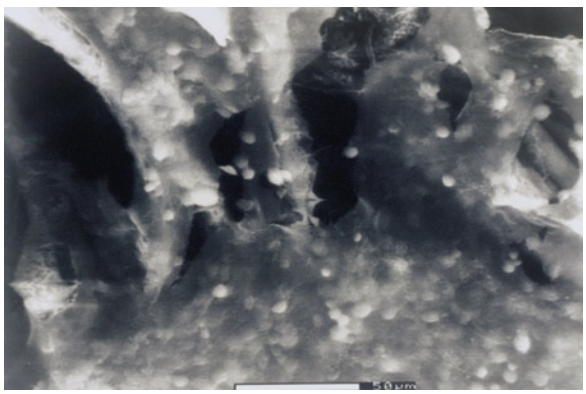

Fig. 26.1 Polymer cell system

26.3.2 Cell Transplantation Devices

A prerequisite for the successful application of the technique of tissue engineering in OMFS, dentistry, craniofacial surgery, plastic and reconstructive surgery, otolaryngology, and orthopedic surgery is to provide 3D bioresorbable matrices [55]. They are constructed, so that the cells may be nourished by diffusion, and keep their specific 3D form during neomorphogenesis and provide the right intrinsic stability and flexibility for the production of new tissue with normal histoarchitecture. During neomorphogenesis of tissues, the polymeric matrix of the cell transplantation devices will resorb [2, 16].

The biodegradable polyesters of the family of polyglycolic acid (PGA) and polylactic acid (PLA) and their copolymers are used as resorbable sutures, osteosynthesis materials, and as drug delivery devices [12, 59].

For tissue engineering of structural tissues, different methods of production of individual cell transplantation devices have been developed using a polyglycolic fiber network with low intrinsic stability and an average fiber thickness of 14 μm, an interfiber distance of 75–100 μm, and a porosity of 97% as a starting material [46].

Cell transplantation device modifications:

1. Coating technique
2. Spraying technique
3. Framing technique
4. Sandwich technique

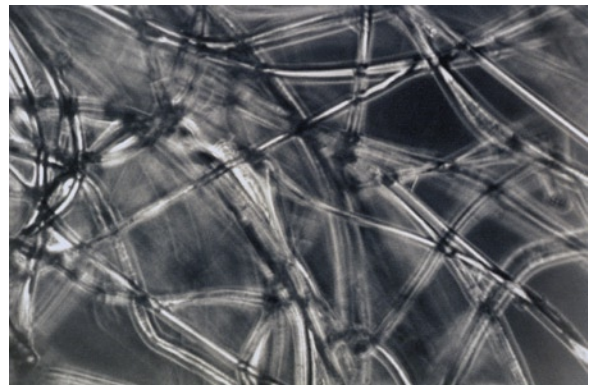

Fig. 26.2 Light microscopy: non woven mesh of polyglycolic acid fibers (fiber diameter :14 micrometer)

26.3.2.1 Coating Technique

The physical properties of PGA and PLA allow the production of inforced, combined composite materials.

A nonwoven fiber network (fiber diameter ~ 14 μm, interfiber distances ~ 100 μm) of PGA (which is not soluble in methylene chloride) was covered by a film of coating polymer PLA.

Depending on the concentration of the solution of PLS in methylene chloride, the porosity was between 90 and 96%. While 20% solutions showed an incomplete closing of the interconnecting spaces, a 5% solution showed an excellent microporosity and a sufficient reinforcement to stabilize the scaffold.

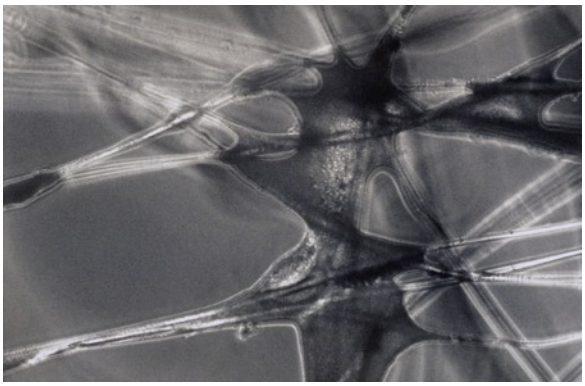

Fig. 26.3 Light microscopy: polyglycolic acid fibers bonded at their cross points using polylactic acid (fiber diameter 14 μm)

26.3.2.2 Spraying Technique

Resorbable cell transplantation devices designed like sponges or using coating technique may provide a reduced porosity of the cell transplantation device, resulting in an inhomogeneous distribution of the seeded cells and the creation of tissues with a variable histoarchitecture.

To avoid these potential disadvantages, we developed the spraying technique. Nonwoven fibers (PGA) were sprayed with PLA dissolved in methylene chloride. Miniaturized, self reinforcing pillars of PLA were placed in the periphery of the cell transplantation devices.

The spraying technique is described for generating anatomically shaped matrices used for the neomorphogenesis of cartilage.

After performing magnetic resonance imaging (MRI), 3D models of nasal cartilages were produced using spraying technique and used as cell transplantation devices.

The individually formed models of nasal cartilage were sprayed with a 5% solution (weight/volume) of PLA dissolved in methylene chloride. Per cm^2 surface 1.1 mg PLA at a temperature of 26°C has been applied. After vacuum drying and disinfection, the devices were sterilized using ethylene oxide. The cell transplantation devices showed a porosity of 96%. The fiber density was 168 g/cm^3.

Spraying PGA fibers with PLA caused a linking of the fibers (PGA) at their crossing points [71, 76].

26.3.2.3 Framing Technique

Using this technique, the highly porous PGA-fiber constructs were surrounded by a PLA-frame, which reinforced the construct. Topographically, the frame was located peripheral to the cell transplant area.

The use of flexible, highly porous polymer constructs reinforced by solid polymer struts and spraying technique is particularly attractive for the fabrication of individually created, 3D, biodegradable polymer constructs for cell attachment and transplantation because of its flexibility and simplicity [103]. If complex anatomical structures are to be created, the spraying technique may be used to connect different modular structures. Using 3D imaging techniques, the computer generation seems possible.

26.3.2.4 Sandwich Technique

For in vitro cell augmentation in number and potential, differentiation cell culture techniques using several layers of resorbable biomaterials are used.

Respecting the maximum diffusion distance, several layers of thin cell-polymer constructs are formed and transplanted. To induce angiogenesis, cocultures of alternative cell types like endothelial cells may be used.

The image-guided tissue engineering of anatomically shaped implants via MRI and Micro-CT, for example, injection molding demonstrates the potential to apply current techniques to engineer a variety of tissues.

Compared to self-assembly and phase separation techniques, electro spinning may provide a simpler and more cost-effective means to produce scaffolds with an interconnecting pore structure and fiber diameters in submicron range.

26.3.3 Cell Sources, Growth, and Differentiation

26.3.3.1 Autologous Cells for Cartilage Regeneration

Chondroprogenitor Cells

Cartilage, basically consisting of one cell type, the chondrocyte, shows a limited regenerative capacity clinically. Chondrocytes can be easily isolated by enzymatic digestion in great numbers, show high viability, are readily multiplied in vitro and most importantly, there is no significant vascularization of cartilage. The chondrocytes are nourished by diffusion and exhibit a low oxygen requirement compared to other tissues [43].

Autologous chondrocyte implantation (ACI) using the periostal flap technique in the human knee joint demonstrated the therapeutic potential of autologous cell transplantation. However, complications like periostal graft hypertrophy, graft delamination, or calcification were observed.

To avoid these complications in temporomandibular joint (TMJ) tissue engineering, to decrease surgical invasiveness, reduce operating time and minimize donor site morbidity small intestine submucosa (SIS R) as a carrier for ACI as oligolayers for TMJ disk reconstruction has been used. We have demonstrated that chondrocytes seeded onto SIS produce abundant intercellular matrix after 21 days in vitro incubation using a semiautomatic perfusion incubator. The biocompatibility of the SIS scaffolds transplanted intraarticularily has been demonstrated to be superior to periosteum. Collagen type II was present after ACI; the histoarchitecture demonstrated the presence of fibroblast-like cells and chondrocytes besides intercellular matrix formation [57].

26.3.3.2 Autologous Cells for Bone Regeneration

Osteoprogenitor Cells

Bone marrow and periosteum are known to contain osteoprogenitor cells. Therefore, substantial efforts have been focused on to isolate and culture-multiply osteoprogenitor cells from humans and various animals, followed by the development of strategies for generating site-specific skeletal tissue regeneration.

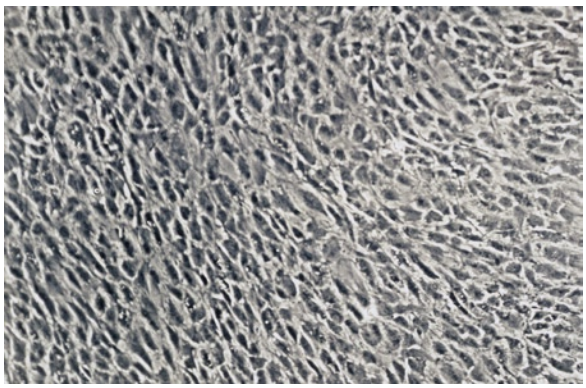

Fig. 26.4 Light microscopy (phase contrast): Chondrocytes after 14 days of in vitro culture forming a cell sheet on the bottom of the cell culture well (magnification 100 x)

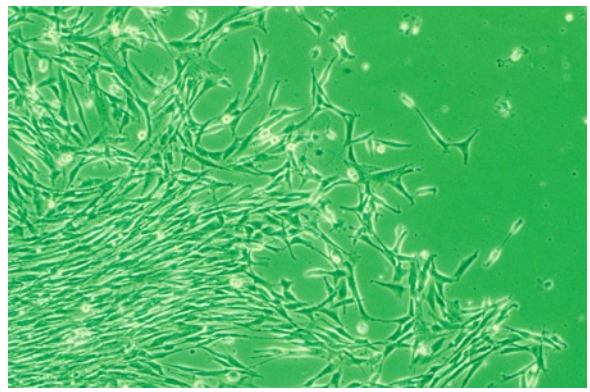

Fig. 26.5 Light microscopy (phase contrast) osteoprogenitor cell population growing out from periosteum (magnification 200 x)

Mesenchymal Stem Cells

Human mesenchymal stem cells (hMSCs) have become the most widely used seeding cells in structural tissue engineering, because there are no ethical problems, they are readily obtained and show multipotency in generating osteogenic, chondrogenic, and adipogenic lineages [14, 24, 50, 64, 68]. Autologous serum for clinical application of cell cultures for tissue engineering applications is recommended, because fetal bovine serum (FBS) used as a supplement in the medium for the expansion of human (mesenchymal stem) cells shows a potential risk of transmitting viral and prion diseases and may provoke immunological rejection [91].

The osteogenic potential of adipose-derived SCs has been demonstrated in nonhealing critical size skull defects [23, 110].

The comparative analysis of cartilage and bone engineered from different perinatal mesenchymal progenitor cells (MPCs) demonstrated that the ECM profile of cartilage and bone engineered from MPCs depends highly on the cell source, although specific differentiation was evident in bovine MPCs isolated from amniotic fluid, new natal bone marrow, and preterm umbilical cord blood [9]. Our group demonstrated the isolation and characterization of perinatal progenitor cells using human term placental tissues, probably, an unlimited source of cells for tissue engineering applications [85].

26.3.3.3 Autologous Cells for Oral Mucosa Regeneration

Several authors recommend autologous tissue-engineered mucosal cells in order to create oral mucosa or mucosa-lined flaps for oral rehabilitation [19]. Today tissue-engineered oral mucosal lining fulfills most of the requirements for clinical application [54, 63, 83].

Dentoalveolar Tissue Progenitor Cells

Dentoalveolar hard and soft tissues form a functional unit in connection with the 3D anatomical structure of the upper and lower jaw. Enamel, dentin, pulpal tissues, and periodontium (consisting of periodontal ligament, cementum, alveolar bone, and gingiva) are the essential tissues of the dentoalveolar regions. Dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), periapical follicle stem cells (PAFSCs), and mandibular bone marrow stem cells (MBMSCs) have been isolated from human dentoalveolar tissues [107].

Periodontal Progenitor Cells

A number of surgical techniques have been described for periodontal tissue regeneration including GTR, bone grafting, the use of antibiotic supplemented collagen matrix (ASCM), enamel matrix derivative, or PRP [67].

Mizuno et al. (2006) describe a novel approach to regenere periodontal tissue by grafting autologous cultured periosteum [60]. Autologous adipose-derived stem cells (ASCs) may be easily available and highly useful in periodontal tissue regeneration as shown by Tobita in 2008 in an animal model [97].

Dental Pulp Progenitor Cells

Human and rat bone marrow-derived stromal cells (BMSCs) and dental pulp-derived stromal cells (DPSCs) were capable of proliferating and producing a calcified ECM. Mature bone formation was found only in ceramic implants loaded with rat BMSCs but not using DPSCs. The authors concluded that the clinical potential using human BMSCs and DPSCs is questionable, until more suitable cell culture and expansion methods are developed [65, 113, 119].

After BMP2 gene transfection, DPSCs demonstrated the ability to differentiate into an odontoblast-like phenotype [113].

Neural Crest-Derived Progenitor Cells

Craniomaxillofacial tissues are derived from the cranial neural crest (ectomesenchyme).

This assembly of transient embryonic tissues yields pluripotent cells with migratory properties generating complex soft and hard tissue structures. One might speculate that the development of teeth comes from

neural crest-derived ectomesenchymal SCs [39]. As a consequence, progenitor cells derived from the apex of developing teeth may be used as tools to regenerate neural crest-derived tissues within the craniofacial region [21].

Salivary Gland Progenitor Cells

The in vitro formation of salivary acinal cell spheroids has been described [18].

The initial feasibility research for an artificial salivary gland has been accomplished till now [1] using cell-based strategies for salivary gland regeneration [81].

Skeletal Muscle Progenitor Cells

Yan et al. (2007) demonstrated the multilayered culture of skeletal muscle cells, derived from neonatal satellite cells, that are distributed in a 3D pattern of organization, mimicking the features of intact tissue. Although the cell constructs showed that they tetanize, their developed force per cross-sectional area was below that of native rat skeletal muscle. With time, the satellite cells fuse into myotubes that align and become elongated, characteristic of skeletal muscle cells [112].

Studies trying to increase the volume and thickness of the culture and to induce hypertrophy by stretching the constructs are in progress [112].

Placenta-Derived Multipotent Cells

Because there are ethical concerns and biological limitations using embryonic stem cells (ESCs), the search for alternative sources of multi- and pluripotent cells continues. The human term placenta is an available source of tissue presenting no major ethical issue concerning its use. Placenta-derived cells potentially demonstrate their usefulness for clinical tissue engineering applications because of their self-renewal abilities and differentiation capabilities into multiple types of cells. Placenta-derived multipotent cells (PDMCs) can differentiate into mesenchymal tissue lineages including osteoblasts, chondrocytes, and adipocytes, as well as endothelial cells [85].

Embryonic Stem Cells

ESCs are a potential source for tissue engineering because of their virtually unlimited supply of cells. They differentiate into various cell lineages under appropriate culture conditions. ESCs are described to be capable of unlimited proliferation in vitro, while their differentiation potential is maintained. The differentiation into cells associated with all three germ layers (endoderm, mesoderm, and ectoderm) seems possible [25].

Various publications have described their chondrogenic differentiation capacity. Jukes et al. (2008) could demonstrate that ESCs show chondrogenic differentiation in pellets, on scaffolds and in gels. However, homogenous cartilaginous tissue masses could not be obtained [41].

26.3.4 Bioreactors for Engineering of Structural Tissue

For tissue engineering of structured tissues like cartilage and bone, an enhanced cell growth and proliferation as well as the formation of ECM is crucial for the generation of natural tissues [35].

The dynamic and controlled environment of a bioreactor should enhance mass transfer, promote uniform distribution of cells in the scaffold, provide exchange of nutrients and wastes between cells and the culture medium, and supply mechanical stimulation for cell growth [28].

26.3.4.1 Stirred Spinner Flask

In the spinner flask, the fluid flow is induced by a magnetic stirred bar at the flask bottom, which significantly increase the exchange rate of oxygen and nutrients between the medium and the inner scaffold, resulting in an augmented number of cells and ECM in comparison to static culture.

In cartilage tissue engineering, a fibrous capsule was observed surrounding the scaffolds and native tissue explants in the stirred spinner flask after 6 weeks of in vitro culture, whereby a continuous cartilaginous matrix was formed in the inner part of the scaffold.

Accumulation of mineralized matrix at the periphery due to different mechanical stimulation on the surface has been described in bone tissue engineering using the spinner flask resulting in a reduced homogeneity of histoarchitecture [28].

26.3.4.2 Rotating Bioreactors

The laminar flow in the rotating wall bioreactor generates a small shear force in order to promote cell proliferation and biosynthesis and avoid the shear-related damage to cell growth.

Increased sulphated glycosaminoglycan (S-GAG) and collagen content have been demonstrated for cartilage regeneration in rotating wall bioreactors. Extending the culture time (up to 7 months) the S-GAG content has achieved the same wet weight fraction as fresh native cartilage. The rotating bioreactor did not induce uniform distribution of ECM or a significant increase of compressible stiffness in comparison to static culture [92].

26.3.4.3 Perfusion Bioreactors

Mass transport in perfusion bioreactors is enhanced in the interior part of the scaffold with the forced flow penetrating the interconnecting spaces within the highly porous scaffolds. Perfusion bioreactors produce a pressure gradient to drive the nutrient and waste transport via convection.

Perfusion of a scaffold seeded with chondrocytes has been shown to upregulate cell growth and ECM biosynthesis in correlation to the perfusion time and medium flow rate. The distribution of cells on and throughout the cell transplantation matrix using chondrocytes on small intestine submucosa membranes showed a multilayer band of organized chondrocytes after three weeks of perfusion using a semiautomatic bioreactor providing intermittent change of flow rate and hydrostatic pressure. The cyclic hydrostatic pressure during the culture of human chondrocytes using autologous serum enhances not only the cartilaginous matrix formation, but also redifferentiation of dedifferentiated chondrocytes [117].

Using a perfusion bioreactor for bone marrow stromal osteoblast culture, significantly more mineralized matrix deposition on porous bioresorbable PLLA scaffolds has been observed.

26.4 Experimental and Clinical Applications of Tissue Engineering in OMFS

26.4.1 Cartilage Tissue Engineering

26.4.1.1 Experimental Tissue-Engineered Growth of Cartilage

Cell Concentration Study

Construction of cartilage implants with chondrocytes and polymer scaffolds appears as an attractive alternative to sculpted cartilage, silicone, or hydroxyapatite implants [66].

In vivo cartilage regeneration has been reported with systems including isolated chondrocytes, cartilage progenitor cells, organic support matrices, or synthetic, biodegradable polymer matrices. The technology of tissue engineering has created an increased demand for viable chondrocytes respectively progenitor cells, necessating effective utilization of cells [71].

The highly porous biodegradable polymer constructs proved to be valuable matrices for the generation of cartilage in an animal model, since the chondrocytes readily adhered to the fibers, and the openness of the templates allowed nutrient, gas, and waste exchange between adherent cells and environment [32]. The polymer templates appeared to guide the development of new cartilage, as the shape of the cartilage formed corresponded to the original shape of the polymer delivery device [70].

A density greater than 20 million cells/cc was required to ensure engraftment and formation of new cartilage in specific shapes of predetermined weight and thickness. The attachment of seeded cells to the polymer fibers allowed the formation of a structurally integrated system for the transduction of mechanical forces. The maturation of the newly formed cartilage-like tissue was brought about by increasing amounts of sulfated S-GAGs. Compression studies demonstrated that the compliance of neocartilage seeded with a concentration of 20 million cells/cc is similar to that of native articular control cartilage [61]. Type II collagen, which is considered to be indicative of cartilage formation, could been deducted in specimens implanted for more than 6 weeks. The effect that the cell explants of the new cartilage appeared to be avascular might be

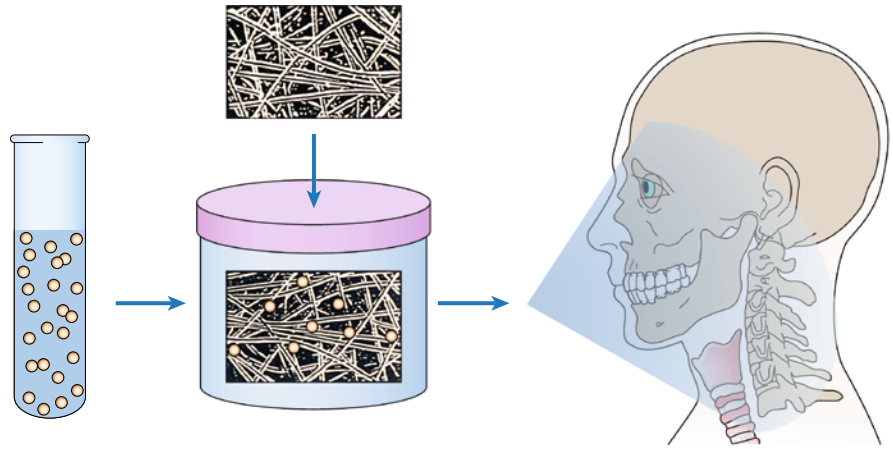

Fig. 26.6 Principles of production of three-dimensional, multi cellular systems for tissue engineered growth of structural tissue: isolated cells are seeded onto polymer constructs, cultured in vitro and implanted into recipients

attributed to the chondrocyte secretion of an angiogenesis inhibiting factor [26].

About 20–100 million cells/cc allow to produce neocartilage implants with an appropriate form and function. These numbers correspond to the known value of bovine articular cartilage of 30–100 million cells/cc [89, 90].

Injectable Cartilage

The idea to have injectable cartilage or bone for clinical applications would be a fascinating alternative to the use of autologous sculpted cartilage [111].

In this endeavor, it has been demonstrated that the subcutaneous injection of concentrations higher than 5×10^6 cells/cc induce the neomorphogenesis of cartilage in an animal model [75]. Using the same amount of cells seeded onto a 3D, highly porous polymer construct, resulted in a significant bigger mass of cartilaginous tissue [37].

Quality Control Using Magnetic Resonance Imaging (MRI)

MRI investigation is a noninvasive method, which allows differentiating between cartilaginous and neighboring topographic structures. This technique has been used to watch the neomorphogenesis of cartilage, which is demonstrated in a mouse model generating 3D cartilaginous structures (Fig. 26.8).

The MRI signal characteristics of the human TMJ disk are those of fibro cartilage; a very low signal intensity on both T_1, and T_2-weighted images. The signal characteristics of the 12 weeks in vivo generated neofibrocartilage explants appeared most similar to those of epiphyseal cartilage [37].

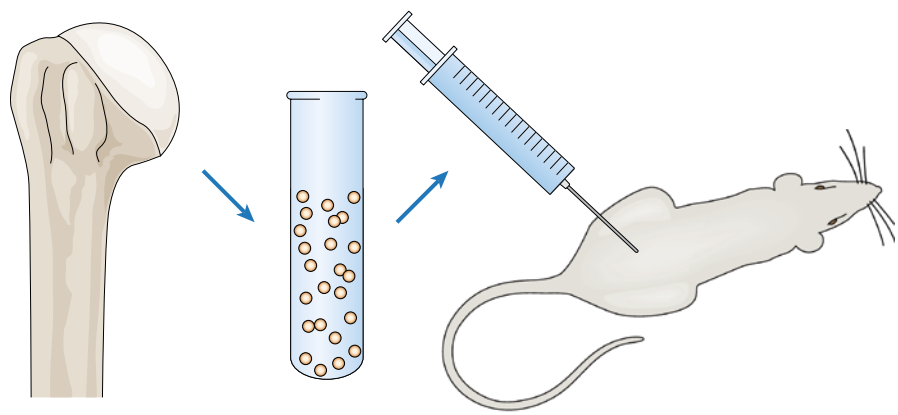

Fig. 26.7 Injectable cartilage: Isolated chondrocytes suspended in culture media are injected subcutaneously into test animals

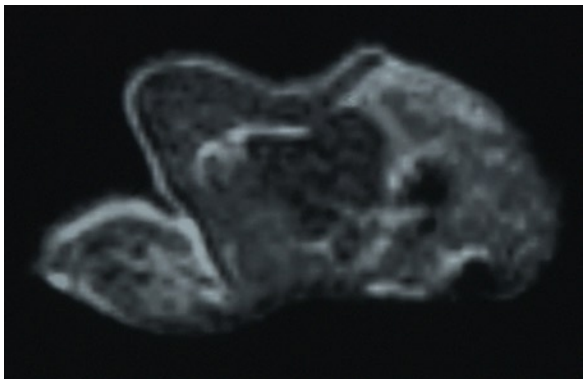

Fig. 26.8 Axial Magnetic Resonance Imaging section 12 weeks after implantation of a chondrocyte polymer construct implanted to the back of a nude mouse, showing the concave-convex form of the temporomandibular joint disc replacement

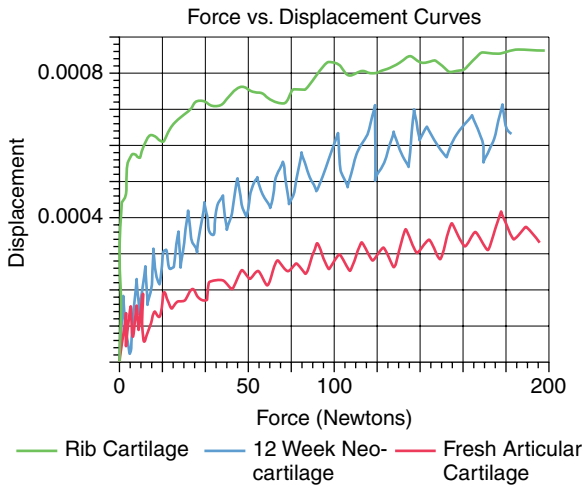

Fig. 26.9 Force-displacement curves of: rib cartilage, 12 weeks old tissue engineered neocartilage and articular cartilage (n = 72)

Biomechanical Testing of New Cartilage Generated by Tissue Engineering

Meyers and Van Mow described hyaline cartilage as poroviscoelastic, whereby the cell-collagen-proteoglycan complex is described as elastic and the interstitial fluid as incompressible [5]. Unidirectional closed chamber compression has been used to test the intrinsic mechanical properties of cartilage [58].

Using biomechanical compression testing, the force/displacement curves showed that the change in disk thickness (or displacement) increased to a maximum point with increasing load. The ascending portion of the curves was interpreted as cartilage compliance during the aqueous phase of compression. This closed chamber compression demonstrated that newly created cartilage was more resistant to compression than native rib cartilage. The ability to recover following compression testing under varying loading conditions improved with increasing time of in vivo incubation and correlated with the augmentation of S-GAGs during neomorphogenesis of cartilage. The composition of the newly created tissue has been manipulated by varying the cell density and cell type seeded onto the biocompatible polymer materials before in vivo implantation [108].

The model of dynamic compression testing of native cartilage and neocartilage produced by tissue engineering demonstrated that the newly formed cartilage is more resistant to compression than native rib cartilage, indicating that the biomechanical properties of neocartilage appears sufficiently mechanically stable for potential applications in reconstructive craniomaxillofacial surgery (after being implanted for a period of 12 weeks).

Facial Implants Generated by Tissue-Engineered Growth of Cartilage

The nasoseptal, the upper lateral cartilages, the alar cartilages, and sesamoid cartilages form the nasal cartilaginous skeleton. These large 3D structures are relatively thin, so that they seemed ideal candidates for the tissue-engineered production of anatomically shaped 3D neocartilage implants, as the reconstruction of the cartilaginous subunits play a major role in repair during both reconstructive and esthetic rhinoplasty.

No ideal biomaterial for facial augmentation has yet been found, although there is a considerable amount of implantable materials available in Plastic and Reconstructive Surgery, including synthetics, autografts, homografts, and heterografts. Most alloplastic materials are thought to be nonabsorbable. However, the problems of infections and graft extrusion make these prostheses particularly vulnerable.

Using the technology of tissue-engineered growth of cartilage, chondroprogenitor cells were transplanted into recipients after augmenting them in number and seeding them onto synthetic polymers [73].

Results showed, both, during in vitro incubation and in vivo implantation, the sprayed PGA-fiber

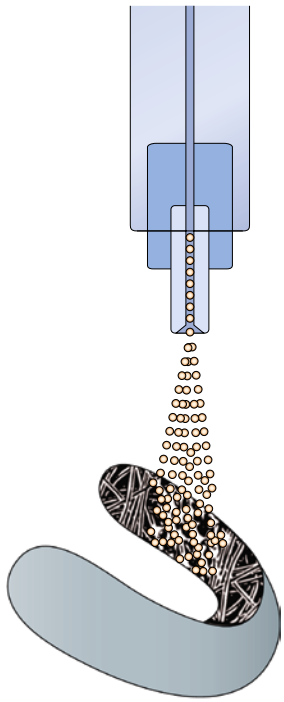

Fig. 26.10 Alar cartilage Cell transplantation device created by spraying technique using 5% solution of polyglycolic acid (PGA) solved in methylenechloride

constructs additionally reinforced with PLLA framework, to present sufficient structural stability during neomorphogenesis of cartilage. The transplantation of the individually created polymer constructs loaded with chondrocytes into animals resulted in the formation of histologically organized hyaline cartilage in predetermined 3D shapes and the maintenance of the configuration and dimensions of the original cartilage scaffold [72, 73].

The constructs presenting a high porosity and a corresponding surface volume ratio were able to support $20–100 \times 10^6$ chondrocytes per cm^3 volume [108]. The biodegradable scaffolds allowed the adhesion of dissociated cells, cell growth, and performance of differentiated cell function. During in vitro incubation, the cells proliferated faster at lower cell densities in order to establish a critical mass prior to expressing a differentiated phenotype. Seeding chondrocytes at an optimal density on the appropriately designed resorbable polymer matrices will promote cell growth and retention of the differentiated function indicated by extensive matrix production, increasing thickness, weight, and amounts of S-GAGs [20]. An increasing production of collagen type II, seen as indicative for hyaline cartilage was observed after the progressing time of in vivo implantation.

After experimental in vivo implantation, the new cartilage formed was surrounded by vascularized tissue [52]. These observations suggest the possibility of surgical transfer of the cartilage implants, not only as free grafts, but also as prefabricated musculo-chondro-cutaneous flaps, consisting of musculature, cartilage, and skin [36, 44].

The creation of a sufficient amount of living tissue forming cells and appropriate cell transplantation devices will be one of the main goals in research, before the creation of new cartilage implants with appropriate form and function will become a routine procedure in reconstructive surgery.

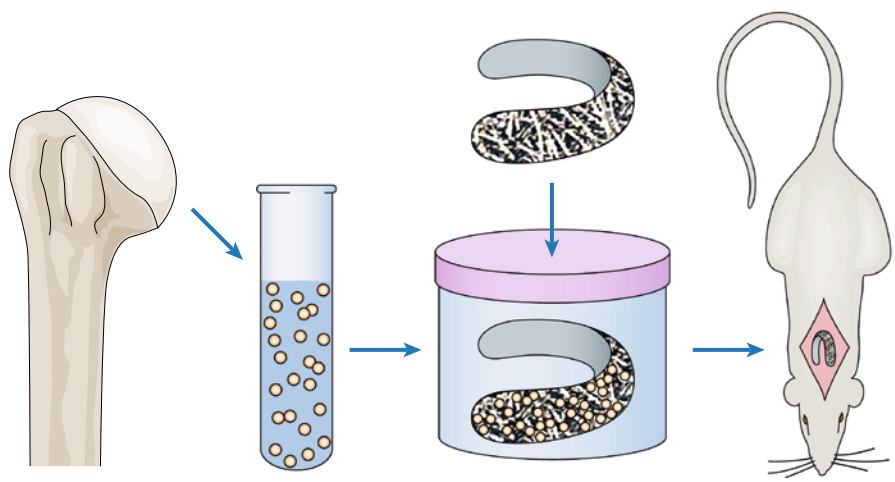

Fig. 26.11 Tissue engineered growth of nasal alar cartilage implants: After harvesting of bovine articular cartilage chondrocytes are isolated and suspended in cell culture media and seeded onto anatomically shaped cell transplantation devices. After implantation into recipients for 6 weeks cells produce new cartilage while the polymeric matrix is resorbed

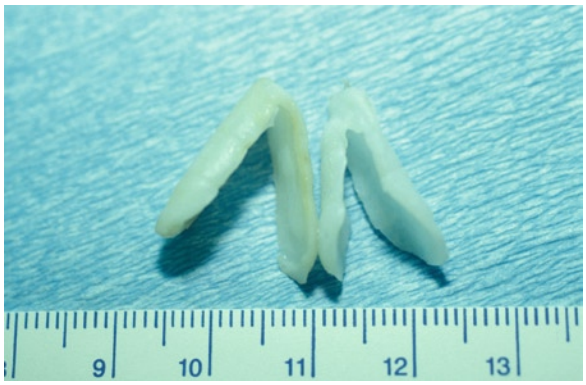

Fig. 26.12 Newly created cartilage in the shape of nasal alar cartilages

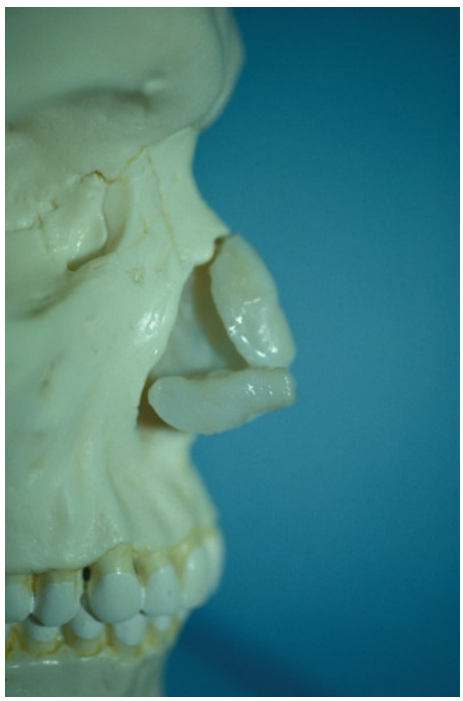

Fig. 26.13 The nasal cartilaginous skeleton is created using tissue engineered growth of bovine chondrocytes in a nude mouse model

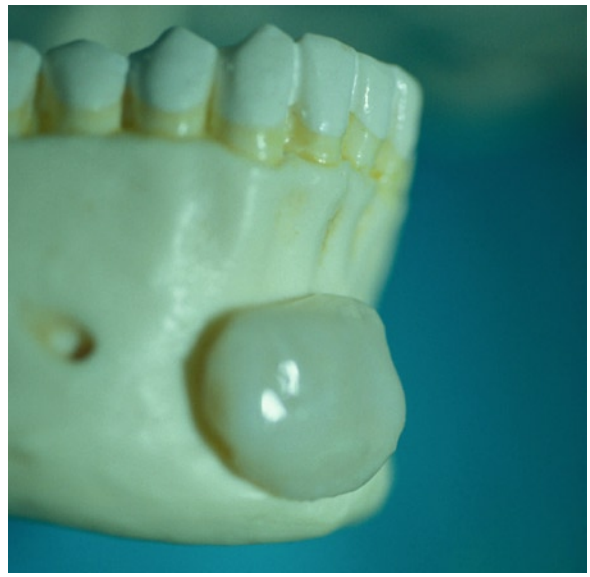

Fig. 26.14 Cartilaginous implant for potential chin augmentation

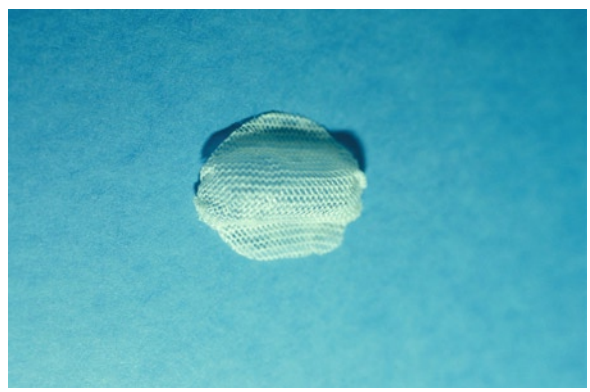

Fig. 26.15 Tissue engineered growth of temporomandibular joint disc replacement : polymer construct in the shape of temporomandibular joint disc

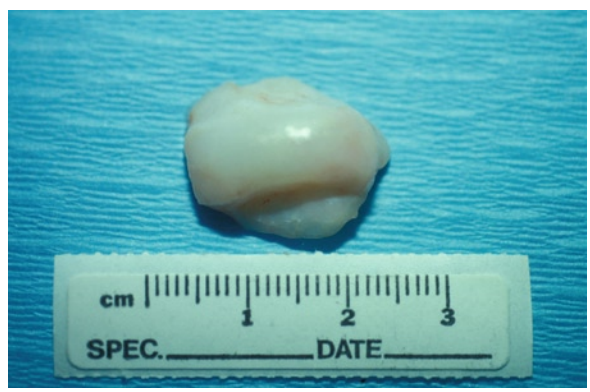

Fig. 26.16 Temporomandibular joint disc replacement created by tissue engineered growth of chondrocytes in an animal model

Transplantation of Neocartilage Created by TE Techniques

Cartilage transplants play an important role in Plastic and Reconstructive Surgery. Fresh autologous cartilage transplants show good clinical results, although self-bending of fresh cartilage transplants or resorption are still major problems. In this endeavor, we transplanted tissue-engineered cartilage to the craniofacial and subcutaneous region of animals. The cartilage demonstrated sufficient stability of form during a period of 5 months after transplantation [66].

26.4.1.2 Clinical Applications of Cartilage Engineering in OMFS

Tissue-Engineered Growth of a Temporomandibular Joint (TMJ) Disk Replacement

Both animal and human studies have shown evidence of degenerative changes of the TMJ structures associated with the use of alloplastic materials. The reconstruction of the TMJ remains a challenging problem for the oral and maxillofacial surgeon.

The variety of grafts used after discectomy – including temporalis muscle, temporalis fascia, dermis, ear cartilage, or lyophilized dura indicates that an ideal TMJ disk replacement has not yet been found [22, 118].

The construction of a functional replacement equivalent to the native tissues in cellular and biochemical composition and organization, and in biomechanical properties and behavior is the ultimate goal of TMJ tissue engineering, presenting a revolutionary novel treatment to TMJ surgeons [4, 79].

The development of a TMJ disk replacement respectively articular cartilage from the subject's own joint is still a challenge. Autologous rib cartilage appears a suitable source for TMJ tissue engineering allowing the repair of the diseased tissue with an immunologically, histologically, and biomechanically natural biomaterial [49, 76].

Puelacher et al. [76] have demonstrated the creation of a TMJ disk replacement made by tissue-engineered growth of cartilage in an animal model using polyglycolic and polylactid acid fiber constructs and chondrocytes.

The following research activities were directed to establish protocols for clinical applications.

The naturally occurring biomaterial SIS R is an acellular matrix composed predominantly of collagen Type I with glycosaminoglycans and growth factors including bFGF. SIS is considered a naturally derived biomaterial with the potential to fulfill the criteria for meniscal engineering showing cell proliferation, diffusion of nutrients, access to cytokines, simultaneous mobility and flexibility, and natural degradation as host cells produce ECM [77]. SIS-treated meniscal defects resulted in superior functional outcomes, when compared to untreated controls (in partial menisectomy). In consecutive series of experiments, the attachment and ingrowth of chondrocytes into oligolayers of SIS has been tested in vitro, resulting in cellular proliferation and ECM production (Figs. 26.17 and 26.18).

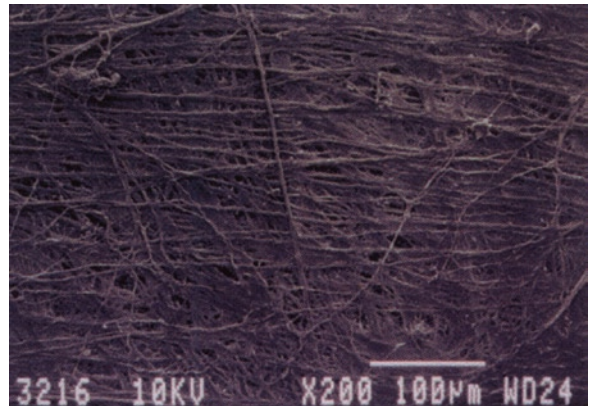

Fig. 26.17 Small intestine submucosa (SIS R) (electron microscopy). Biologic resorbable matrix

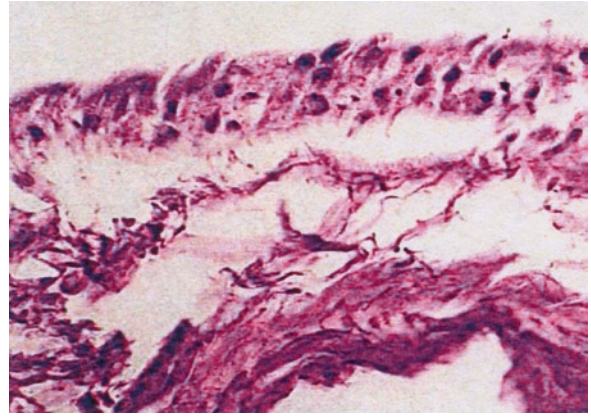

Fig. 26.18 Histologic section (hematoxylin and eosin staining, magnification 100×): oligolayer of organized chondrocytes seeded onto a SIS R after three weeks in vitro perfusion culture

In this endeavor, we performed the tissue-engineered creation of TMJ disk replacements using bioresorbable SIS-polymer constructs and autologous cartilaginous cells.

The ACI Technique [33] in combination with SIS polymer as cell housing matrix has been used in patients presenting TMJ pathology. Patients suffering from severe clinically and radiologically confirmed recurrent TMJ ankylosis and patients with TMJ arthrosis in combination with TMJ disk perforation were treated [69]. The patients were assessed using multiple validated clinical and instrumental function analysis, MRI, CT, and X-ray investigations. In the cases with second look arthroscopic evaluation histologic specimen were obtained. The TMJ symptoms included restricted mouth opening, chewing and mastication problems, and oral hygiene problems, and dental treatment was not possible.

Biopsies of the patient's own rib cartilage were taken. The cell culturing started after mincing the cartilage into small pieces and digesting it into single cells, followed by augmenting them in number during a period of 21–28 days using autologous serum (IGOR-Laboratory, Wels, Austria). The cultured chondrocytes were seeded onto several layers of bioresorbable SIS polymer after creating the desired shape [88].

In cases with TMJ disk perforations, they were covered by the created biopolymer-chondrocyte patch, which were sutured in place. In cases of recurrent ankylosis, the upper and lower surface of the neoarthrosis were reconstructed using oligolayers of biopolymer-chondrocyte constructs after the resection of the overgrowing bone. Then, the TMJ disk replacement was implanted and sutured in place. The neoarthrosis was embedded in a vascularized temporalis fascia flap to mimic the TMJ capsule. One week postoperatively, physiotherapy was started and the patients instructed to exercise. The patients were examined following a protocol using clinical parameters, X-rays, CT scans, and MRI. In the cases of complete ankylosis, where the osteosynthesis material had to be removed from the region of the newly created TMJ condyle, biopsies of the neoarthrosis were obtained. Histologic sections stained with aldehyde fuchsin alcian blue suggested the presence of S-GAG. The cell populations of the created TMJ disk replacement were fibrochondrocyte-containing cells that resembled both, chondrocyte-like and fibroblast-like cells.

The clinical results using ACI on SIS biopolymers in treating TMJ pathology are stable. The mean follow-up is now 6 years and 3 months. All patients were rated good to excellent by clinical evaluation and presented extensive improvement by self-assessment.

The clinical application of rib cartilage as a source of chondroprogenitor cells and chondrocytes showed excellent results in TMJ reconstructive surgery [40] (Figs. 26.19 and 26.20).

An alternative to rib cartilage would be to obtain shavings of the TMJ articular disk or articular cartilage during diagnostic arthroscopy. The specimen can be dissociated into individual cells, augmented in vitro and seeded onto bioresorbable polymer scaffolds, and reimplanted to achieve TMJ disk or articular cartilage repair [42, 93].

However, the augmentation of TMJ articular cells without loss of function is still a major problem, when larger tissue-engineered constructs are to be produced and a relatively high initial cell density is necessary to obtain sufficient amount and quality of cartilage for clinical use.

On the other hand, great efforts have been made to use SCs in cartilage and bone tissue engineering applications. Exposed to specific growth factors, SCs

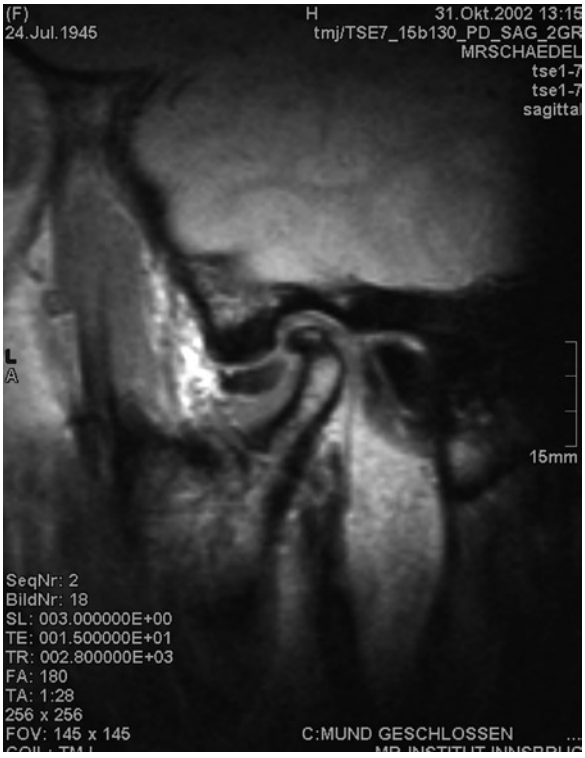

Fig. 26.19 Temporomandibular joint (TMJ) magnetic resonance imaging (MRI) section preoperatively (disk perforation)

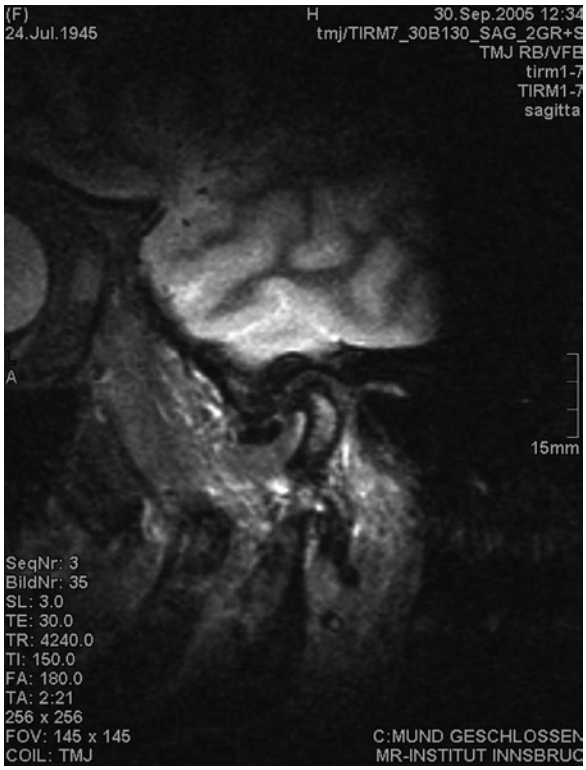

Fig. 26.20 Temporomandibular joint MRI section postoperatively (integrated tissue-engineered TMJ replacement)

including human adult bone marrow-derived mesenchymal stem cells (hBMSCs), adipose-derived stem cells (hASCs), human umbilical cord matrix (hUCM) and human term placental stem cells (hPCs), and ESCs, showed the capability of differentiating into different cell lineages, which may result in the formation of cartilage, bone, tendon, and other structural tissues [9, 51, 86, 94].

The ability of marrow-derived SCs to differentiate into chondrocytes and osteoblasts for TE of the mandibular condyle has been shown. However, the limited self-renewal and proliferative abilities, the biological age-dependent availability of sufficient cell numbers, and donor site morbidity present potential limitation for their use.

Adipose-derived stem cells (ASCs) have been used as a source of full thickness cartilage repair. Adipose tissue may be an attractive cell source in the future because of its abounding quantity in the body, limited donor site morbidity, and ease of harvesting [24].

UCM SCs, cultivated after isolation from extraembryonic mesoderm forming the umbilical cord matrix, respectively SCs derived from adult term placenta appear as a potential alternative to ESCs, as they have been demonstrated to be multipotential SCs. The significant absence of major histocompatibility complexes suggests the umbilical cord cells to have an innate mechanism to evade the immune system.

The ultimate goal of TMJ tissue engineering in the future will be the construction of a functional replacement equivalent to the native tissues in cellular and biochemical composition and organization as well as in biomechanical properties and behavior [120].

26.4.2 Bone Tissue Engineering

26.4.2.1 Experimental Tissue-Engineered Growth of Bone

The treatment of continuity bone defects in the craniofacial skeleton or of segmental defects in the long bones involves appropriate fixation techniques and biologic graft materials [8]. Autogenic or allogenic bone grafts have been used for many years [7]. They act as a matrix for new osseous ingrowth and creeping substitution, when nonvascularized bone grafts or other resorbable biomaterials were implanted into the body. When other grafts (dead bone from another individual) are used as a transplant, replacement occurs also via creeping substitution, an often time-consuming process. The potential transmission of infectious diseases is associated with this technique. Free autologous bone grafts with microsurgical vascular anastomoses can close bony defects with excellent results. The availability of donor sites and patient morbidity are potential limiting factors of this approach.

Attempts to replace a lost bone by the stimulation of mesenchymal tissue differentiation into bone [121] are made by means of polypeptides (e.g., BMP; PRP), demineralized bone powder, or a combination of both, hydroxyapatite, or membrane techniques.

A tissue engineering alternative is to use biodegradable polymers – serving as scaffolds for cell transplantation and regeneration of structural tissues.

The Laboratories for Tissue Engineering at Harvard Medical School in Boston, USA and at Leopold Franzens Medical School in Innsbruck, Austria have used bioresorbable synthetic polymers in the family of

PGA, polylactid acid (PLA), their copolymers, as well as biologic absorbable and non absorbable materials to design constructs, onto which structural cells have been seeded. The cells have been augmented in number and transplanted into recipients to create 3D implants.

The implantation of periosteum-derived cells seeded into synthetic polymer scaffolds resulted in the repair of nonhealing defects in weight-bearing bone [74].

After testing the model for a nonhealing femoral defect in rods using internal fixation technique, osteoprogenitor cells were isolated under sterile conditions. The harvested specimen were allowed to incubate until they formed a monolayer of cells on the bottom of each well, and thereafter, the cell-polymer complex were implanted into the long bone defects.

The analysis of the supernatant nourishing the cells showed the presence of osteocalcin, indicative of functioning osteoblasts. Biocompatible and biodegradable polymer templates allowed exact engineering of matrix configuration, so that the biophysical requirements of mass transfer were satisfied [14].

Osteoblast-like cells seeded onto synthetic biodegradable polymers and implanted into nonhealing segmental femoral bone defects resulted in the formation of large masses of bone in an animal model. This has been confirmed by radiology as the formation of bone in an orthotopic location originating from periosteal osteoprogenitor cells [74] (Figs. 26.21 and 26.22).

A sufficient number of animal experiments using tissue-engineered growth of structural bony tissues have been performed indicating the usefulness of the cell-based tissue regeneration strategies [53].

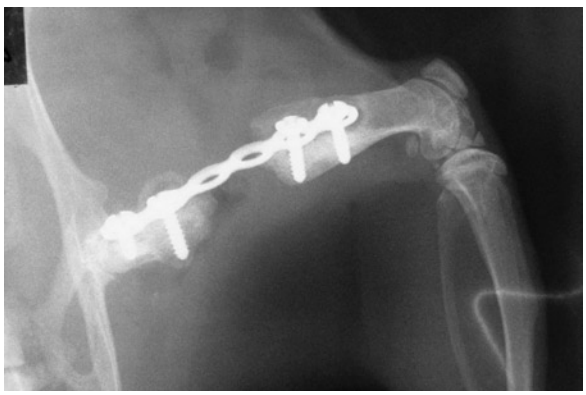

Fig. 26.21 Conventional X-ray postoperative after the implantation of bovine osteoprogenitor cell-polymer construct into the nonhealing defect of a nude rat and bridging the defect using a titanium miniplate

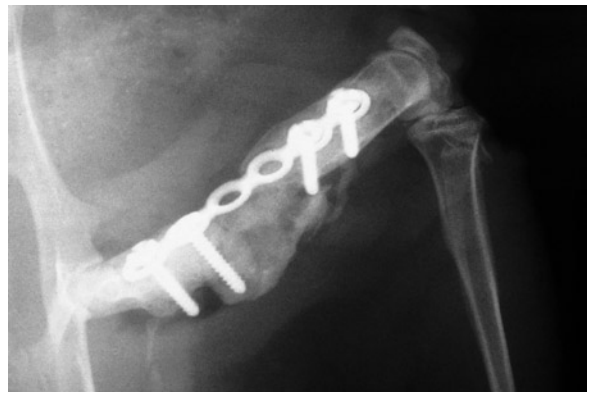

Fig. 26.22 Conventional X-ray 12 weeks after the implantation of bovine osteoprogenitor cell-polymer construct. The defect is bridged by large masses of new bone

26.4.2.2 Clinical Application of Bone Engineering in OMFS

The first clinical investigation of bone progenitor cell transplantation principles has been applied for the tissue-engineered growth of bone in the maxilla [84].

In a prospective clinical study (91 cases), tissue-engineered bone transplants were used for maxillary sinus augmentation. Periosteal tissue patches were harvested [66] from the bony cortex to isolate periosteal cells. The isolated cells were cultured using autologous serum. The augmented cells were seeded onto resorbable polymer constructs and allowed to adhere. The grafting procedure was performed 7.5 weeks after the harvesting of periosteal grafts. The tissue-engineered constructs were placed into the maxillary sinus cavity after elevating the sinus membrane (Fig. 26.23).

The clinical and radiological examination showed sufficient bone formation in the majority of one-step procedure (sinus augmentation and implant insertion at the same time) candidates.

Fifty percent of the two-stage cases (sinus augmentation prior to implant insertion) were available for radiologic follow-up of more than 6 months postoperatively, and an excellent outcome under implant-loading conditions was found. In 19 patients, insufficient bone regeneration was found in 3 months following augmentation.

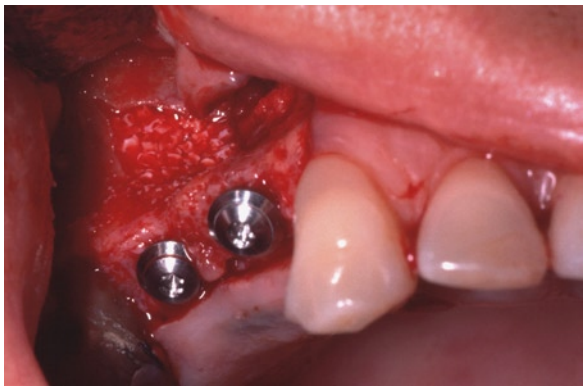

Fig. 26.23 Maxillary sinus cavity after elevating the sinus membrane and simultaneous placement of dental implants

In a recent animal study, Lin et al. (2008) described a missing osteogenic effect of expanded autogenous osteoblast-like cells in sinus augmentation in minipigs [56].

However, a major question concerning in vitro tissue engineering of larger amounts of tissue remains unanswered: For the question of how to supply cells within large cell-polymer constructs with nutrients and oxygen, so that not only survival, but also proliferation and generation of mature tissue may be reached, while the polymer constructs degrades [87], one possible solution might be the application of endothelial growth factor in order to induce sufficient angiogenesis for the growth of vascularized bone.

26.4.2.3 Bone Regeneration and Reformation

For potential clinical applications, there are sources of growth factors for bone regeneration available – including autologous PRP and BMP as a cloned, recombinant human protein, able to induce neomorphogenesis of bone [78].

Platelet-Rich Plasma (PRP)

In clinical terms, PRP represents a concentration of autologous, human platelets in a reduced volume of blood plasma. Several expressed growth factors are responsible for soft and hard tissue healing: platelet-derived growth factor (PDGF; three isomers known as PDGFaa, PDGFab, PDGFbb), transforming growth factor beta (TGFβ; two isomers known as TGFβ_1 and TGFβ_2), vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), vitronectin, fibronectin, and fibrin (cell adhesion molecules are also responsible for cell migration).

For clinical application, PRP may be produced using centrifugation, concentrating platelets from autologous whole blood and resuspending the platelet concentrate in a small amount of autologous plasma. Although PRP is prepared using autologous blood, there are some concerns about the use of bovine thrombin as an activating agent.

The PDGF isomers induce mitosis resulting in the expansion of the endosteal osteoblasts marrow SC populations. The TGFβ-isomers – known as potent morphogen and mitogen – may direct cellular differentiation towards bone formation. Vitronectin, fibronectin, and fibrin provide scaffolding upon which cells can migrate and hence influence neomorphogenesis of bone. VEGF may induce revascularization of the graft by the promotion of capillary cell growth.

An earlier vascularization of the graft material may ameliorate the survival of osteoblasts and osteocytes influencing positively the neomorphogenesis of bone resulting in an increase of the amount of bone formed and the rate of bone formation [66]. In various reconstructive surgical procedures (e.g., sinus grafting with autogenous or xenogenic graft material), PRP may be used as an adjunct that may accelerate bone healing.

Bone Morphogenetic Proteins (BMPs)

In 1965, Urist [99] described ectopic de novo bone formation using crushed and purified bone. Today, more than 13 naturally occurring BMPs, out of the super family of growth factors are known [98].

While the balance between new bone formation, bone remodeling, and resorption is regulated in the grown up, during the development of the fetal skeleton, the BMPs induce cell organization, migration, and bone formation, inducing recruitment, proliferation, and differentiation of (marrow) SCs into functioning osteoblasts.

Today – as an example – recombinant BMP (rhBMP2) is used in orthopedic surgery inducing recruitment, proliferation, and differentiation of (marrow) SCs into functioning osteoblasts.

The rhBMP, developed in the specialized molecular biology laboratory is delivered to the clinician (mainly

orthopedic surgeons) as a product, which has to bind to a potential carrier before implantation. Absorbable collagen sponge (ACS) used as a carrier for rhBMP2 showed bone regeneration in a rhBMP2/ACS graft construct beginning at its periphery, resulting in an initial central void, that dissipated over time [95].

Warnke et al. used a titanium mesh cage that was filled with bone mineral blocks and infiltrated with recombinant human BMP7 and the patient's own bone marrow. Transplanted into the latissimus dorsi muscle in a human being, heterotopic bone formation in order to form a mandibular bone replacement was induced. The created neobone was used to regenerate a mandibular defect [104].

Clinical applications in OMFS are to be seen critically, although impressive case reports are published: a case of a female born with a Tessier facial cleft and left mandibular hypoplasia, in whom after unsuccessful mandibular distraction, rhBMP2 was used to generate mandibular bone, which has been distracted again with good consolidation; or clinical cases of alveolar bone repair using recombinant human bone morphogenetic protein (rhBMP2) in patients with clefts [83, 114].

Summarizing, in vivo tissue engineering methods using rhBMP may be seen as a useful adjunct to conventional surgical techniques enhancing the rate and quantity of bone regeneration [13].

26.4.2.4 Guided Tissue Regeneration

Guided Bone Regeneration

In clinical studies, the use of advanced membrane devices that separate tissues during healing [15] has shown evidence of their potential to induce the regeneration of soft and hard tissue healing including periodontal attachment and bone [86].

These technologies have been named GTR and guided bone regeneration (GBR), representing a basic concept in tissue engineering: scar formation is prevented and regeneration from specific tissue sources is allowed by providing vascular supply and maintaining a spacing chamber during the healing period. On the other hand, compromised wound closing or early mechanical wound failure (e.g., exposing of the membrane) may restrict regenerative outcomes even in smaller tissue defects [122].

26.4.3 Periodontal Tissue Engineering

For the generation of an engineered tissue, the main requirements include the appropriate level and sequencing of regulatory signals, the presence and number of responsive progenitor cells and an appropriate ECM or carrier scaffold [29, 30]. As a prerequisite of tissue regeneration can be seen the recruitment of progenitor cells, which, after regenerative specialization and proliferation demonstrate synthesis of the necessary connective tissues to be repaired [15, 38]. The engineered scaffold should be of sufficient stability and form to allow placement and to prevent consequent collapse of the repositioned tissues in the periodontal defect site (Fig. 26.24).

Preliminary studies have shown that periodontal ligament and bone cells can be transplanted into periodontal sites. At present, there is no good evidence to suggest that current regenerative technologies will enhance tooth retention.

Jin et al. [38] used an ex vivo gene-targeting model for BMP delivery to alveolar bone defects.

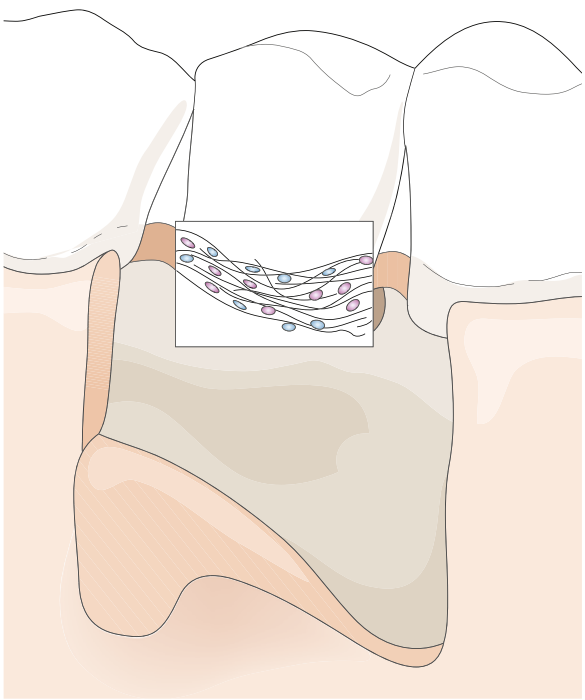

Fig. 26.24 Cell-polymer construct implanted into the periodontal defect of a single tooth

Cells from a small biopsy were expanded in vitro and then transduced by gene therapy vectors. After seeding the transduced cells into gelatine scaffolds, the cell gelatine constructs were transplanted into large periodonto-osseous defects to promote wound healing. Rapid chondrogenesis was followed by osteogenesis, cementogenesis, and bridging of the bone defect. However, there are concerns about vector biodistribution properties in the patient using viral vectors for growth-factor delivery. Another problem is presented by the strong host response to microbial contamination influencing the oral wound tissue environment [10].

Approval of growth factor therapies, as well as the development of advanced gene transfer techniques may present a second-generation growth factor therapy for long-term delivery of recombinant proteins to periodontal and peri-implant wounds [38, 80].

26.5 Expert Opinion

Tissue engineering is a multidisciplinary field involving the development of bioartificial implants and/or the fostering of tissue remodeling with the purpose of repairing or enhancing tissue or organ function. Since tissue engineering includes the recapitulation of the steps undergone in embryological development, this field may be considered a form of applied developmental biology [101]. Important aspects of cell and scaffold-based tissue engineering include cell sources, cell and SC isolation and differentiation, biomaterials, biomimetic materials, drug delivery, bioreactors, 3D scaffolding for cells, cell encapsulation and immobilization, gene therapy, morphogenes and growth factors, regulatory perspectives as well as quality control and analysis of engineered tissue [96, 99, 102, 104].

Different research groups have shown that biodegradable polymer templates for cell transplantation are adhesive substrates for cells, promote cell growth, and allow the retention of differentiated cell function. The high porosity of the templates provides adequate space for cell seeding, growth, and ECM production. A uniformly distributed and interconnected structure is important, so that an organized network of tissue constituents can be formed. In the reconstruction of structural tissues like cartilage, tissue shape is integral to function. Therefore, cell transplantation scaffolds are processed into devices of varying shape, thickness, and microarchitecture. Highly porous biodegradable polymer constructs are suitable matrices for the generation of new tissue, since the cells readily adhere to the fibers, and the openness of templates will allow nutrient, gas, and waste exchange between the adherent cells and the environment. The polymer templates appear to guide the development of the new tissue as the shape of the tissue formed corresponds to the original shape of the polymer delivery device. Different regulatory factors can be used to selectively control the growth rate and improve the composition of the in vitro engineered tissue [1, 2, 4, 27, 28, 41, 50, 122].

The therapeutic potential of multilineage SCs for applications such as transplantation, tissue engineering, and gene therapy is enormous. Conceptually, there are general types of SCs potentially used for the engineering of structural tissue applications: ESCs, fetal stem cells (FSCs) (umbilical blood), autologous SCs, and placentar SCs [85, 115, 116].

Although theoretically appealing because of their pluripotency, the practical use of ESCs is somehow limited due to the potential problems of regulation and ethical considerations.

Autologous SCs by nature are immunocompatible and give no ethical issues related to their use.

Continuous research needs to be performed before perfect results can be reached. Additional characterization of native tissues needs to be done, both biochemically and biomechanically, in a variety of animal models. Current scaffolds need to undergo tests that are specific to different tissue histoarchitecture, structure-function, and even new adequate scaffolds need to be developed. Properties such as degradation time and mechanical and chemical characteristics need to be optimized as does the use of morphogeneses and mitogenes for improving cellular response within these scaffolds. If cells are seeded onto these scaffolds, cell type, seeding conditions, and culturing conditions need to be optimized. Suitable bioreactors need to be further developed, taking into consideration, the biomechanical and biochemical environment along with nutrient transport requirements. Angiogenesis is, up to now, not a completely solved problem for new tissue generation [105]. Sophisticated methods to assess the effectiveness of tissue engineering approaches need to be further developed to ascertain that tissue

regeneration functionally satisfies over an extended period of time [87].

26.6 Five Year Perspective

Tissue Engineering Technologies combine the biological properties of living cells and physicochemical properties of individually designed materials in order to enable the creation of artificial tissues like cartilage, bone, dentin, mucosa, periodontium, etc. for potential use in OMFS [45, 51, 55, 60, 69].

Living cells, 3D cell housing scaffolds, and bioactive factors are used to produce an implantable tissue forming device for regenerative and functional repair.

The aim of the discipline, OMFS, in the next 5 years will be to enhance reconstructive and regenerative tissue engineering technologies. This goal can be reached by surgically oriented research including: cell sources, growth and differentiation, bioreactors, and clinical tissue engineering applications including: cartilage, bone, dental, periodontal as well as mucosal engineering.

Progress in the understanding of fundamental biology associated with tissue regeneration will be essential for the development of approaches to enhance cell function, angiogenesis, and cell differentiation in the next 5 years.

26.7 Limitations/Critical View

Tissue loss or organ failure is one of the most frequent, devastating, and costly problems in human health care. Advances in life sciences and medicine have enabled physicians to restore lost functions in their patients through organ and tissue transplantation, reconstructive surgery with autogenous tissue transfer, or the implantation of alloplastic materials [44].

Tissue engineering as the use of biological and/or synthetic materials in conjunction with cells to create biologic substitutes to serve as functional tissue replacements has been explored by modern scientists for several decades [54, 66, 91]. Cells are seeded onto synthetic and naturally occurring, biocompatible, biodegradable polymers of different chemical compositions and physical configurations. The cell-polymer composites are transplanted for the purpose of generating new functional tissue. The approach ultimately results in successful engraftment of cells with new tissue formation. The polymer scaffolds serve as cell anchorage sites and allow the cell-polymer complex to be implanted with intrinsic structure. This enables precise engineering of a multitude of characteristics, such as surface area and exposure of the attached cells to nutrients (biophysical limitations of mass transfer). The ratio of surface area to mass can be altered, or porosity using different configurations can be changed. If cells anchorage sites and reasonable structural cues are provided in an appropriate environment, the intrinsic ability of cells to reorganize and generate new tissue is enhanced [53].

One might speculate that advances in material sciences and life sciences will enable physicians and scientists to help mankind in the future by being able to replace a multitude of diseased or damaged tissues.

Prerequisites include the isolation and characterization of adult, embryonic, fetal, and placentar (stem) cells, the development of biodegradable scaffolds and cell-polymer tissue engineering constructs, the development of innovative bioreactors, the enhancement of angiogenesis, and the stimulation of cultured cells by developmentally relevant signals.

The multidisciplinary field of tissue engineering has led to clinical success in OMFS including tissues like skin, mucosa, cartilage, and bone. Continued research needs to be performed despite such successes.

26.8 Conclusion/Summary

Tissue Engineering represents an interdisciplinary field with sufficient potential to advance regenerative medicine and basic science. The field of tissue engineering merges the expertise of life sciences, physical sciences, engineering, and medicine to develop functional tissues that can maintain, restore, or improve damaged function. In most cases, tissue engineering attempts to recapitulate certain aspects of normal development to stimulate cell differentiation and organization into functional tissue assembly [47, 105].

In the fields of OMFS, extensive progress has been made in tissue engineering over the past two decades, including the development of biodegradable scaffolds, the combination of cells and biomaterials

for generating tissue engineering constructs, the development of bioreactors designed to stimulate the cultured tissue by developing relevant signals, and the isolation and characterization of embryonic and adult SCs.

Biomaterials have played a crucial role in the development of tissue-engineered structures. They are used to produce bioresorbable scaffolds to place cells in close proximity with each other. Efforts are being made to optimize the mechanical, physical, chemical, and biological properties of scaffolds for applications requiring different microenvironments [16].

An important goal is the generation of vascularized tissues. Tissue vascularization can be used to launch or restore blood flow and thereby meet the universal requirement for establishing blood perfusion through engineered tissues with clinically relevant thicknesses [82]. Methods that show great promise in addressing this challenge include controlled release of angiogenic factors from scaffolds, seeding endothelial cells directly into the scaffold, and engineering the vasculature directly into the tissue [82, 85].

The field of SC biology has developed considerably and presents huge potential for using human adult or embryonic cells as sources for the generation of tissues. Endothelial cells derived from human ESCs have been shown to generate functional vasculature. However, much more research is required to understand the cues and signals that regulate cell differentiation [45].

Summarizing, the understanding of fundamental biology associated with tissue regeneration is essential for the development of approaches to enhance cell function, angiogenesis, cell differentiation, and subsequent tissue formation in all fields of surgical research including the discipline of OMFS.

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Tissue Engineering of Musculoskeletal Tissue

27

Lu Zhang and Myron Spector

27.1 Introduction and Objectives

The musculoskeletal system includes numerous types of tissues of diverse composition and structure, which when acting in concert, enable locomotion: bone, articular cartilage, meniscus, synovium, ligament, tendon, and muscle (Table 27.1). All of these tissues, except muscle are of the connective tissue family. While these tissues share some common features of their cellular behavior and extracellular matrix (ECM) composition, there are profound differences in their properties and potential for regeneration (Table 27.2). Disorders of the musculoskeletal tissues often result in pain, deformity, and/or malfunctions of joints, and are more likely to interfere with the quality than the quantity of life. Tissue engineering with its endpoint of reconstruction of the composition and structure of tissues offers the promise of a more complete and longer-lasting restoration of musculoskeletal function, compared to prosthetic replacement. When compared to reconstructive procedures using autografts, tissue engineering offers the advantage of lessening or eliminating donor site morbidity, and when compared to reconstructive procedures using allografts, tissue engineering offers the advantage of lessening or eliminating rejection and the potential for transmission of disease. Tissue engineering of these musculoskeletal tissues, like the engineering of other tissues, requires an understanding of: the phenotypic characteristics of the cells in the tissues; the make-up of the extracellular matrix; and the regulatory molecules. The objective of this chapter is to compare and contrast the many tissues comprising the musculoskeletal system with an emphasis on those features impacting tissue engineering. There have been several recent reviews of musculoskeletal tissue engineering that have addressed the clinical applications and strategies being taken to address the clinical problems [41, 89].

27.2 Features of Musculoskeletal Tissues Which Impact Tissue Engineering

Tissue engineering has come to apply to two decidedly different approaches to reconstructing tissues. In one approach the tissue is fully formed in vitro, and subsequently implanted into the body. In the alternative approach the majority of the regenerative process occurs in vivo at sites in which a scaffold is implanted alone, seeded with cells in vitro prior to implantation, or impregnated with molecular regulators or genes encoding regulatory proteins prior to implantation. For cell-seeded constructs, the decision must be made regarding the level of maturity to reach in vitro, before implantation. The challenges for fully ex vivo regenerated tissues include: (a) the creation of the tissue-specific stress-induced architecture; and (b) integration of the implanted and host tissues. All

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Table 27.1 Composition and structure of musculoskeletal tissues

Tissue	Cell types	Main ECM components	Architecture
Bone	Osteoblast, osteocyte, osteoclasts	Type I collagen; CaPhos mineral; noncollagenous proteins	Lamellar
Articular cartilage (hyaline cartilage)	Chondrocyte	Types II, IX, and XI collagen; proteoglycans	Rounded cells in lacunae
Meniscus (fibrocartilage)	Chondrocyte	Types I and II collagen	Fiber bundles
Synovium	Types A (macrophage) and B (fibroblast) synovial cells	Types I, III, V, and VI collagen and fibronectin	Layer of cells
Ligament	Fibroblast	Type I collagen	Uniaxially aligned fibers with crimp
Tendon	Fibroblast (tendoncyte)	Type I collagen	Uniaxially aligned fibers with crimp
Muscle	Myocyte, myofibril	Basement membrane	Fiber bundles
Intervertebral disc; annulus fibrosus (fibrocartilage) and nucleus pulposus (hyaline cartilage)	Fibroblast and chondrocyte	Collagen	AF, lamellar

Table 27.2 Properties of the principal musculoskeletal cell types, graded 0-++

					Cell behavior		
Tissue	Spontaneous regeneration	Intraarticular	Vascularity	Progenitor cells	Mitosis	Migration	Synthesis of ECM
Bone: osteoblasts	Yes	No	++	++	++	++	++
Articular cartilage: chondrocytes	No	Yes	0	0/?	0/+	0	+
Meniscus	No	Yes	0/+	0/?	0/+	+	+
Synovium	Yes	Yes	++	++	++	+	+
Ligament: cruciate	No	Yes	0/+	0/?	+	+	+
Ligament: collateral	Partially	No	0/+	0/?	+	+	+
Tendon	No	No	0/+	+	+	+	+
Muscle	No	No	++	+	0	0	0
Intervertebral disc	No	No	0/+	0/?	0/+	+	+

of the musculoskeletal tissues in this chapter have precise arrangements of their ECM molecules (viz., collagen fibers) imparted by the cellular response to mechanical strain (resulting from applied stress). Our incomplete understanding of the strain environment of the tissues during their formation obviates its duplication in ex vivo engineering of the tissues. The integration of the implanted engineered tissue construct will require remodeling of the implanted and host tissues such that the newly formed ECM molecules in the interfacial zone become integrated into the implanted construct and surrounding host tissues. While there is evidence that such an integrative process can occur, it is not yet clear how to insure that it does in all cases.

For approaches in which the majority of the regeneration is to occur in vivo, it is possible to rationalize the tools required for the engineering of select tissues based on the factors which prevent their spontaneous regeneration or impair the compete regeneration of the tissues which do have intrinsic capabilities regeneration. In other words: what elements have to be supplied to a defect in order to facilitate regeneration – a scaffold, cells and/or molecular regulators?

Of the tissues being considered in this chapter, only one has the capability to spontaneously regenerate: bone (Table 27.2). In fact, bone is one of the few tissues in the body with this capability. The determinants of regeneration include: (1) several cellular phenotypic traits; (2) tissue-level features; and (3) environmental factors (Table 27.2). The phenotypic traits include the abilities to: divide in response to injury; migrate into a lesion; and synthesize ECM. In order for regeneration of a tissue, the defect must be populated by cells that migrate into the lesion from surrounding tissue and subsequently increase their cell number through division. These cells may be fully differentiated cells or progenitor cells (perhaps resident "stem cells"). In the latter case the cells must proceed through the additional process of differentiation. The parenchymal cells that populate the defect must then have the capability to synthesis ECM and participate in the organization of the matrix molecules into the tissue-specific architecture. Understanding the limitations of the cellular traits which compromise spontaneous regeneration can serve as a guide to the formulation of tissue engineering constructs. For example, for tissues in which the cells are not able to freely migrate because they are fully contained within their ECM (e.g., articular cartilage), provision may need to be made to supply a sufficient number of exogenous cells to the defect. If the cells that do populate the defect have a low mitotic index or are poorly biosynthetic, growth factors may need to be supplied to stimulate their behavior.

One of the critical tissue-level features that affects the regenerative process is the vascular supply. Of the musculoskeletal tissues being dealt within this chapter, one is among only a handful of tissues in the body that is completely avascular: articular cartilage. Three others – meniscus, tendon, and ligament – have regions which are avascular. The absence of a fibrin clot in response to injury in avascular tissues prevents the migration of cells into the defect, thus rationalizing the implantation of a scaffold. In tissues which normally have a rich vascular supply, such as bone, compromises to that supply as a result of disease (e.g., osteonecrosis) can necessitate the supplementation of the scaffold with angiogenic factors, or alternatively include a vascular network in the implanted construct, to be connected with the host vascular supply. These same approaches to deal with a limited vascular supply may also need to be taken when treating large defects in tissues such as bone, where the normal revascularization response may not be adequate to oxygenate deep into the defect.

One of the severe environmental factors affecting the potential of musculoskeletal tissues to regenerate applies to the tissues within the synovial joints: articular cartilage, meniscus, and the cruciate ligaments (Table 27.2). In addition to being avascular or only poorly vascularized, these tissues are bathed in synovial fluid which can interfere with the formation of, or dissolve, a fibrin clot, which serves the important role of a provisional scaffold. Moreover, joint fluid contains the lubricating and anti-adhesive glycoprotein, lubricin, which when coated onto the surfaces of the host tissue or implanted construct can interfere with integrative binding.

27.3 Bone Tissue

27.3.1 Bone Composition and Structure

Bone is characterized physically by hardness and rigidity of structure imparted by its mineral phase, and microscopically by a high ratio of extracellular substance to a relatively sparse cell population. The extracellular material of bone consists of crystals of hydroxyapatite and an organic part that is largely collagen and some glycosaminoglycans. The cells entrapped in the mineralized ECM, osteocytes, communicate among themselves and with osteoblasts at the bone surfaces by means of thin cellular processes that lie in minute tubules, the canaliculi.

Bone has several functions, the most obvious one of which is its mechanical support for the insertion of muscles and a lever arm through which muscle contraction can act. Another important function of bone is as a storehouse for calcium and phosphorus. The large surface area of the minute calcium phosphate crystallites (of the mineral, hydroxyapatite) of bone makes available an enormous supply of calcium which can be readily mobilized, and of growth factors with calcium-binding affinity. The third role of bone is its function as a containment of the marrow elements for the formation of new blood cells. Bone also serves as a protective covering for the nervous system and other vital organs. The goal of bone–tissue engineering almost exclusively relates to restoration of the mechanical

function of bone; i.e., there is little need to restore its functions of storing calcium and growth factors, and of containing marrow.

Over the past 10 years there have been numerous reviews of bone–tissue engineering. Some have emphasized growth factors (viz., the BMPs) [24], other reviews have addressed the scaffolds [91], and still other reviews have highlighted the cells being investigated [53]. Of note are reviews of bone–tissue engineering from reconstructive surgeons' point of view [51] that place the new therapeutic approaches in the context of the grafting procedures routinely used by surgeons for many decades.

27.3.2 Bone Reconstruction and State of the Art

There are reports of surgical procedures employing implants for bone reconstruction dating back thousands of years [29]. Careful and systematic documentation of the use of autogenous and allogeneic bone for reconstruction began to be found in the literature in the 1900s [27]. Procedures for the regeneration of bone in trauma, orthopedic, and spinal surgery have significantly advanced in the past 10 years, but a number of clinical challenges remain. Autologous iliac crest bone graft (ICBG) continues to be considered the "gold standard" for bone reconstruction, despite its limited supply and the complications associated with donor site morbidity, including: infection, seroma, hematoma, herniation, vascular and neurologic injury, and iliac wing fracture. In addition, donor site pain has been reported in the literature in ranges of 25–49%, with 19–27% of patients experiencing chronic donor site pain 2 years postoperative. Research, therefore, has focused on developing bone–tissue engineering techniques that could avoid autograft harvesting.

27.3.3 Investigative Approaches to Advance Bone–Tissue Engineering

In recent years there have been an increasing number of investigations of exogenous cells and growth factors, as well as scaffolds, for bone regeneration. The following provides a brief review of select studies investigating various aspect of bone–tissue engineering to provide examples of the methodologies and results being obtained.

27.3.3.1 Biomaterial Scaffolds

Numerous bone growth substitute materials have been employed into orthopedics for many years for the treatment of a wide variety of problems [5, 6, 26, 64, 82, 83]. In the context of tissue engineering, we would now consider these materials to be tissue engineering scaffolds.

While some polymers have been investigated as matrices to be used as implants to facilitate bone regeneration, most materials comprise calcium-containing substances. Calcium sulfate [54] and tricalcium phosphate [15, 25, 72, 78] were two of the calcium-containing substances first implemented as bone graft substitute materials. These materials undergo physiochemical dissolution relatively quickly, disappearing in days to weeks in some cases. However, there is still some question as to the rate at which these materials become absorbed by the body. Differences may be related to variations in the composition and structure, and the different physiological characteristics of the implant sites and animal models. Uncertainty about the bonding of bone to these substances may be due to the fact that in many situations the tricalcium phosphate undergoes physicochemical dissolution at a rate which precludes the precipitation of biological apatite and subsequent bone formation on its surface; the dissolving surface does not allow for protein adsorption and cell attachment.

Much of the early work with hydroxyapatite began in the mid 1970s [47]. Many studies focused on the use of hydroxyapatite, in dense and porous forms of block and particles [37]. With the increasing interest in investigation of bone graft substitute materials has come an advance in our understanding of the bone response to these substances. Mechanisms underlying the bone-bonding associated with incorporation of these substances in osseous tissue are beginning to be revealed.

The mechanism of breakdown of calcium phosphates in vivo is not fully understood. The appearance of macrophages and multinucleated foreign body giant cells around some types of calcium phosphates suggest that particles might provoke the activation of phagocytes that in turn stimulate other cells and an

inflammatory response. Agents produced by these cells could accelerate the degradation process.

While it is generally been considered a nonresorbable material, synthetic hydroxyapatite has also been found to undergo physicochemical dissolution, albeit at a very slow rate. Because it is only slightly soluble in biological fluid, synthetic hydroxyapatite substances can functionally be considered as long-lasting implants, especially when they are incorporated into bone.

27.3.3.2 Growth Factors

Perhaps the most notable recent advance in bone reconstruction based on the principles of tissue engineering is a resorbable collagen scaffold incorporating the differentiation and growth factor, bone morphogenetic protein (BMP)-2 [10, 14]. This product, currently marketed under the trade name, Infuse (Medtronic Spine, Memphis, TN), is approved in several countries for specific clinical indications including lumbar spine fusion. The principal claim is that it can be used as an off-the-shelf replacement for autogenous bone graft. The clinical utilization of Infuse for spine fusion has prompted a wide array of "off-label" uses of the construct. Another growth factor-impregnated scaffold has recently been introduced into the clinic for the treatment of select oral and dental applications, viz., periodontal defects: platelet derived growth factor (PDGF)-BB incorporated into a porous tricalcium phosphate scaffold [58].

27.3.3.3 Exogenous Cells

While there may normally be a ready supply of osteoprogenitor cells around the site of a defect in bone, there have been numerous studies of the use of exogenous cells for bone–tissue engineering. The calvarial bone, "critical size," defect is a commonly used model to test the feasibility of bone engineering and repair in different animal studies, which define the roles that seeded cells or scaffold materials play in the bone repair process. In one mouse study [65] for example, human mesenchymal stem cells (hMSC) were first isolated and seeded on a porous silk fibroin scaffold, grown in vitro five weeks in a bioreactor, and then transplanted to repair the calvarial bone defects created in nude mice with a diameter of 4 mm. The results showed that hMSC could undergo osteogenic differentiation on this scaffold and that in vitro engineered construct could achieve better reparative results than the cell-scaffold complex without growth in a bioreactor. In another study [23], osteogenic-induced adipose-derived stromal cells and osteoblasts were seeded into polylactic/glycolic acid (PLGA) scaffolds which were implanted into critical-sized calvarial defects in mice. The results showed that apatite-coated PLGA seeded with either type of cells could completely heal the bone defects and thus demonstrated that adipose-derived stromal cells could serve as a cell source for bone regeneration as well as bone marrow stromal cells.

In other studies exogenous cells have been transduced with the gene for an osteogenic factor prior to implantation. One such investigation [12] transduced rabbit periosteal cells with retrovirus containing a human BMP-7 gene and then seeded them on polyglycolic acid (PGA) scaffold for bone repair. After 12 weeks, better bone formation was observed both histologically and radiographically in the transduced cell group than in the nontransduced group. In a similar study [21], BMP-2-gene-transfected bone marrow stem cells were found to repair a calvarial bone defect better than nontransfected stem cells when applied with biomaterial to the bone defect site. In several other studies, the rabbit calvarial model was used to test scaffold materials for bone formation. The bone engineering studies in mouse and rabbit calvarial models provide convincing data that critical-sized defects of flat bone in small animals can be completely healed with a tissue-engineering approach. However, these results may not necessarily represent the bone formation and repairing process in human being, because low-level mammals usually have a stronger ability than human beings for tissue regeneration. In contrast, large mammals, such as the sheep, dog, and pig, may reflect better than small animals the actual bone formation process in human beings, which will provide a guide to clinical application.

In a study of sheep cranial bone defect repair [93], bone marrow stromal cells (BMSCs) were isolated, expanded, and osteogenically-induced with dexamethasone, β-glycerophosphate, and vitamin D3. The induced cells were then mixed with calcium alginate to form a cell-scaffold construct for implantation. In bilateral cranial defects (20 mm in diameter), the experimental side was repaired with a bone graft comprising in vitro-induced autologous BMSCs and

calcium alginate. Histology demonstrated new bone formation at six weeks post-implantation, which became more mature by 18 weeks. In contrast, the control defect, which was transplanted with calcium alginate alone, remained unrepaired. Furthermore, chemical analysis showed that the engineered bone tissues contained a high level of calcium (71.6% of normal bone tissue), suggesting that engineered bone can achieve good mineralization. Of note, the engineered bone formed cancellous bone structure instead of lamellar structure of natural calvarial bone.

Of interest is that engineered flat bone did not form a typical lamellar structure, as natural flat bone has. Instead, a cancellous bone structure was observed in engineered cranial bone. This phenomenon is probably due to the lack of proper mechanical loading during engineered bone formation in vivo. Unlike the bone-engineering process, during development and growth a natural calvarial bone may sustain a continuous and long-term pressure from growing brain tissue and eventually forms a lamellar structure.

Therefore, an in vivo model that allows for bone remodeling by mechanical loading is particularly important for generating a bone that has the structure and mechanical strength similar to those of natural bone counterpart. Different from flat bone, weight-bearing long bones usually sustain mechanical loading in their physiological activities during their lifetime. These kinds of bones, including femur, tibia, and metatarsus, might be fit for such a model for engineering weight-bearing bone. In 2000, one investigation [86] succeeded in engineering a metatarsus in a sheep model using coral and bone marrow stromal cells and showed the feasibility of generating a weight-bearing bone in large animals.

The femur is the largest weight-bearing bone in animals and human beings. One study [116] tested the possibility of engineering femoral bone in a goat model by using BMSCs and coral scaffold. As other studies reported, BMSCs were expanded in vitro and osteogenically induced and then seeded onto a natural coral scaffold and cultured in vitro for 1 week before implantation in vivo. To provide a mechanical loading for the remodeling of the engineered bone, a special internal fixation device was developed that contains an internal fixation rod, with a diameter a little smaller than that of marrow cavity, and two sets of interlocking nails for fixing the rod at each end. After engineered bone formation, the interlocking nails can be removed, leaving the rod in the marrow cavity and thus allowing for mechanical loading by body weight. In the surgical procedure, 22 adult goats were randomly divided into experimental group (implanted with cell-scaffold construct, $n=10$), scaffold control group (implanted with scaffold alone, $n=10$), and blank control group (without implantation, $n=2$). After anesthesia, the aspect of the right femoral shaft was exposed and a 25-mm-long (20% of the femur's total length) osteoperiosteal segmental defect was created with proper fixation by using the device. The defects then were treated with either cell-loaded scaffold, or scaffold alone or were left untreated. Animals were sacrificed at either 4 months or 8 months, respectively, for gross observation and histological and biomechanical analysis. In order to enhance tissue remolding, interlocking nails were removed at 6 months in animals that would be sacrificed at 8 months, to give the newly formed bone a mechanical loading.

As shown in Fig. 27.1, radiography examination demonstrated new bone formation 1 month after implantation at the site where BMSCs were implanted with coral. At 6 months, bone union was achieved at the defect site by engineered bone tissue, and so the interlocking nails were removed. After 2 months of mechanical loading, the engineered femur was further remodeled to become a mature bone. As observed radiologically, cortex bone was formed at 8 months post-implantation. Interestingly, marrow cavity formation inside the engineered bone was also observed at this time point. Grossly, the engineered bone also exhibited an appearance similar to that of natural femoral bone (Fig. 27.2). Histology demonstrated the formation of trabecular and woven bone at 4 months. At 8 months, the engineered bone became obviously more mature. Although still being an irregular pattern, the formed Haversian system became observable, indicating that mechanical loading plays an important role in remodeling of engineered bone. In contrast, implantation of scaffold alone without cell seeding could not generate a femoral bone tissue sufficiently to achieve a bone union both grossly and histologically (Fig. 27.3).

In addition to structural restoration by engineered bone, functional recovery is an important requirement for engineered bone repair. In this study, a three-point bending test demonstrated that engineered femoral bones at 8 months were much stronger than those of 4 months. In addition, no significant difference was found with respect to bending load strength and bend

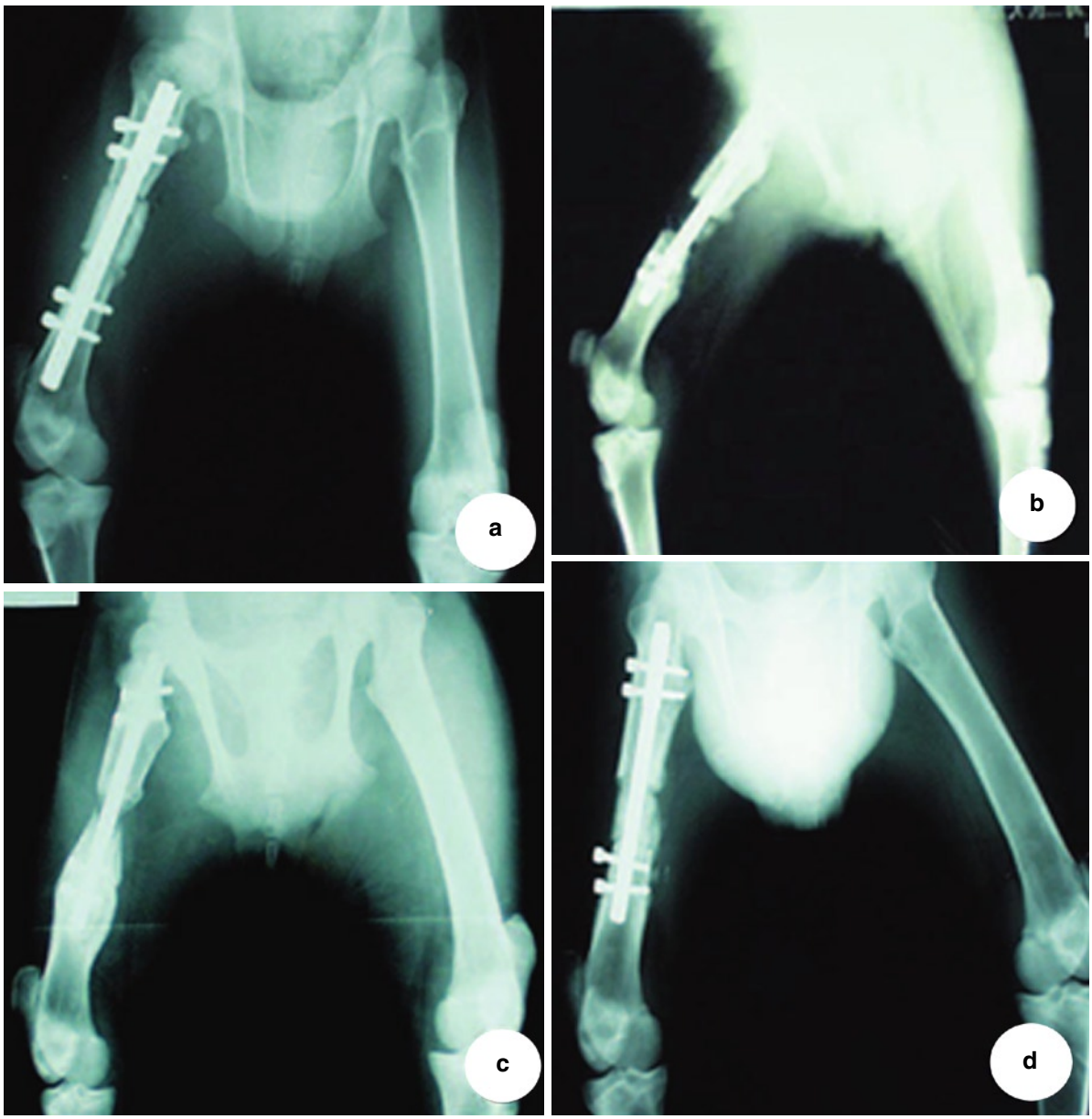

Fig. 27.1 Radiographs taken of the treated goat's femur at different time frames. (**a**–**c**) Femur defects treated with coral alone. (**d**–**f**) Femur defects treated with coral loaded with culture expanded and osteo-inducted BMSCs. (**a**) Femur defect treated with coral alone at day 1 after implantation. The cylinder with a density less than that of cortex can be found. (**b**) Coral density mostly disappeared at 1 month after implantation. (**c**) At 8 months after implantation, minimal bone formation at the bone cut ends without bridging defect. (**d**) Femur defect treated with coral loaded with culture expanded and osteo-inducted BMSCs at 1 month after implantation. The shape of the implant was maintained with callus formation at the interface to the bone cut ends. (**e**) At 6 months after implantation, newly formed bone can afford weight-bearing after removing interlocking nails. (**f**) At 8 months after implantation, the density of newly formed bone is equal to that of cortex

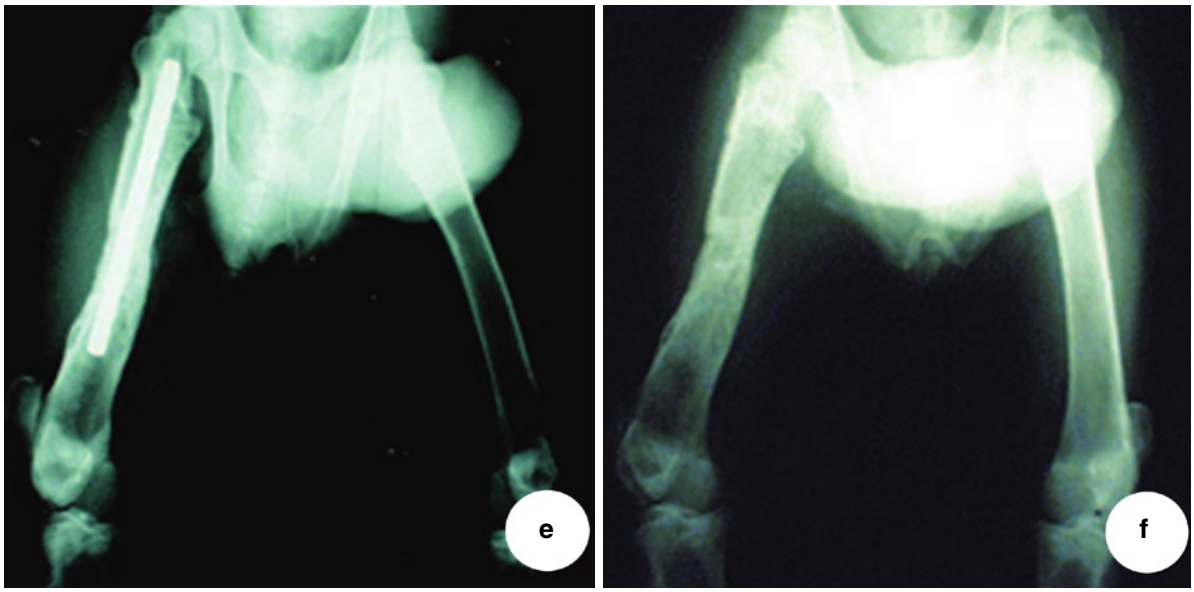

Fig. 27.1 (continued)

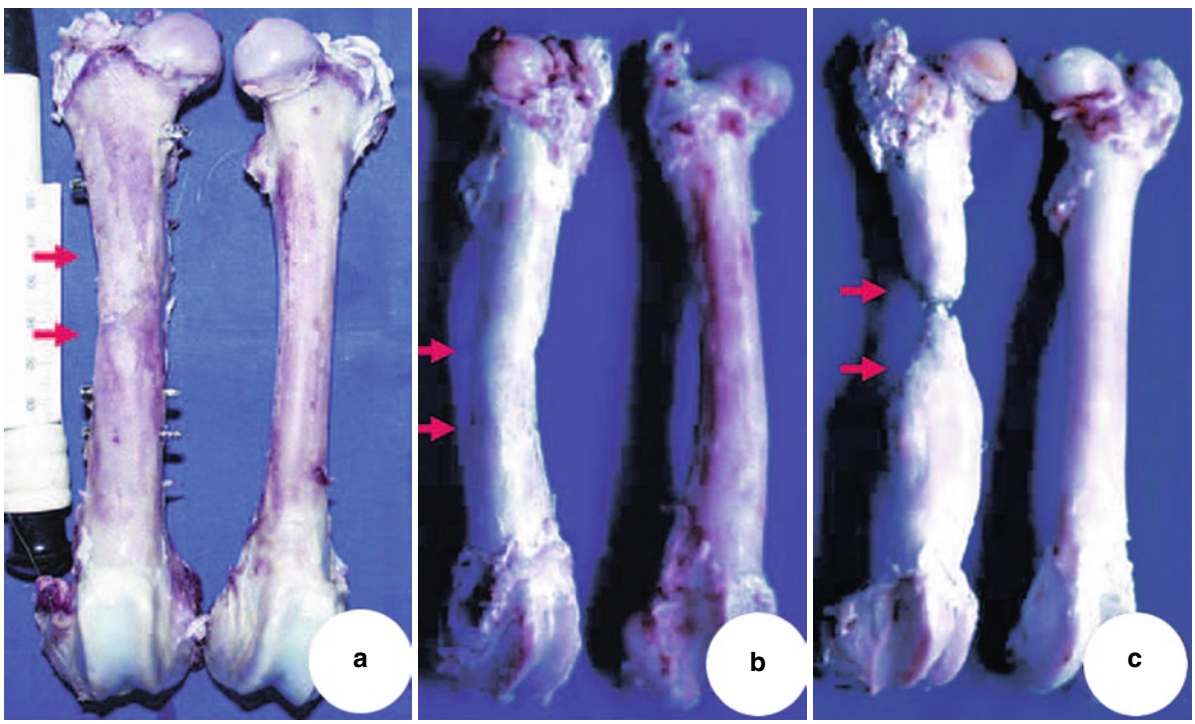

Fig. 27.2 Gross view of repaired defects in experimental and control groups. (**a**) Femur defect implanted with coral loaded with culture expanded and osteo-inducted BMSCs at 4 months. Newly cancellous bone formed with red color. (**b**) Femur defect implanted with coral loaded with culture expanded and osteo-inducted BMSCs at 8 months. White-colored, remodeled cortex formed. (**c**) Femur defect implanted with coral alone at 8 months. Non-union formed. (**a**–**c**) *Left*, experimental bone defects; *right*, normal bone serving as a positive control

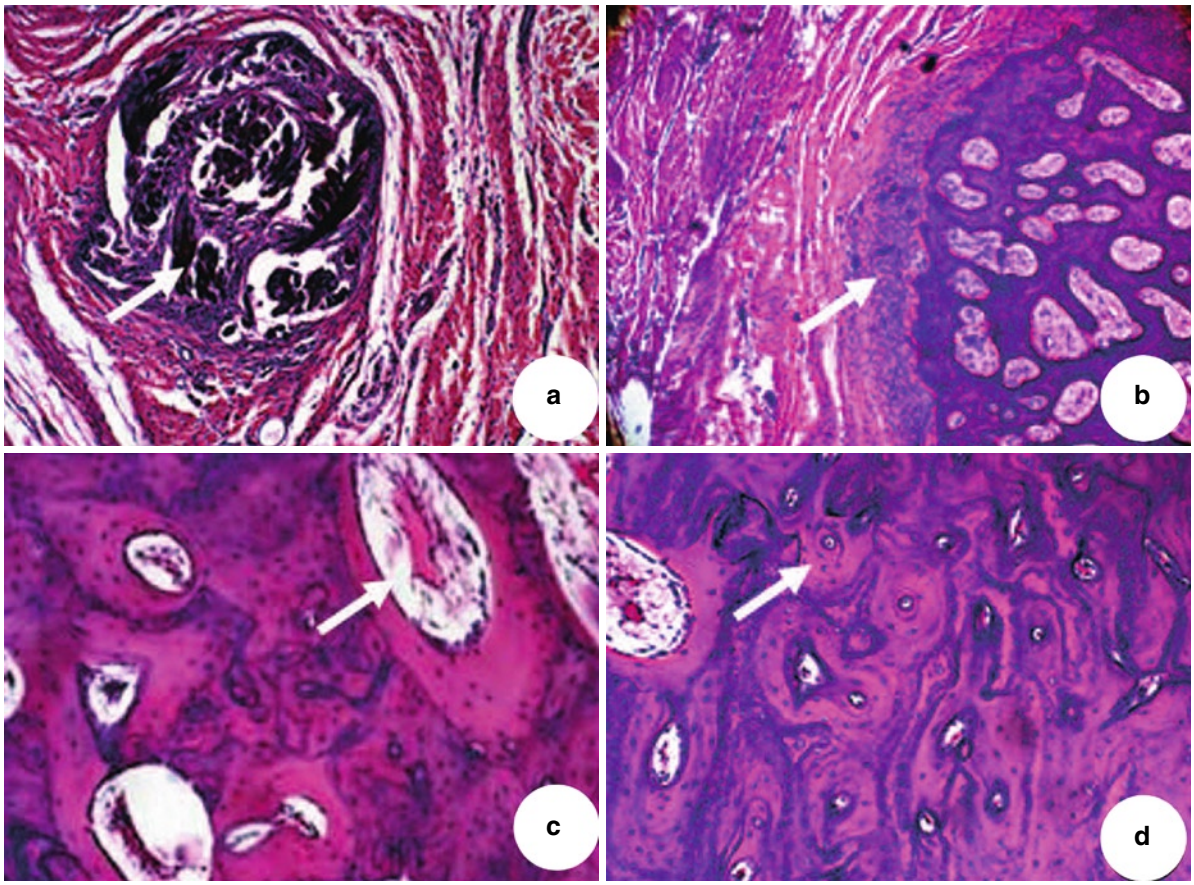

Fig. 27.3 H&E staining of repaired bone tissues. (**a**) Femur defect treated with coral alone at 4 months after implantation. Residual coral particle remained, surrounded by fibrous tissue (*arrow*). ×100. (**b**) Femur defect treated with coral alone at 8 months after implantation. Coral particle disappeared completely and a certain level of bone formed at bone cut end (×100, *arrow*). (**c**) Femur defect implanted with coral loaded with culture expanded and osteo-inducted BMSCs at 4 months. Coral completely disappeared and woven bone was formed. White regions indicated by arrows are newly formed medullary canal (×100). (**d**) Femur defect implanted with coral loaded with culture expanded and osteo-inducted BMSCs at 8 months. Irregular bone was formed (×100, *arrow*)

rigidity between the 8-month engineered bones and natural counterpart bones. However, the engineered bones were still inferior to natural bones with regard to bend intention and bend displacement.

27.3.4 Clinical Applications of Bone–Tissue Engineering

One of the first clinical trials of engineered bone [96] involved replacement of an avulsed phalanx with tissue-engineered bone. The construct was fabricated using autologous osteogenic precursor cells isolated from periosteum. Recently [109], the successful clinical treatment of a large mandibular bone defect in a male patient with tissue engineered bone was reported. A titanium mesh cage with the mandibular shape of the patient was first filled with bone mineral blocks infiltrated with recombinant human BMP-7 and bone marrow mixture, and then implanted into the patient's latissimus dorsi muscle for 7 weeks to form a vascularized bone graft. The combined engineered bone graft was then transplanted functionally to repair the mandibular defect. Bone remodeling and mineralization was also observed using scintigraphy.

Other clinical work focused on the repair of human cranio-maxillofacial bone defects using autologous

BMSCs and allogeneic bone matrix [20]. In one case a patient with a small-size cranial defect was treated. Bone marrow was harvested from the patient to isolate BMSCs. After in vitro expansion and osteogenic induction, cells were seeded on demineralized bone matrix (DBM) scaffold and cultured for 7 days prior to implantation into the defect. Computed tomography (CT) demonstrated the bone formation at 3 months post-implantation, and this was found to remain stable when followed up at 6 and 18 months post-operation. During a secondary revision operation, a bone biopsy was taken at 18 months post-implantation, and the histology showed a typical structure of cancellous bone. Therefore, it was shown that engineered bone could form and remain stable long term in a human subject.

Another craniofacial bone defect of larger size and more complicated 3D structure was also repaired with engineered bone. The patient had a craniofacial bone defect involving both frontal and orbital roof bones. To proceed with the repair, the patient's 3D CT scanning data were first obtained and then used to 3D-print a mold that was fit exactly into the defect site. According to the 3D shape of the mold, DBM materials were trimmed and assembled to form a 3D-shape scaffold. After cell-seeding and culture for 7 days, the cell-scaffold construct was implanted to repair the defect. The patient has been followed up for up to 3 years with 3D CT scanning, which demonstrated that engineered bone not only satisfactorily repaired the defect, but remained stable without absorption even after 3 years. These clinical trial results demonstrated the promise of bone–tissue engineering for complex reconstructive applications.

27.3.5 Limitations

In the early 1990s, advances in bone cell biology, combined with expertise in material science, fueled the notion of engineering bone chips in Petri dishes. The initial concept consisted of taking cells from a patient, seeding them in vitro to create bone chips that would look and feel very much like autologous bone chips, and then reimplanting them in the patient as needed. This approach was based on the idea that cells harvested from a patient's bone marrow would generate unlimited volumes of tissue, which would also be devoid of risks of rejection or disease transmission. These concepts were, in part, confirmed by advances in stem cell biology that demonstrated the potential of bone marrow aspirates to grow in culture and to generate significant numbers of stem cells.

While this design seemed to address some of the key limitations related to the use of autograft, it brought along its own challenges. From a purely logistical perspective, the in vitro phase of the tissue-engineering design was riddled with complications, for it would require a long and potentially complex regulatory path to market, carried risks of culture contamination, and was not easily optimized for large-scale applications. The economics of this approach could also prove particularly unfavorable since the majority of cases seen by orthopedic surgeries are known to respond with excellent clinical outcomes while employing fairly basic technology; the market for a true "tissue-engineered" approach would therefore be limited to a relatively small number of high-risk patients.

In addition to these logistical and financial considerations, parallel progress in medical devices, pharmacology, and cell biology further modified the bone regeneration landscape with the development of new point-of-care cell concentration devices, recombinant growth factor technologies, or allogeneic stem cell banks. The concept of an in vitro engineered bone chip may thus seem obsolete. Instead, current approaches focus on creating the right biological environment in vivo to promote bone healing.

27.3.6 Conclusions

Significant progress has been achieved over the past several decades in bone–tissue engineering. Specifically, in-depth understanding of bone marrow cells was critical to optimize the use of bone marrow in bone defects and develop methods of concentrating these osteogenic components to further enhance the healing potential of novel devices. Moreover, the success in clinical trials of repairing patient's craniofacial defects with engineered bone reveals the promising future of tissue engineering as a favored approach for bone reconstruction.

27.4 Cartilage Tissue

27.4.1 Types of Cartilage

The three types of cartilage – elastic, hyaline, and fibrocartilage – comprise various structures in the body, including the ear (auricular, AU), articular cartilage (AR), and the meniscus (ME), respectively.

Elastic cartilage contains large amounts of elastic fibers (elastin) scattered throughout the matrix. It is stiff yet elastic, and is important to prevent tubular structures from collapsing. Elastic cartilage is found in the pinna of the ear, in tubular structures such as the auditory (Eustachian) tubes and in the epiglottis. Hyaline cartilage is a rather hard, translucent material rich in type II collagen and proteoglycan. It covers the end of bones to form the smooth articular surface of joints. It is also found in the nose, the larynx and between the ribs and the sternum. Fibrocartilage is the most common form of cartilage by weight. It is characterized by a dense network of type I collagen. It is a white, very tough material that provides high tensile strength and support. It contains more collagen and less proteoglycan than hyaline cartilage and is present in areas most subject to frequent stress like joint meniscus, intervertebral discs, the symphysis pubis and the attachments of certain tendons and ligaments. Prior studies have described the isolation of chondrocytes from each of these three types of cartilage for expansion in monolayer and subsequent tissue engineering: ear [18, 19]; meniscus [74, 75]; and articular cartilage [102].

27.4.2 Cartilage Cells Isolated for Tissue Engineering

Cartilage tissue repair is an important task for plastic and orthopedic surgeons. One of the disadvantages of the traditional surgical repair procedure is the need to harvest autologous tissues from a donor site of the human body for the repair of a defect on another site, thus leaving a secondary tissue defect. Tissue engineering offers an approach to tissue repair and regeneration with less donor site morbidity.

Prior work has revealed differences among chondrocyte sources [114]. Higher cell yields and faster proliferation rates were obtained for AU chondrocytes when compared to AR chondrocytes [98]. Source-dependent differences were also found in the resulting engineered cartilage tissues, including differences in construct size, gene expression, biochemical content and mechanical properties. A comparison study of bovine nasal, AR, costal and AU chondrocytes grown on poly(L-lactide-ε-caprolactone) scaffolds [46], showed that AU chondrocyte-seeded constructs had the largest diameter, while costal chondrocyte-seeded constructs had the greatest thickness. In addition, costal chondrocytes, followed by nasoseptal, AR and AU chondrocytes, had the highest expression of type II collagen and aggrecan. Other work showed that AU chondrocyte-seeded samples produced neocartilage with greater biochemical and histological similarity to that of native cartilage than AR counterparts when implanted in vivo [81]. Later studies also showed that AU chondrocytes encapsulated in fibrin exhibited the highest equilibrium modulus compared to those encapsulated with AR and costal chondrocytes [113]. Such information is important toward isolating a specific cell source. Regardless source, behavior of the cells is dependent on the passage number, biomaterial scaffold and regulators used. The passage number and growth factor supplementation of the medium can affect AR chondrocyte behavior in tissue engineering scaffolds [49, 101]: P_0 and P_1 cells continued to increase in number and the P_2 cells decreased. In another study [100], FGF-2 treated AR chondrocytes seeded onto type II collagen displayed more qualitative signs of chondrogenesis. Other studies utilizing collagen-based matrices found that pore diameter and collagen type affected the morphology and biosynthetic activity of the seeded chondrocytes, with a greater percentage of the cells seeded in type II collagen scaffold retaining a chondrocytic phenotype than those seeded in a type I collagen scaffold [75, 77].

27.4.3 Articular Cartilage Tissue Engineering

27.4.3.1 Aim of the Discipline

With regard to clinically relevant models for cartilage engineering and repair, articular cartilage repair is the most commonly involved subject. The need for engineered cartilage arises from the fact that while the tissue often functions well through a lifetime of use, 9% of the US population age 30 and older have osteoarthritis (OA) of the hip or knee. The total cost of OA is estimated at $28.6 billion per year in the USA alone, with over 200,000 knee replacements performed each year. The intrinsic healing capacity of the native tissue is limited, and, given the increasing incidence of OA and the increasing life expectancy of the population, there is a growing demand for novel repair strategies. Effective treatment of cartilage injuries may eliminate or forestall the need for joint replacement, thus enhancing the quality of life.

27.4.3.2 State of the Art

There have been numerous reviews of articular cartilage tissue engineering [34, 97, 103].

The repair of articular cartilage defect using tissue engineering was reported many years ago. While some studies focused on repairing a cartilage defect in small animal models using allogeneic chondrocytes engineered hyaline cartilage, Liu et al. tried to repair the osteochondral defect in large mammals using autologous chondrocytes engineered cartilage [57]. In a porcine model, autologous articular cartilage at a non-weight-bearing area was harvested from the knee joint on one side. On the other side, an 8 mm full thickness articular cartilage defect deep to the underlying cancellous bone was created at both weight-bearing areas of the medial and lateral femoral condyles. The cell-scaffold construct containing (PGA), polyethylene-polypropylene hydrogel, and chondrocytes was then transplanted to repair the defects in the experimental group. In the control group, the defects were either transplanted with scaffold material alone or left unrepaired. After 4 weeks transplantation, cartilage tissues were formed in the defects of the experimental group in the gross view. Histological examination demonstrated the presence of hyaline cartilage tissue. At 24 weeks postrepair, gross examination revealed a complete repair of the defects by engineered cartilage, shown by a smooth articular surface indistinguishable from nearby normal cartilage. A cross section revealed ideal interface healing between the engineered cartilage and the adjacent normal cartilage. Histological analysis of the tissue harvested from repaired defects further revealed a typical structure of cartilage lacuna and ideal interface healing to adjacent normal cartilage as well as to underlying cancellous bone. Moreover, the engineered cartilage exhibited an enhanced extracellular matrix production and improved biomechanical properties, indicating that engineered cartilage resembles the native articular cartilage not only in morphology and histology, but also in biochemical components and biomechanical properties as well. Unfortunately, the subchondral bone defect was also repaired by the engineered cartilage rather than by engineered bone, suggesting that chondrocytes may not be an optimal cell source to achieve a physiological repair of both articular osteochondral defect.

Although chondrocytes are a useful seed cell source for cartilage engineering and are relatively safe for clinical treatment, immune rejection will still be the major obstacle to the use of allogeneic chondrocytes [60, 73], and the use of autologous chondrocytes will face the challenge of donor site morbidity and limited cell source due to their limited expansion capacity, cell aging, and dedifferentiation during in vitro expansion [84, 85]. In addition, it has been indicated that chondrocytes are not appropriate to bony repair of an osteochondral defect. Therefore, chondrocyte-based therapy may not be optimal for clinical applications. In contrast, mesenchymal stem cell-based therapy may potentially avoid these disadvantages. Of note, one current surgical strategy for enhancing the repair of cartilage defects is via microfracturing of the subchondral bone. This technique provides entrance for marrow elements, including MSCs, to the wound site, and has been shown to generate an enhanced, albeit fibrous, repair response. Recent studies showed that BMSCs have become a reliable replacement for chondrocytes due to their capability for chondrogenic differentiation and rapid proliferation [33, 87]. There have been many reports in the past decade of in vitro chondrogenic induction of BMSCs, and the induced cells could express chondrocyte-specific molecules, such as type II collagen, aggrecan, and Sox9 [42, 44, 63]. Moreover, several reports have demonstrated that induced BMSCs actually formed cartilage in vivo [32, 43, 104, 110].

The repair of articular cartilage with BMSCs has been reported for many years. However, most of these studies focused on small animal model, such as rats and rabbits. Due to the differences in species and defect size, the data obtained from small animals might be inappropriate to elucidate the repair mechanism in human beings. In addition, there is still controversy regarding the true cell source for the engineered repair of osteochondral defects, although some related studies have been performed in small animals using labeled seed cells. This is particularly true in large animal model studies, in which no direct evidence has yet been provided. Therefore, two issues remain worthy to explore in this area: (1) discovering whether the approach is feasible or not in large animals for engineered repair of an articular osteochondral defect using autologous BMSCs, especially for a long-term study; and (2) providing more convincing evidence to demonstrate the true cell source of repaired cartilage and its subchondral bone. To address these concerns, Zhou et al. performed a study of repairing articular osteochondral defect with autologous BMSCs

in a total of 18 pigs using the previously established porcine model [115]. BMSCs were isolated from hybrid pigs' marrow and treated with dexamethasone and transforming growth factor-β1. The cells were then seeded, respectively, onto polylactic acid-coated PGA scaffolds to generate cell-scaffold constructs. Four osteochondral defects in each animal were created at non-weight-bearing areas of knee joints (two defects/side). Chondrogenically induced BMSC-PGA/PLA constructs were implanted in the defects of the experimental group (Exp). In control groups, the defects were repaired with dexamethasone-treated BMSC-PGA/PLA constructs (Ctrl 1) or PGA/PLA construct alone (Ctrl 2) or left unrepaired (Ctrl 3). To trace the implanted BMSCs, cells were retrovirally labeled with green fluorescent protein (GFP) before implantation.

The results showed that cartilage tissue was formed in the experimental group defects as early as 3 months after transplantation, and an improved result was achieved after 6 months. Additionally, the experimental group achieved the best reparative results among all tested groups. Grossly, most of the defects in this group were repaired by newly formed cartilage-like tissue with relatively regular surfaces (Fig. 27.4a). Cross sections showed fine interface healing between repaired tissue and normal osteochondral tissues. The thickness of repaired cartilage approached a level similar to that of adjacent normal cartilage. Interestingly, both cartilage and bony defects were satisfactorily

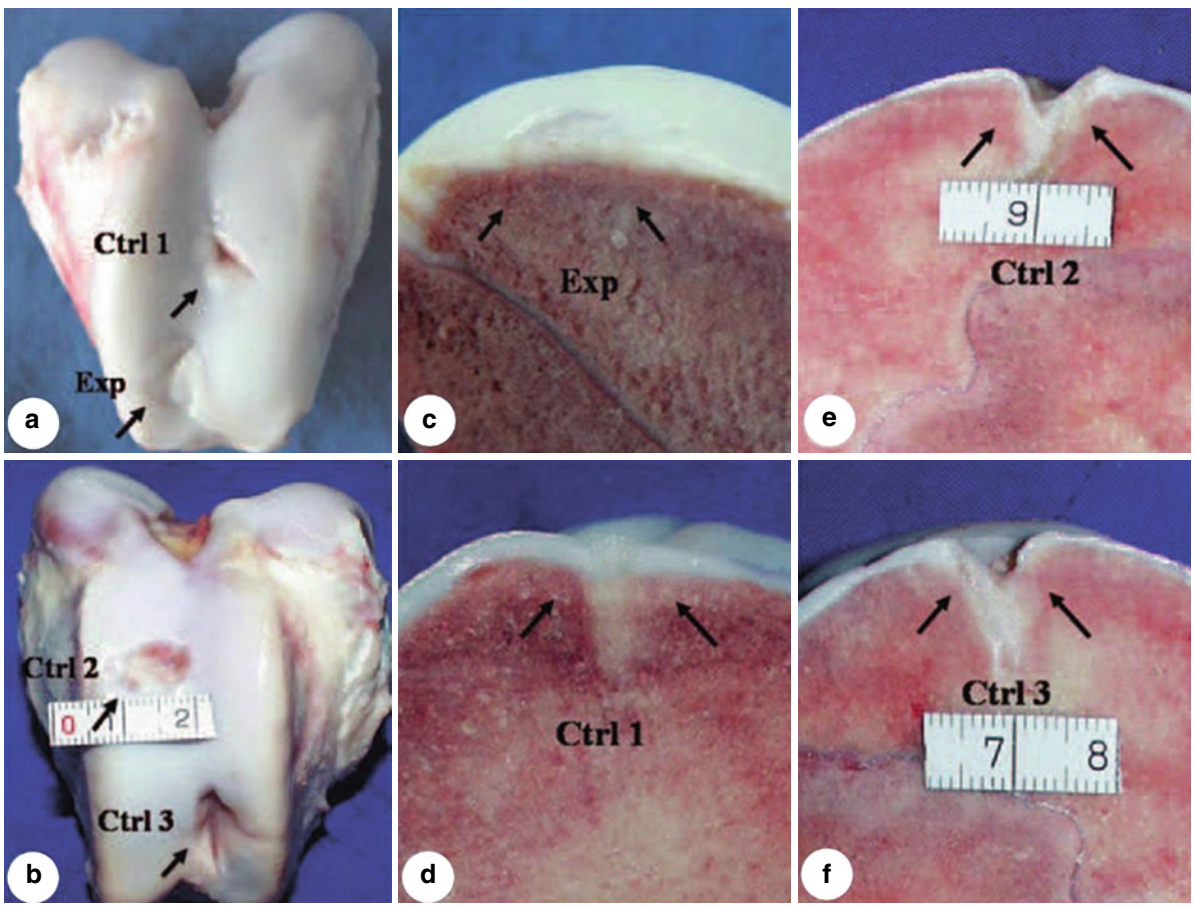

Fig. 27.4 Gross and cross section view of repaired defects at 6 months post-repair. *Arrows* indicate the repaired regions. The experimental defect exhibits a relatively regular surface (*Exp*, **a**) and the osteochondral defect is completely repaired with both engineered cartilage and bone when observed at the cross section (**c**). The repaired surface of control 1 group remains irregular (*Ctrl 1*, **a**), but the osteochondral defect is mostly repaired at the cross section (**d**). The defects in control 2 group (*Ctrl 2*, **b**, **e**) and control 3 group (*Ctrl 3*, **b**, **f**) remain largely unrepaired at both cartilage and bony layers

repaired by engineered cartilage and bone, respectively (Fig. 27.4c). However, in control group 1, only some defects surfaces and relatively normal osteochondral structures, but most defects showed irregular surfaces in the central area of the defects and poorly repaired subchondral bone (Fig. 27.4a, d). As expected, defects of control group 2 and control group 3 healed poorly, showing unrepaired defect and partial formation of osteochondral tissue only at the interface between the defect areas and the native tissue areas (Fig. 27.4b, e, f).

Histology also showed that the experimental group achieved the best results. Most defects exhibited the structures of mature hyaline cartilage and cancellous bone, with cellular density and cartilage thickness similar to those of normal tissues (Fig. 27.5a1). Importantly, a nearly normal tidemark could be observed between repaired cartilage and cancellous bone. Strong metachromatic matrix production and satisfactory interface healing were also observed, despite the fact that a tiny difference between repaired tissue and native articular tissue remains observable (Fig. 27.5a2). The results were less satisfactory in control group 1 because only some of the defects were repaired by the mature hyaline cartilage and cancellous bone. Yet the tissue quality remained inferior to that of the experimental group. In addition, a larger part of the defects in control group 1 showed fibrocartilage repair and the lack of an obvious tidemark at the osteochondral junction (Fig. 27.5b1, b2). In contrast to the experimental group and control group 1, the defects of control group 2 and 3 showed mainly fibrotic repair, with poor interface healing and poor metachromatic matrix staining at the area with the most defects (Fig. 27.5c1, c2). All these results indicate that implanted cells play an important role in tissue regeneration and repair and that in vitro

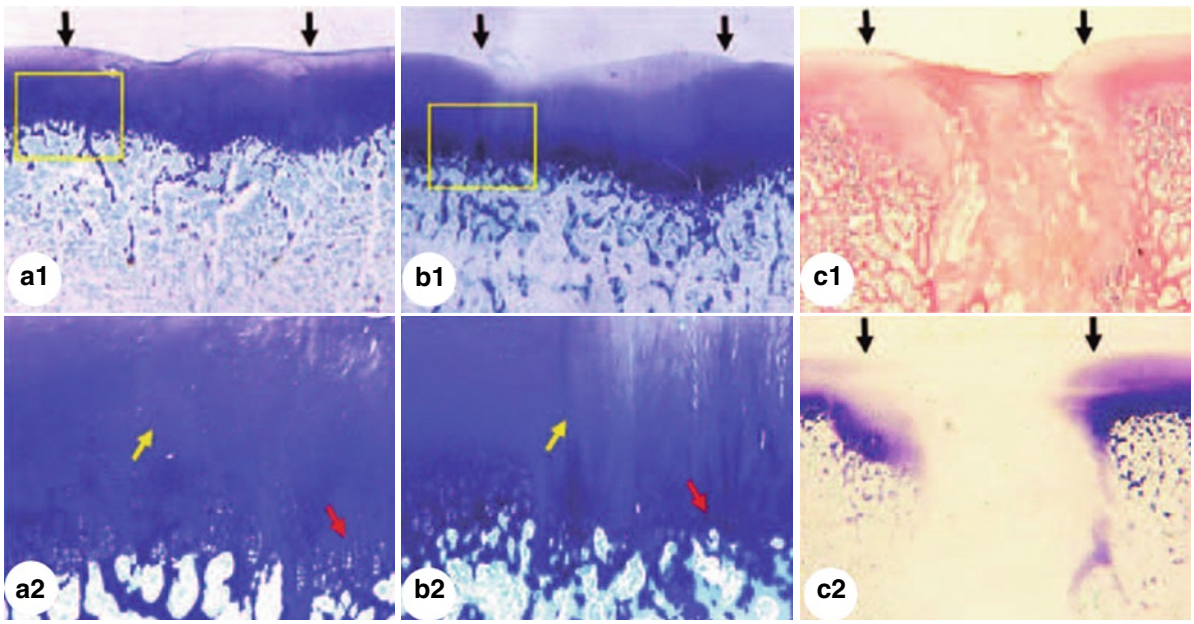

Fig. 27.5 Histology of repaired tissue at 6 months post-repair. The repaired regions are indicated between arrows. (**a**) Histologic view of repaired tissue in Exp via toluidine blue staining, representing complete repair. The rectangular region indicates the interface area (A1, ×10), which lies between the normal and engineered tissues. In the amplified view (A2, ×40), a yellow arrow indicates the interface line between native (*left*) and engineered (*right*) tissues. Interestingly, a nearly normal tidemark structure is observed in the engineered tissue as indicated by the *red arrow*. (**b**) Histologic view of repaired tissue in Ctrl 1 via toluidine blue staining, representing incomplete repair, which shows a relatively poor tissue structure in the cartilage repair area (B1, ×10). In an amplified view (B2, ×40), normal articular tissue structure (hyaline cartilage and cancellous bone) is observed at a normal region (*left side* of the interface as indicated by the *yellow arrow*) with the obvious tidemark structure. At the repair area (*right side* of the interface), however, the repaired tissue is composed mainly of fibrocartilage and cancellous bone. In addition, no mature tidemark structure is observed at the repaired osteochondral junction area (*red arrow*). (**c**) Histologic view of repaired tissue in Ctrl 2 via H&E and toluidine blue staining, representing no repair. The defect area is filled by fibrotic tissue (C1, H&E, ×10) and shows no metachromatic matrix staining (C2, toluidine blue, ×10)

chondrogenic induction is beneficial for that in vivo cartilage regeneration.

To further determine the fate of implanted BMSCs and the cell source for osteochondral tissue formation, confocal microscopy was used to examine the tissue sample in which GFP-labeled BMSCs were implanted. As shown in Fig. 27.6, GFP-labeled cells remained observable at both cartilage and subchondral bone layers, even at 7 months postoperation. Importantly, these GFP-labeled BMSCs actually formed typical lacuna structures (Fig. 27.6a1), suggesting that the implanted BMSCs have differentiated into both chondrocytes and osteoblasts, respectively, in their respective in vivo environments and that implanted BMSCs are the vital cell source for the osteochondral tissue repair.

Biomechanical property analysis of the repaired cartilage showed that the compressive moduli increased with postrepair time. At 3 months, the moduli reached 43.82 and 30.37% of normal cartilage level, respectively, in the experimental group and control group 1, and they further increased to 80.27 and 62.69% at 6 months postrepair. In addition, the glycosaminoglycan

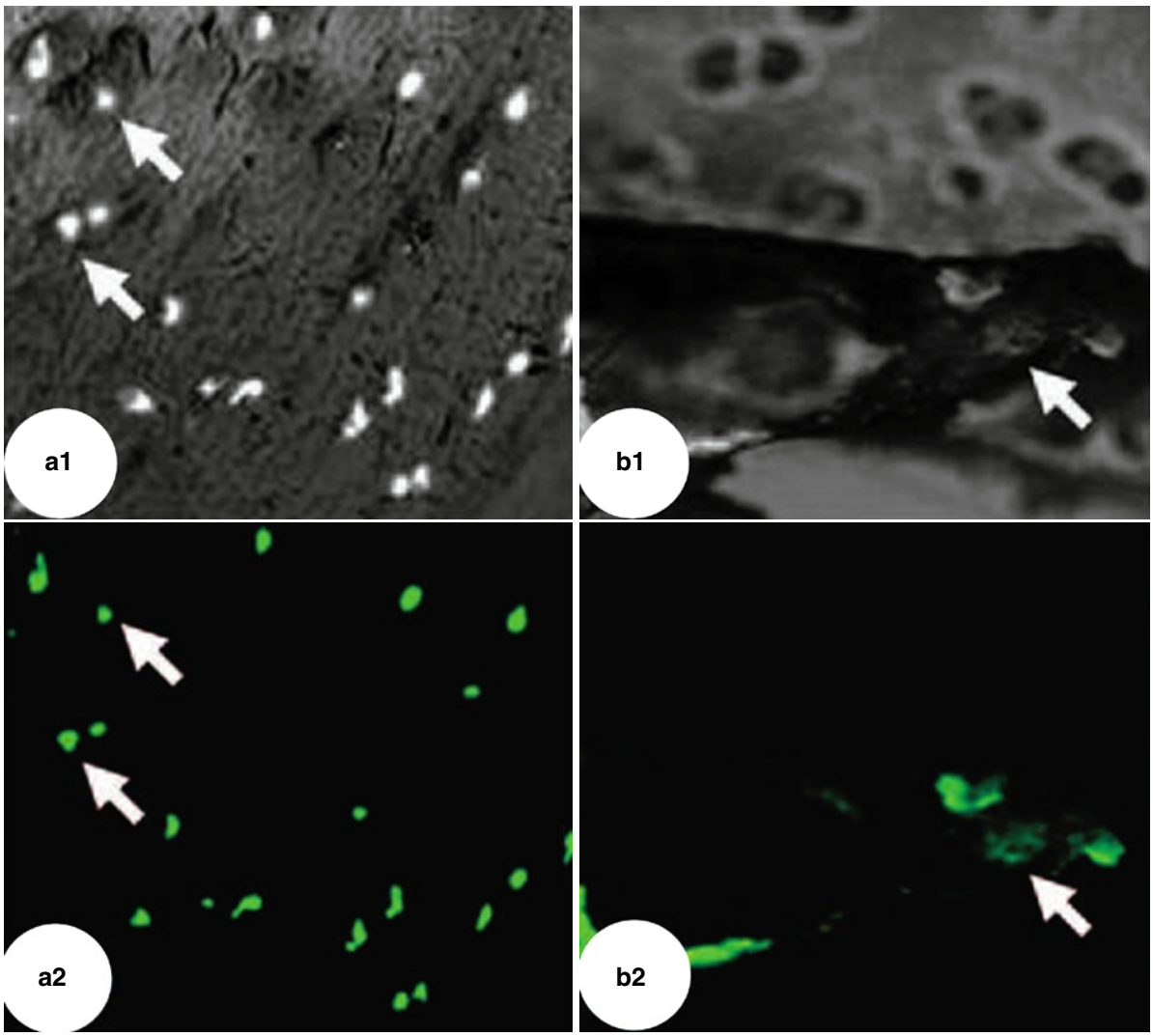

Fig. 27.6 GFP expressions in the repaired cartilage and cancellous bone at 7 months post-repair by laser confocal microscopy (×200). (**a**) GFP-labeled BMSCs are evenly distributed in repaired cartilage area and form typical lacuna structures (*arrows*). (**b**) GFP-labeled BMSCs also contribute to the bony repair of subchondral bone. *Top panel*: Images under regular light (**a1**, **b1**); *bottom panel*: images under fluorescence light (**a2**, **b2**)

content of the experimental group reached 93.25% normal content, with no significant difference between the native cartilage group and the experimental group ($p > 0.05$). Thus, BMSC-mediated repair not only restores the osteochondral tissue structure, but also generates the tissues with mechanical property and biochemical components similar to those of natural osteochondral tissues.

The foregoing study demonstrated that BMSCs are a better cell source than chondrocytes to achieve tissue engineered repair of an articular osteochondral defect. In addition, it also proves that implanted BMSCs can differentiate into both chondrocytes and osteoblasts in their related in vivo niches.

27.4.3.3 Clinical Applications

In recent years, a few clinical trials of matrix-induced autologous chondrocyte implantation have been conducted in the treatment of articular cartilage lesions [4, 61]. Positive outcomes have been achieved in short-term and long term follow-up (up to 5 years). The encouraging clinical results obtained indicate that autologous chondrocyte implantation is a safe and effective therapeutic option for the treatment of articular lesion. Furthermore, there are a few clinical trials of repairing articular cartilage defect using BMSC-engineered cartilage as well [52, 105, 106], although the therapeutic effect needs to be further follow up.

27.4.3.4 Conclusion

Many studies have demonstrated the feasibility of cartilage reconstruction in small and large animals using tissue engineering techniques. BMSCs have been shown to be a preferred cell source for tissue reconstruction. In addition, clinically relevant tissue defects should be repaired by engineered cartilage in large mammals.

27.4.4 Fibrocartilage

Fibrocartilage is the most common form of cartilage by weight. It is characterized by a dense network of Type I collagen. It is a white, very tough material that provides high tensile strength and support. It contains more collagen and less proteoglycan than hyaline cartilage. Thus, its properties are closer to those of tendon than hyaline cartilage. It is present in areas most subject to frequent stress like joint menisci, intervertebral discs, the symphysis pubis and the attachments of certain tendons and ligaments.

27.4.4.1 Meniscus

The menisci are unique wedge-shaped semi-lunar discs present in duplicate in knee joint. Its function is to load bearing, load distribution, shock absorption, joint lubrication and stabilization of the knee joint. Meniscus lesions are among the most frequent injuries in orthopedic practice and they will inevitably lead to degeneration of the knee articular cartilage. Tissue engineering of meniscus using meniscal cells and polymer scaffolds could be an alternative option to treat meniscus injury. One animal study [48] reports on the regeneration of whole medial meniscus in a rabbit total meniscectomy model using the tissue engineering technique. Biodegradable scaffolds in a meniscal shape were fabricated from PGA fiber meshes that were mechanically reinforced by poly(lactic-co-glycolic acid). Allogenic meniscal cells were isolated from rabbit meniscus biopsy and cultured in vitro. The expanded meniscal cells were seeded onto the polymer scaffolds, cultured in vitro for 1 week, and transplanted to rabbit knee joints from which medial menisci were removed. After 10 or 36 weeks of transplantation, the implants maintained their original scaffold shape approximately. Histological staining of the sections of the neo-menisci at 6 and 10 weeks revealed the regeneration of fibrocartilage, which was similar to that of normal meniscal tissue. But tissue-engineered meniscus showed differences in collagen content and aggregate modulus in comparison with native meniscus. This study demonstrated the feasibility of regenerating whole meniscal cartilage in a rabbit total meniscectomy model using the tissue engineering method. Recently, another animal study in sheep model used hyaluronic acid/polycaprolactone material for total meniscal regeneration with expanded autologous chondrocytes. There have been a wide variety of animal models that have been used in meniscus [13, 22, 107], each with certain advantages and disadvantages, but no model has yet been found that is similar to human meniscal tissue in all aspects.

27.4.4.2 Intervertebral Disc, IVD

As a single unit, IVD represents a biologically complex structure, but it can be separated macroscopically into three specific tissue components: annulus fibrosus (AF), nucleus pulposus (NP) and end-plate: (1) AF consists of a series of loosely connected concentric lamellae of highly oriented collagen (mainly type I) tissue that enclose the NP. Within these lamellae, the collagen fibers lie parallel to each other at an angel of approximately 60° to the spine axis and are oriented in opposite directions in successive layers. The peripheral area of the AF attaches to the posterior longitudinal ligament and inserts into the vertebral body via Sharpey's fibers. The innermost region merges horizontally with the NP, and the collagen fibers continue vertically through the end-plate. (2) The NP comprises a more random network of collagen fibrils (predominantly type II) that are loosely embedded in a proteoglycan-rich gelatinous matrix. This tissue is confined within the annular lamellae and exhibits a high affinity for water due to its highly negative sulfated charge. (3) End plates lie at the cranial and caudal interface between the IVD and the vertebral body. These plates are connected directly to the lamellae in the inner region of the AF, but there is no sign of collagenous connections with the underlying vertebral body. They consist of translucent, hyaline cartilage and an osseous component. The central region of the end plates is thinner and more permeable than the thicker, impermeable peripheral region. These structures contain nutrition pathways that connect to vascular buds lying in the medullary space and facilitate diffusion of molecules into and out of the disc.

Because of the complicated structure of the IVD, it is not surprising that there are fewer examples of cell-based regeneration of the IVD with multiple components ex vivo. In studies conducted by Bonassar et al. [68, 69], IVD regeneration was attempted with a fully integrated scaffold combining poly(glycolic co-lactic acid) as a scaffold for annulus fibrosus and a cross-linked alginate hydrogel as a scaffold for nucleus pulposus tissue. Primary cells for culture within each scaffold region were derived from the corresponding native IVD tissues, and the resultant cell-laden scaffolds were implanted subcutaneously into athymic mice for a period of 8 or 16 weeks. Results illustrate spatially directed matrix regeneration with extracellular matrix that exhibited distinct morphologies and contained both collagen and glycosaminoglycans. In biomechanical tests, the composite tissue engineered disc was found to have a compressive modulus about one order of magnitude lower than that of the native tissue, with a permeability to fluid flow that fell between values for the nucleus pulposus and the annulus fibrosus. Thus, this approach illustrated an ability for cell-laden scaffold folds to regenerate extracellular matrix with some of the functional and compositional features of the native tissue. In another integrative tissue-engineering study of note, Hamilton et al. [39] placed bovine articular chondrocytes on the top surface of a porous calcium polyphosphate (CPP) construct and allow it to form cartilage in vitro. Nucleus pulposus cells were then placed onto the in vitro-formed hyaline cartilage, in order to determine whether it was possible to form in vitro a triphasic construct consisting of NP, cartilage EP, and a CPP bone substitute, which mimics the natural integration of the nucleus pulposus against the vertebral endplate. Additional work will be required in adapting these integrative tissue-engineering approaches to ensure that mechanical integration with adjacent tissues is adequate, but these studies focused on generating integrated nucleus-endplate or nucleus-anulus are an important step in illustrating feasibility for this approach.

27.5 Synovium Tissue

27.5.1 Synovium Composition and Structure

The synovial "membrane" is the capsule that contains the joint fluid, which lubricates and provides nutrients to the articulating tissues of the diarthrodial joints. As such, it forms a continuous envelope about the joint and is the lining of the synovial cavity. The membrane is usually smooth and glistening, and it may be thrown into folds or processes, some of which are called villi. It is richly supplied with blood vessels, lymphatics, and nerves. The richness of blood capillaries and their proximity to the inner surface explains the hemorrhage into joints that may follow minor injuries. Although it lines a cavity, the synovial membrane is a layer of fibrous connective tissue and has no epithelial component (i.e., no epithelial cells or basement membrane).

The two types of synovial cells (type A macrophage-like and Type B, fibroblast-like) are surrounded by adhesions proteins which maintain the membranous structure of the tissue.

The synovium tissue is very variable, being composed of several layers, any of which may be present or absent at a given site in human body. The anatomical and histological boundaries of the tissue are often hard to identify. In terms of function, synovium facilitates skeletal movement by the maintenance of a fluid-filled space around cartilage or tendon surfaces. The presence of the fluid is dependent on three factors: the presence of a compact uninterrupted superficial synovial tissue layer; the subatmospheric pressure within the cavity at rest, which encourages the entry of plasma dialysate and the hyaluronic acid produced by synovial cells. There are two types of synoviocytes: macrophagic cells (type A cells) and fibroblast-like cells (type B cells). Type A synoviocytes are non-fixed cells that can phagocytose cell debris and wastes in the joint cavity, and possess an antigen-presenting ability. These type A cells, derived from blood mononuclear cells, can be considered resident macrophages like hepatic Kupffer cells. Type B synoviocytes are characterized by the rich existence of rough endoplasmic reticulum, and dendritic processes which from a regular network in the luminal surface of the synovial membrane. Type B synoviocytes are involved in production of specialized matrix constituents including hyaluronan, collagens and fibronectin for the intimal interstitium and synovial fluid.

27.5.2 Aim of the Discipline

Tendon sheath adhesions are a common postoperative complication in orthopedic and hand surgery. They result from scar formation because of extrinsic tendon healing. Creating a barrier between the repair sites and surrounding tissue layers may prevent adhesions. The ultimate tissue should produce fluid for lubrication as well.

27.5.3 State of the Art

Numerous substances consisting of either permanent or biodegradable materials have been used in tendon surgery to create either mechanical or biological barriers between surrounding tissues or the repair site [38, 66, 76, 94, 95]. For example, hyaluronic acid (HA) is a glycosaminoglycan polymer that has been found to have some beneficial effects on the prevention of adhesions in primary tendon repairs.

One early study [45] reported the fabrication of a HA membrane after drying in the refrigerator and tested it in the animal model. The flexor profundus tendons of chickens were made partial cuts at zone II in the second, third and fourth toes, and repaired using a modified Kessler type suture. HA-membranes were applied around the repair sites in the third toes while hyaluronic acid and saline were poured on to the repair sites in the second and fourth toes, respectively. At the third month, there were few adhesions in HA-membrane group microscopically while dense adhesions were seen in the saline treated group. These findings suggest that HA membrane acting as a physicochemical barrier can prevent restrictive adhesions in primary tendon repairs, but the resulting tissue cannot fully reconstruct the structure and function.

Another study [80] recently fabricated a biomembrane that may provide natural lubrication for tendon gliding, nutrition for tendon tissue, and act as a barrier to adhesion formation. Synovial cells harvested from the tendon sheath and the knee joint of a rat were cultured for 2 weeks, and then impregnated into a collagen type 1 matrix for another 2 weeks. Cells originating from both tendon and synovium demonstrated cell growth and layer formation on the surfaces of the matrix 2 weeks after impregnation. Alcian blue staining using Scott's method demonstrated the presence of acidic mucopolysaccharide, indicating hyaluronic acid production. This provides indirect evidence of functioning synovial cells on the membrane. It is possible to culture synovial cells and engineer a synoviocyte-collagen membrane that synthesizes endogenous HA. Application of this biomembrane to tendon repair sites may help to prevent adhesions after tendon repairs.

In animal model, one study in Shanghai tissue engineering center was designed to engineer tendon sheath using synoviocytes and PGA. Synoviocytes were digested from Leghorn chicken tendon sheath, and expanded in vitro. During the period of cell amplification, the ratio of type A cells became less, and it was almost the type B cells in the second passage, which were seeded onto PGA mesh to form a piece of cell-PGA construct. After 6 days in vitro culture, the

construct was wrapped on a silica gel tube and implanted in nude mouse subcutaneously. Meanwhile, the construct was used to directly repair the defected epitenon of chicken claw. Silica gel tube only encased with PGA was considered as control group. The constructs were harvested after 6 weeks for morphological and histological analysis. At the 6th week, there was dense tissue neogenesis of the subcutaneous constructs implanted into the nude mouse, and the tissue was flexible and could retain its original shape, while the control group with only PGA implanted had little tissue neogenesis (Fig. 27.7). Meanwhile, the complexes implanted into the chicken's claws had smooth surfaces along the inner wall of the membrane sheath and did not show signs of tendon adhesion, and it formed the normal synovium like tissue at the 6th week (Fig. 27.8). So it is quite possible to construct a tissue engineered tendon sheath that is similar to the natural one.

27.5.4 Conclusion

Compared to other kinds of tissue engineering research, the studies of synovium tissue engineering are much less at present. It still has to be further investigated especially in animal models for future clinical use.

27.6 Tendons and Ligaments

27.6.1 Introduction

Tendon and ligament defects are often seen in the clinical practice of plastic and orthopedic surgery, their repair remains a challenge to surgeons because of the lack of a proper tissue source for grafting. The similarities in the structure and function of tendons and ligaments warrant their being considered together in a discussion of possible tissue engineering strategies for the regeneration of these types of tissues. Undoubtedly, autograft is an ideal tissue source for repairing tendon and ligament defects. Unfortunately, only limited donor sites are available. Besides, harvesting autologous tendons and ligaments may lead to some minor functional disability. Tendon and ligament allograft or xenograft is not practical for repairing tendon and ligament defects because of immune rejection [67]. Most prosthetic replacements also fail to achieve a satisfactory long-term result of tendon and ligament repair. Therefore, seeking a biological tendon and ligament replacement that possesses both the natural structure and function became the ultimate goal in tendon and ligament repair. The tissue engineering technique, however, can generate different tissues using autologous cells and thus may provide an optimal approach to address this concern.

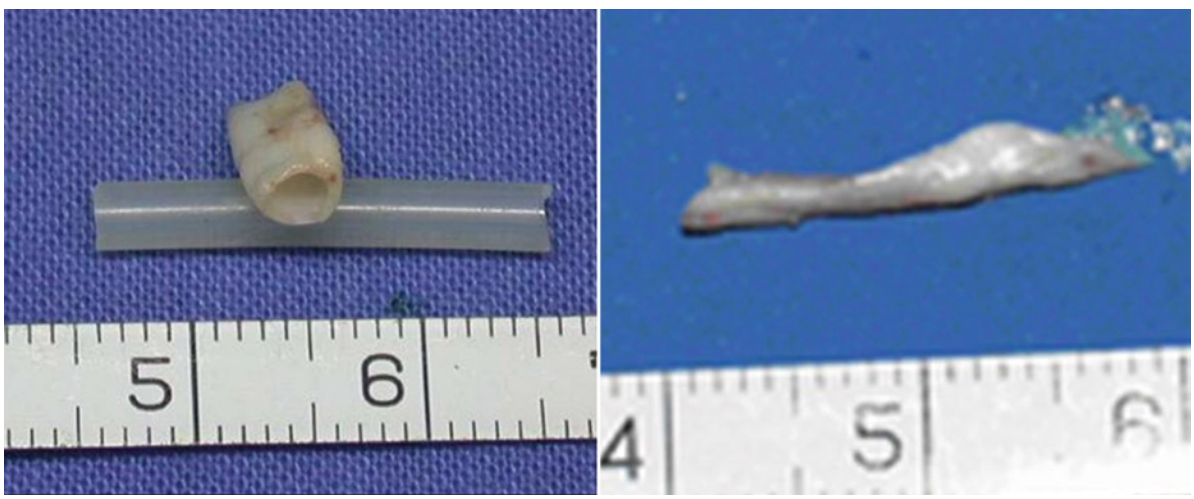

Fig. 27.7 Gross view: synoviocytes-PGA group after 6 weeks in vivo culture in nude mouse subcutaneously (*left*) and PGA-only control group (*right*)

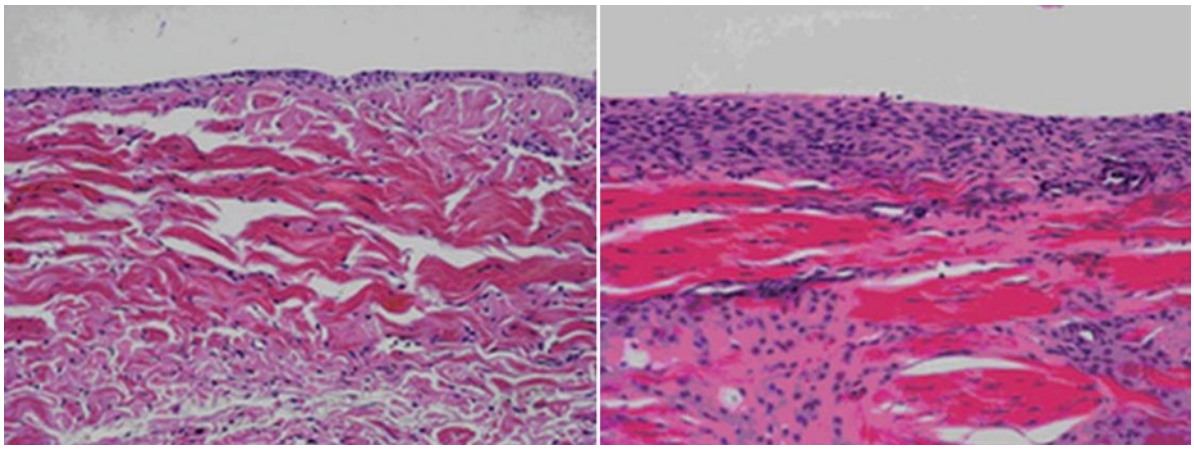

Fig. 27.8 HE staining: normal synovium tissue (*left*) and tissue engineered synovium tissue in hen model (*right*) ×100

The nature of the junction between a muscle and its tendon may not be known with certainty, and the available descriptions seem to reflect the method of study employed. It is reported that collagenous fibers of the tendon are continuous with the endomysium, perimysium, and even the epimysium of the muscle; it is also reported that muscle fibers and myofibrils gradually change into one or several collagenous fibers at this junction.

A tendon is composed of heavy, parallel bundles of collagenous fibers packed to form an exceedingly dense tissue. Between bundles of fibers are located fibroblasts and nerves and blood vessels that supply the tendon. The bundles of tendon fibers are held together by connective tissue resembling perimysium. Surrounding the entire tendon is a dense but thin fibroelastic membrane, the peri- or epitendineum, a counterpart of the epimysium.

27.6.2 Aim of the Discipline

Tissue engineering approaches applied to tendon and ligament, as with other tissues, are founded on the use of cells, biomaterial scaffolds and soluble regulators, alone or in combination. Also, as with other applications, one of the two goals might be set: to engineer tendon and ligament in vitro for subsequent implantation or to develop an implant to facilitate regeneration in vivo. Regeneration should result in tissue that is indistinguishable from the original tissue, that is, the newly formed tissue has morphological, ultrastructural, biomechanical functions identical to those of the original tissue.

27.6.3 State of the Art

Early work has focused on the implementation of absorbable synthetic and natural matrices, alone, as implants for tendon and ligament engineering by the host [40, 55]. While the results of this approach have been promising, there are indications that exogenous cells will be necessary for more complete regeneration. This has led to several in vitro investigations for the purpose of assessing the influence of the candidate cells on the scaffold for tendon and ligament regeneration.

Cao et al. [17] explored the feasibility of in vitro tendon engineering using tenocytes and PGA. Tenocytes were extracted from the tendons of a hen's foot with enzyme digestion. Unwoven PGA fibers were arranged into a cord-like construct and fixed on a U-shape spring, and tenocytes were then seeded on PGA fibers to generate a cell-PGA construct. In experimental group 1, cell-scaffold constructs were fixed on the spring with no tension and collected at weeks 4, 6 and 10; in experimental group 2, cell-scaffold constructs were fixed on the spring with a constant strain and collected after 6 weeks of culture. In the control group, cell-free scaffolds were fixed on the spring without tension. The gross and histological results showed that tendon tissue

could be generated during in vitro culture. In addition, the tissue structure and mechanical property became more mature and stronger with the increase of culture time. Furthermore, application of constant strain could enhance tissue maturation and improve mechanical property of the in vitro engineered tendon. Nevertheless, tendon engineered with constant strain appeared much thinner in its diameter than tendon engineered without mechanical loading. Additionally, its collagen fibers were highly compacted when compared to natural tendon structure, suggesting that constant strain may not be the optimal means of mechanical load.

It is unclear whether in vitro engineered tendon would become more mature after in vivo implantation particularly under mechanical loading, an important niche for tendon development after birth. In addition, less is known about the mechanism of mechanical loading in human tendon development and maturation. In a recent study [108], an ex vivo approach was developed to investigate these issues. Human fetal extensor tenocytes were isolated, expanded and seeded on PGA fibers that formed a scaffold with a shape mimicking human extensor tendon complex. After in vitro culture for 6 weeks, cell-scaffold constructs were further in vitro cultured with dynamic mechanical loading for another 6 weeks in a bioreactor. The other constructs were in vivo implanted subcutaneously to nude mice for another 14 weeks. Half of them were implanted without loading, whereas the others were sutured to mouse fascia and animal movement provided a natural dynamic loading in vivo. The results demonstrated that human fetal cells could form an extensor tendon complex structure in vitro and become further matured in vivo by mechanical stimulation. In contrast to in vitro loaded and in vivo nonloaded tendons, in vivo loaded tendons exhibited bigger tissue volume, better aligned collagen fibers, more mature collagen fibril structure, and stronger mechanical properties. These findings indicate that an extensor tendon complex like structure is possible to generate by an ex vivo approach and in vivo mechanical loading might be an optimal niche for engineering functional extensor tendon.

In animal models, one study [16] was to test the feasibility of engineering tendon tissues with autologous tenocytes to bridge a tendon defect in a hen model. In a total of 40 Leghorn hens, flexor tendons were harvested from the left feet and were digested with collagenase. The isolated tenocytes were expanded in vitro and mixed with unwoven PGA fibers to form a cell-scaffold construct in the shape of a tendon. The constructs were wrapped with intestinal submucosa and then cultured for 1 week before in vivo transplantation. On the feet, a defect of 3–4 cm was created at the second flexor digitorum profundus tendon by resecting a tendon fragment. The defects were bridged either with a cell-scaffold construct in the experimental group or with scaffold material alone in the control group. Specimens were harvested at 8, 12, and 14 weeks postrepair for gross and histologic examination and for biomechanical analysis. In the experimental group, a cordlike tissue bridging the tendon defect was formed at 8 weeks postrepair. In the control group, PGA constructs were mostly degraded at 8 weeks and disappeared at 14 weeks. At 14 weeks, the engineered tendons resembled the natural tendons grossly in both color and texture. Histologic examination at 8 weeks showed that the neo-tendon contained abundant tenocytes and collagen; most collagen bundles were randomly arranged. The undegraded PGA fibers surrounded by inflammatory cells were also observed. At 12 weeks, tenocytes and collagen fibers became longitudinally aligned, with good interface healing to normal tendon. At 14 weeks, the engineered tendons displayed a typical tendon structure hardly distinguishable from that of normal tendons. Biomechanical analysis demonstrated increased breaking strength of the engineered tendons with time, which reached 83% of normal tendon strength at 14 weeks. However, the breaking strength of the scaffold materials accounted for only 9% of normal tendon strength. The results of this study indicated that tendon tissue could be engineered in vivo to bridge a tendon defect. The engineered tendons resembled natural tendons not only in gross appearance and histologic structure but also in biomechanical properties.

Harvesting autologous tenocytes for tendon engineering may cause secondary tendon defect at the donor site. Dermal fibroblasts are an easily accessible cell source and do not cause major donor site defect. Another study [56] aims to explore the possibility of tendon engineering using dermal fibroblasts. A total of 45 hybrid pigs were randomly divided into three groups: experimental group: repair of tendon defect with a dermal fibroblast engineered tendon; control group 1: repair of defect with tenocyte engineered tendon; and control group 2: repair of defect with scaffold alone. Both autologous dermal fibroblasts and tenocytes were seeded on PGA unwoven fibers to form a

cell-scaffold construct and cultured in vitro for 7 days before in vivo implantation to repair a defect of flexor digital superficial tendon. Specimens were harvested at weeks 6, 14, and 26 for gross, histological, and mechanical analyses. Microscopy revealed good attachment of both dermal fibroblasts and tenocytes on PGA fibers and matrix production. In vivo results showed that fibroblast and tenocyte engineered tendons were similar to each other in their gross view, histology, and tensile strength. At 6 weeks, parallel collagen alignment was observed at both ends, but not in the middle in histology, with more cellular components than natural tendons. At weeks 14 and 26, both engineered tendons exhibited histology similar to that of natural tendon. Collagens became parallel throughout the tendon structure, and PGA fibers were completely degraded. Interestingly, dermal fibroblast and tenocyte engineered tendons did not express type III collagen at 26 weeks, which remained observable in normal pig skin and control group 2 tissue using polarized microscopy, suggesting a possible phenotype change of implanted dermal fibroblasts. Furthermore, both fibroblast and tenocyte engineered tendons shared similar tensile strength, about 75% of natural tendon strength. At 6 weeks in control group 2, neo-tissue was formed only at the peripheral area by host cells. A cord-like tissue was formed at weeks 14 and 26. However, the formed tissue was histologically disorganized and mechanically weaker than both cell-engineered tendons. These results suggest that dermal fibroblasts may have the potential as seed cells for tendon engineering.

27.6.4 Critical View

Repair, in the classical use of the term, results in fibrocollagenous tissue which is distinguishable from the original tissue and is known as "scar." Scar tissue tends to be more vascular and have increased cell density, in comparison to tendon and ligament. Reparative scar has a higher cell metabolism and the presence of inflammatory cells can be seen. The collagen fibrils are smaller in scar and there are defects in collagen packing, when compared to tendon and ligament. The cross-links between the collagen fibers are also immature. Biochemically, repair tissue contains more type III and type V collagen than normal tendons [59]. Scar tissue also has larger proteoglycans, an excess of other glycoproteins and immature elastin in comparison to normal ligaments. Some studies have claimed regeneration of tendon; however, there were still differences from the original tendon. In this ongoing attempt to regenerate tendon, we would inevitably move from a spectrum from complete scar to 100% regenerated tendon. Controversy, however, exists as to which parameters serve as the best measure for tendon regeneration. Some authors have measured differences such as crimp pattern, average fibril diameter, and distribution of the collagen fibrils within the tissue. Other measures include using polarized light to determine the orientation of the collagen fibers [50].

27.6.5 Conclusion

In the past decade, significant progress has been made in tendon and ligament tissue engineering including cell source, biological effect of mechanical stretch on cells, on in vitro cultured cell-scaffold constructs and on in vivo tendon formation, scaffold development for tendon and ligament engineering. Apparently, these accomplishments were achieved individually in different systems, and further integration of these achievements might be the future direction in tendon and ligament research in order to provide valuable information to guide functional tendon and ligament engineering and practical application.

27.7 Skeletal Muscle

27.7.1 Introduction

In keeping with its function of contraction, muscle has morphological attributes that make contraction possible. Equally important, muscle has attributes that make it possible for its contraction to be translated into mechanical work. Irrespective of its shape, muscle has a dimension of length; an object without length cannot shorten or change in length. As seen with the naked eye, muscle is fibrous in nature, and the fibers are oriented parallel to the long axis of the muscle.

The reconstruction of skeletal muscle tissue using tissue engineering methods holds promise for the treatment of a variety of muscle diseases, including skeletal myopathies such as muscular dystrophy or spinal muscular atrophy. In addition to traumatic injury, aggressive tumor ablation and prolonged denervation are

common clinical situations that often result in significant loss of muscle tissue and require subsequent surgical reconstruction. Until now, only few alternatives exist to provide functional and esthetic restoration of lost muscle tissues except free muscle flap transfer. This reconstructive technique, although a common practice, is associated with significant donor site morbidity causing functional loss and volume deficiency. Therefore tissue engineered skeletal muscle could be a promising approach. Even though some tissue engineering techniques attempt to regenerate human tissues have recently entered into clinical practice such as skin, bone and cartilage. However, the engineering of skeletal muscle tissue still remains a challenge.

27.7.2 Aim of the Discipline

Skeletal muscle tissue engineering depends on the regenerative properties of satellite cells and their potential for proliferation and differentiation, since these primary skeletal muscle cells can be harvested from adult muscle and successfully grown in vitro [2, 9]. Important requirements of engineering functional skeletal muscle are a parallel alignment of myofibrils with myosin/actin filaments, intracellular calcium-storage and acetylcholine receptors, which are needed for direct forces and functional use. Moreover, the neotissue should be biocompatible, vascularized and finally innervated [3, 99].

27.7.3 State of the Art

In order to obtain large volumes of tissue engineered skeletal muscle, we need huge amount of myoblast cells. It is the fact that when passaging primary myoblast cells, the differentiation ability of these cells will lose. To overcome these problems, many studies were focusing on in vitro generation of muscular tissue cell lines such as C2C12 which is an established cell line of satellite cells from skeletal muscle of C3H mouse [1, 79]. However this approach seems to have disadvantages, the myogenesis of established cell lines is less closely than that of primary myoblasts. Therefore primary cultures of satellite cells from between the basal lamina and sarcolemma are the preferred source of myoblasts because they recapitulate muscle development more precisely than immortal myogenic cell lines [8, 62].

Studies on the replacement of muscular tissues using tissue engineering methods have only recently started and many investigators have focused on the creation of functional muscle tissues in vitro. However, few studies on differentiation of myoblasts within a 3-D matrix have been reported and living tissue substitutes for functional skeletal muscle replacement have not yet been developed successfully. To achieve this goal it is necessary to investigate novel approaches for culturing functional, differentiated skeletal muscle tissue in vitro using primary myoblasts for autologous transplantation. An understanding of the molecular control mechanisms of muscle development and differentiation is therefore an important prerequisite. Many steps involved in the development of myoblasts from mesodermal precursor cells and their subsequent differentiation into multinucleate muscle fibers correspond to the expression of specific transcription factors and signaling systems controlling each developmental event. The factors which play a major role in controlling the events leading to skeletal muscle development are MyoD, myf-5, myogenin and myf-6/MRF4/herculin, a family of myogenic basic helix-loop-helix transcription factors [35, 70, 71, 112]. The proper spatial and temporal expression of these transcription factors is critical for successful myogenesis.

There are several attempts to induce fusion of myoblasts to myotubes in vitro, imitating the in vivo conditions during myogenesis. Critical issues include an understanding of the effects of mechanical and electrical stimulation on cultured myoblasts and the role of the extracellular matrix in the migration, proliferation and differentiation of the cells.

Mechanical stimulation is one of the important factors during myogenesis which influences gene regulation, endogeneous protein expression, protein accumulation and metabolic activity [35, 36]. Both passive and active mechanical forces play an important role in the transition of skeletal muscle from the embryonic to the mature state. Directed mechanical tension is important to organize myoblasts into functional aligned myotubes and provides a stimulus for the expression of mature isoforms of myofibrillar proteins. It has also been shown that mechanical forces have important impact in mature skeletal muscle on myofiber diameter, cell number and myofiber compositon. Based on this knowledge, Powell et al reported the development of 3-D scaffold based on collagen and Matrigel by mechanical stimulation [88]. This model allows determining the cellular effects of mechanical stimulation, particularly those associated with cytoskeletal rearrangements. In order to improve the ratio of

muscle fibers and extracellular matrix, Powell et al. created a mechanical cell stimulator that is able to stretch and relax the cell cultured in vitro. A force transducer was able to measure passive forces and viscoelastic properties. The mechanical stimulation improved the structure of the engineered skeletal muscle. However, the tissue that resulted in this kind of studies is still not an appropriate substitute for functional implantation in vivo. To summarize, the cellular effects of applied mechanical force seem to be an important aspect to the in vitro development of differentiated functional muscle tissue.

The application of electrical stimulation [30, 111] is another approach of developing a higher differentiated and more functional skeletal muscle tissue. It mimics the nerve stimulation during myogenesis and during regeneration of injured skeletal muscle. Induced contractile activity was shown to promote the differentiation of myotubes. Results on directly stimulated, aneural myotubes indicated that neurally transmitted contractile activity may be an important factor in modulating phenotype expression of secondary myotubes. In one study [28], rat myoblasts were cultured, suspended in fibrin gel, and implanted within silicone chambers around the femoral vessels and transected femoral nerve of syngeneic rats for 4 weeks. Neurotized constructs generated contractile forces five times as high as the non-neurotized controls. Indirect stimulation via the nerve elicited contractions of neurotized constructs. Curare administration ceased contraction in these constructs, providing physiologic evidence of neuromuscular junction (NMJ) formation. Histology demonstrated intact muscle fibers, and immunostaining positively identified NMJs. These results indicate that neurotization of engineered skeletal muscle significantly increases force generation and causes NMJs to develop, allowing indirect muscle stimulation.

The composition of the extracellular matrix also plays a key role in the alignment and differentiation of myoblasts. The matrix must be biocompatible and should be bioresorbable. The matrices used in tissue engineering are divided into synthetic and biologically-derived biomaterials. Saxena et al. used PGA meshes seeded with myoblasts to transplant cells in vivo [92]. After 6 weeks a vascularized muscle like tissue could be noticed. Various other biomaterials including Matrigel, collagens and alginate hydrogels have been used to replace the ECM in vitro [31, 90], either to enhance the attachment of myoblasts or to alter their growth. However, these matrices are not totally biodegradable and some are potentially immunogenic. Furthermore, Borschel et al. produced engineered skeletal muscle using an acellularized mouse extensor digitorum longus muscle as a scaffold [11]. C2C12 myoblasts were injected into the acellular muscle matrix and isometric contractile force testing of the constructs demonstrated production of longitudinal contractile force on electrical stimulation. Electron microscopy studies demonstrated recapitulation of some of the normal histologic features of developing skeletal muscle. Since in vitro skeletal muscle tissue engineering involves culturing isolated primary myoblasts in an environment leading to the formation of a 3-D tissue construct, ideal matrices for such an approach should provide a high surface area for cell-matrix interactions, sufficient space for extracellular matrix generation, and a minimal diffusion barrier during in vitro culture. Moreover, the matrix should be absorbable once it has served is purpose of providing a primary structure for the developing tissue. In many studies fibrin has been shown to provide these basic conditions as an ideal cell culture matrix: it is biocompatible and biodegradable and consists of key proteins of the ECM. Since cellular growth and differentiation depend on a structured environment which the cells need to interact with, fibrin supports the migration capacity of cells, allows the diffusion of growth and nutrition factors and has a high affinity to bind to biological surfaces. These properties are basic features of the hybrid skeletal muscle tissues which were developed: the incorporation of the myoblasts into a 3D fibrin matrix. In one study, Beier JP introduced a way of injectable muscle [7]. They injected expanded primary male myoblasts into muscle defects in female syngeneic rats using a two-way syringe (Duploject) within a three-dimensional fibrin matrix. The result showed increasing integration into host muscle fibers in a time-dependent manner, exclusively at the injection site. Antidesmin staining revealed a conserved myogenic phenotype of transplanted cells. The fibrin matrix resolved over a period of 12 weeks, with no indication of an inflammatory reaction. The results of this study confirmed that obviously the presence of a 3D environment and of neuronal tissue is required for the understanding of the control mechanisms which are essential for in vitro regenerating of highly differentiated skeletal muscle tissue. However, aside from the induction of the differentiation of muscle cells, issues such as vascularization and innervation of in vitro generated muscle tissue constructs have to be more addressed, to provide functional muscle tissue in large volumes for clinical applications.

27.7.4 Critical View

Therapeutic treatments for acquired and inherited skeletal myopathies and loss of functional muscle tissue require the ability to either the implantation of differentiated muscle tissue constructs or the injection of muscle-precursor cells into sites of dysfunction or tissue deficiency for subsequent formation of new muscle tissue. The implantation of engineered myoblasts has been utilized as a potential therapy for genetic muscle diseases such as Duchenne's muscular dystrophy or for the repair of damaged myocardial tissues. The rationale behind this strategy is that the implantation of large numbers of myoblasts results in the fusion of these cells to the affected tissue, thereby improving the functional status of the muscle. Early results have demonstrated that exogenously introduced myoblasts are incorporated into local target sites and fuse with existing myofibers. However, these techniques, although shown to improve the architecture and function of muscle as the myoblasts incorporate and differentiate, is limited by the large numbers of cells required and sites that must be injected. Nevertheless implanted and in vitro transfected myoblasts might serve as vehicles for the delivery of other recombinant proteins such as angiogenic factors and growth factors as insulin like growth factor 1, erythropoietin and VEGF. This myoblast-targeted gene therapy with the potential for local production and release of needed therapeutic proteins holds promise for the treatment of several myopathies as well as other diseases, lacking important functional protein.

In contrast to these myoblast transfer strategies, other researchers in the field of muscle tissue engineering are more focusing on in vitro differentiation and maturation of satellite cells harvested from adult skeletal muscle. This approach of in vitro development of bioartificial muscle could be an alternative source for treating muscular disorders as described above. One approach uses in vitro designed and pre-fabricated artificial muscle tissue equivalents to reimplant the new-tissue after differentiation has taken place (in vitro tissue engineering). The second approach uses the application of isolated satellite cells, after expansion of cells in vitro using an appropriate transport matrix, which allows differentiation into myotubes in vivo to occur. Future developments and decision regarding which approach is more promising depend on the elucidation of the relationships among cell growth and differentiation, the cell integration capacity in the host in in vivo experiments and the capability to induce vascularization of tissue equivalents in vitro.

27.7.5 Conclusion

Tissue engineering (regenerative medicine) is an exciting interdisciplinary field, which applies the principles of engineering and biology to the development of viable substitutes that restore the function of damaged tissues and organs. As the techniques of tissue engineering become more sophisticated, the usefulness of these methods for reconstructing a muscle will hopefully become true.

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