Stem Cell Biology and Regenerative Medicine

Anna C. Berardi *Editor*

Extracellular Matrix for Tissue Engineering and Biomaterials

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Stem Cell Biology and Regenerative Medicine

Series editor

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Our understanding of stem cells has grown rapidly over the last decade. While the apparently tremendous therapeutic potential of stem cells has not yet been realized, their routine use in regeneration and restoration of tissue and organ function is greatly anticipated. To this end, many investigators continue to push the boundaries in areas such as the reprogramming, the stem cell niche, nanotechnology, biomimetics and 3D bioprinting, to name just a few. The objective of the volumes in the Stem Cell Biology and Regenerative Medicine series is to capture and consolidate these developments in a timely way. Each volume is thought-provoking in identifying problems, offering solutions, and providing ideas to excite further innovation in the stem cell and regenerative medicine fields.

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Extracellular Matrix for Tissue Engineering and Biomaterials

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Preface

The extraordinary amount of attention being paid to extracellular matrices, and to tissue engineering in general, reflects the enormous potential for significant medical breakthroughs in this field for the foreseeable future.

A vast number of experts from a variety of specialized areas have already contributed to the understanding of the nature and processes involved in the extensive field of tissue regeneration and repair and initial steps have been taken in the attempt to imitate, control, or stimulate some of the ordinary, yet complex, biological processes, and interactions, in living cell-structures.

This book, which attempts to "photograph" the state of the art of extracellular matrices and tissue engineering, features contributions from some very eminent minds involved in this field of research and from those who will most probably be helping to carry this investigative work forward over the next several decades. The variety of content aims to provide both detailed specific information on salient points and a broad context of the range of issues pertinent to the subject area, with the hope of enhancing understanding and stimulating further interest.

Greater awareness of the specific roles of the extracellular matrix in tissue growth and regeneration is continually furthering our ability to design and create artificial matrices and to develop and refine the necessary techniques and materials to facilitate their use in a wide range of clinical practice.

It is hoped that by perhaps reaching out across neighboring disciplines the present work might in some small way contribute to the great developments yet to come.

Pescara, Italy — Anna C. Berardi

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Abbreviations

DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
GAG	Glycosaminoglycan
HA	Hyaluronan
LPS	Lypopolysaccharide
MMP	Matrix metalloproteinase
NO	Nitric oxide
NOS	Nitric oxide synthase
PPAR	Peroxisome proliferator-activated receptor
TLR	Toll-like receptor
TSP	Thrombospondin
TIMP	Tissue inhibitor of metalloproteinases

Part I
Extracellular Matrix

Chapter 1
The Extracellular Matrix, Growth Factors and Morphogens in Biomaterial Design and Tissue Engineering

Caterina Bason, Marialucia Gallorini and Anna C. Berardi

Abstract Cells, morphogens, growth factors, and custom scaffolds are the critical ingredients for successful tissue regeneration in which morphogens and growth factors function sequentially. Extensive studies, in vitro and in vivo, have been made to explore the mechanisms and the roles played by these molecules. As a consequence, precise, localized control over the signaling of these factors and appropriate strategy selection, depending on the tissue or organ to be repaired or regenerated, is known to permit specific management of regenerative processes. The first part of the chapter examines natural ECMs which are a set of molecules secreted by cells that provide structural and biochemical support to the surrounding cells. ECMs also perform many other functions, such as actively regulating cell function through the control of biochemical gradients, cell density, spatial organization, and ligand attachment, thus influencing various types of cell processes. Subsequently, growth factors and morphogens are examined in greater depth to clarify to what degree progress has been made into improving methodologies and functionality and, perhaps, to hint at what remains to be done for the future of tissue engineering.

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Introduction

Over recent years, the therapeutic approach to repairing, regenerating, or replacing tissues or organs damaged by disease or injury has evolved, developing a specific biomedical field called "tissue engineering" (TE) in which cells, scaffolds, and biologically active molecules are combined to functional tissue and organs [1].

Thus, the TE prototype depends on the successful interaction between three components: (1) autologous or xenogenic cells from which the tissue itself will be formed, (2) synthetic scaffolds that hold the cells together and shape the physical form of the tissue, and (3) biological signaling molecules, such as growth factors, which instruct cells to express a desired phenotype.

Adult stem cells or induced pluripotent stem cells have most recently been favored, which can directly provide replacement cells for tissue and organs. Coupling these with synthetic or natural biomaterials which mimic the tissue/organ architecture and signaling permits cell adhesion, proliferation, and differentiation into specialized cells. These TE scaffolds should be porous, three-dimensional structures (3D) so as to match the porosity, pore size, and interconnectivity of native tissue/organs, allowing and enhancing cellular attachment and proliferation, and providing space in which new tissue growth and vascularization can occur.

The critical ingredients for tissue regeneration are cells, morphogens, growth factors, and scaffolds. In tissue regeneration, morphogens and growth factors function sequentially. Extensive studies, in vitro and in vivo, have been made to explore the mechanisms and the roles played by these molecules. As a consequence, precise, localized control over the signaling of these factors and appropriate strategy selection, depending on the tissue or organ to be repaired or regenerated, is known to permit specific management of regenerative processes.

Examples of engineered tissues are artificial skin and cartilage, and musculoskeletal substitutes that have been approved by the Food and Drug Administration (FDA); however, currently they have limited use in human patients. Many other examples have been detailed in the literature including: ligaments, tendons, and bone, as well as more complex organs like hearts, lungs, kidneys, pancreases, and liver tissue. All of these exhibit structural, mechanical, or metabolic function [2]. The ultimate goal of tissue engineering is to orchestrate body regeneration by specifically controlling the biological environment.

Key Molecular ECM Components

Tissue and organ regeneration in the adult are complex processes that often represent a recapitulation of embryonic development and patterning processes and which seek to repair tissue and/or to maintain integrity and function. Increasing

understanding of how cells and biological systems respond to stimuli continues to inspire attempts to successfully apply biological concepts in tissue engineering. In order to optimize the properties of synthetic materials, greater comprehension of the native microenvironment of the cells is required, since it is the dynamic interaction of tissue-generating cells with the surrounding microenvironment, which is largely made up of the extracellular matrix and other cell types (including fibroblasts, macrophages, and plasma), which regulates tissue formation characteristics.

Various strategies including a variety of materials and modulating agents, such as ECM-inspired, biomaterial scaffolds, cells, morphogens, and growth factors, have been progressively investigated and explored [3], in the attempt to stimulate regeneration and repair damaged sites [4].

Natural ECMs are a set of molecules secreted by cells that provide structural and biochemical support to the surrounding cells. Not only do ECMs structurally support the cells, but they also perform many other functions. They actively regulate cell function through control of biochemical gradients, cell density, spatial organization, and ligand attachment, influencing various types of cell behavior, such as proliferation, adhesion, and migration, and regulating cell differentiation and death [5]. They, further, contribute to the mechanical properties of tissues and even actively participate in the establishment, maintenance, and differentiation of tissue and organs, modulating growth factors, the level of hydration, and the pH of the local environment. These provide a degradable physical environment which permits neovascularization and remodeling as a response to dynamic tissue processes such as wound healing [6, 7]. All these varied functions are achieved by means of complex chemical composition and organization. Despite the fact that the main constituents of ECMs are water, proteins, and polysaccharides, ECMs are highly specific in their structural and biochemical composition for each tissue and organ. All this is a result of the innate properties of the interaction of those constituent molecules with the activities of resident cells. Furthermore, the structure and the composition of ECMs are not static but dynamic in response to environmental stimuli which may be physiological in origin or stress-related.

An ECM is a complex network composed of fibrous proteins (including the different types of collagen and elastin), and an interstitial component formed by glycoproteins of adhesive nature (such as fibronectin, laminins, vitronectin, thrombospondin, chondronectin, osteonectin, and fibrin). This is embedded in a well-hydrated, viscoelastic gel, consisting of anionic proteoglycan polymers and glycosaminoglycans (such as heparan sulfate, chondroitin sulfate, dermatan sulfate, keratin sulfate, and hyaluronic acid) [8]. Precise categorization into only structural or functional components of the ECM is impossible because many molecules play a role in both aspects in healthy functioning processes and in disease states.

Prominent Role of the Proteoglycans and Glycosaminoglycans

Proteoglycans (PG) are composed of a protein axis to which one or more glycosaminoglycan chains (GAGs) are covalently linked. Usually the same protein binds to a single type of GAG. However, more than one type of GAG exists, such as those found in cartilage-based proteoglycans, which contain similar quantities of chondroitin sulfate and keratan sulfate [8]. GAG variety depends on the location of the ECM, age, and gender of the individual. GAGs promote water retention and contribute to gel-like properties of the ECM. GAGs also bind cytokines and growth factors and retain them in the ECM [9]. Around 36 ECM PGs have been identified in mammals with multiple, diverse functions, which also vary in different types of tissue. There are three main families of PG: (1) small leucine-rich proteoglycans (SLRPs), (2) modular proteoglycans, and (3) cell surface proteoglycans [10]. SLRPs, which are ubiquitously expressed in most ECMs, are the largest class of PGs. They can function as structural components and are involved in multiple signaling pathways. SLRPs bind with various collagens, tyrosine kinase receptors (RTK), and innate immune receptors, and, in so doing, they participate in several biological functions [11], being able to regulate fundamental processes like migration, proliferation, innate immunity, apoptosis, autophagy, and angiogenesis [11].

Furthermore, they bind to and activate epidermal growth factor receptors (EGFR), insulin-like growth factor 1 receptor (IGF1R), and low-density lipoprotein receptor-related protein 1 (LRP1).

Hyalectans, which are modular PGs, are key structural components of central nervous systems, cartilage, and blood vessels and can modulate cell adhesion, migration, and proliferation, by binding hyaluronan to form complexes of high viscosity. The hyalectan family, found in interstitial membrane matrices, contains aggrecan, versican, neurocan, and brevican [10]. The essential role of aggrecan, the principal load-bearing proteoglycan in cartilage, has been confirmed by several studies. Versican, a large chondroitin sulfate proteoglycan, forms long filaments in the ECM and has been shown to play an important role in modulating inflammatory responses to tissue injury. Basement membrane modular PGs (perlecan, agrin, and collagen type XVIII) have a dual function as pro- and anti-angiogenic factors [12], mediate ligand, cell–matrix and cell–cell interactions, and interaction with integrins and RTK.

Heparan sulfate (HS) binds a large number of extracellular proteins. The functions of heparan sulfate-binding proteins range from extracellular matrix components to enzymes and coagulation factors, as well as most growth factors, cytokines, chemokines, and morphogens. For example, heparin sulfate proteoglycans (HSPGs) bind FGFs and sequester these molecules for storage. HS regulates a wide range of biological activities, including developmental processes, coagulation, and angiogenesis [13].

The heparan sulfate (HS) proteoglycan, perlecan, is ubiquitously found on the apical cell surface and in the basement membrane of both vascular and avascular tissues. Perlecan regulates a variety of biological processes, such as modulating cell adhesion, thrombosis and cell death, skin and endochondral bone formation, and is involved in the biomechanics of blood vessels and cartilage [14]. Perlecan binds and modulates the activity of several growth factors and morphogens. It is a complex regulator of vascular biology and tumor angiogenesis [11, 15, 16]. Moreover, several studies confirm the role of HS perlecan in modulating pro-angiogenic factors such as FGF2, VEGF-A, and PDGF.

Cell surface PGs (syndecans and glypicans) connect the surface of cells to the underlying extracellular matrix and can act as co-receptors, facilitating ligand encounters with signaling receptors [10].

Fibrous Proteins and Adhesive Glycoproteins

ECMs are critical modulators of connective tissue remodeling by binding to and activating TGF-β and bone morphogenic proteins (BMPs), which may then influence health or contribute to disease. TGF-βs, a family that includes TGF-β-1, -2, -3 and BMPs, have a prominent role in ECM metabolism, as a major inducer of collagen synthesis [17–19]. Collagens are the most abundant proteins in the mammalian ECM. Collagen consists of molecules that form a triple-stranded helix to form stretch-resistant fibers that provide tensile strength to tissues. To date, 28 different types of collagen have been identified and described. Type I collagen is the major protein present in ECM of most tissues, the other collagens being expressed in lower quantities. For example, in tendons and ligaments, greater than 90% of the connective tissue is composed of type I collagen. These different compositions provide distinct mechanical and physical properties to the ECM and contribute to the interaction between ligands and local cell population. Moreover, collagen is closely associated with glycoproteins, growth factors, and other structural proteins, such as elastin and laminin, in the generation of tissue types with specific characteristics and/or properties.

Elastin is another significant component of elastic fibers in matrix tissue and permits tissues to return to their original form after stretching or contracting. Elastin is one of the most chemically resistant and durable proteins in the body because of its high content of hydrophobic amino acids. PGs, including glycosaminoglycan and in particular the heparin sulfate, have been detected within the elastic core and they have been reported to regulate the assembly of elastin [20, 21]. Water management plays an important role in three-dimensional elastin organization and in determining the degree of hydration and elasticity in tissue.

Fibronectin is second only to collagen within the ECM and may enhance cell position, and, by spreading, support cell division and migration. Fibronectin is necessary for the development and morphogenesis of a number of tissues and organs throughout embryogenesis. Indeed, some studies report that, during cardiac

development, precursor cells require fibronectin to complete their migration and in the absence of this matrix protein epithelial organization is disrupted [22, 23]. It probably also plays a role in cellular morphology, in directing cellular differentiation [24], and wound repair. Fibronectin is important in cell adhesion through the alpha5beta1 integrin, since it is secreted in an inactive soluble form and is activated by interaction with an integrin [25]. The important role of this protein in matrix assembly lies in its ability to bind simultaneously to cell surface receptors, such as integrins, and to collagen, to PGs, and to other adhesion proteins. Fibronectin domains have been found to bind to a number of different growth factors, including vascular endothelial growth factor (VEGF) and hepatic growth factor (HGF). Laminins are made up of a glycoprotein family with a cross-shaped structure, and their interaction creates a network that provides adhesion between dissimilar tissues. These proteins are required for the correct healing of tendons and other connective tissues. Furthermore, laminins are an important, biologically active part of the basal lamina, and they play important roles in tissue structure and maintenance and in cell signaling, adhesion, and migration, among other functions [26–28].

Thrombospondins (TSPs) are a group of five modular glycoproteins and are considered to be "adhesion-modulating" components of the ECM which are able to modulate cell functions in a variety of tissues [29]. TSP-1 plays an important role in wound healing by activation of TGF-β during early tissue repair, but in later stages persistent production of TSP-1 may lead to fibrosis. Moreover, TSP-1 regulates the activity of several other growth factors, such as VEGF, EGF, and PDGF. TSP-1 binds to a number of cell membrane receptors, integrins, and HS and has been shown to upregulate type I collagen expression [30]. Contrastingly, TSP-2 is involved in collagen fibril assembly, but is not able to activate TGF-β.

Fibrinogen is a complex fibrous glycoprotein and is converted by thrombin into an insoluble fibrin polymer. Fibrinogen and fibrin bind specifically to a variety of other proteins, like fibronectin, thrombospondin, von Willebrand factor, fibulin, FGF-2, VEGF, and interleukin-1 (IL-1). This ability to bind to different compounds, particularly to growth factors, gives fibrinogen an important role in cardiovascular and extracellular matrix physiology [31].

Since it enables cellular attachment, proliferation, and three-dimensional arrangement, fibrin is generally considered to be a good basic scaffold material. On one hand, fibrin's high biocompatibility is advantageous; however, on the other hand, it suffers from poor biomechanical stability.

Growth Factors

Growth factors (GFs) (soluble signals) are the major regulators of cell behavior; they promote cell proliferation, migration, and differentiation through specific GF receptor binding which stimulates cellular signal transduction pathways. GFs are involved in several physiological and pathological processes, such as tissue repair and maintenance [32]. GFs are secreted from the cells directly or are sequestered by

ECM for presentation to cell surface receptors. Some studies demonstrate that GF can be released from ECM by the degradation of ECM proteins, of GAGs, or of PGs. The ability of growth factors to interact with the extracellular matrix is a dynamic, tissue-specific property [33–35]. GFs, ECM components, and cell surface receptors form complexes that may lead to additive or synergistic cell signaling events. The ECM can regulate GF-mediated cell function [36] through many insoluble (ECM-bound) and soluble (un-bound) cell secretions, through cell surface proteins and through proteoglycans. Specifically, the ECM regulates GF activity by sequestering soluble GFs and by cell-demanded release via enzymatic degradation of the ECM, eliciting a variety of effects on GF signaling that are dependent on the context and presentation of the GF to cells [36–38]. Both cytokines and growth factors are present within the ECM in very small quantities, but act as potent modulators of cell behavior. The list of GFs found within the ECM is substantial and includes vascular endothelial cell growth factor (VEGF), transforming growth factor beta (TGF-β), the fibroblast growth factor (FGF) family, insulin-like growth factor (IGF), epithelial cell growth factor (EGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs), and many others. Multiple isoforms of these molecules are present, each with its own specific biological activity. VEGF, which has been the subject of a number of studies, is the major inducer of endothelial cell homeostasis and of angiogenesis and is an example of context-dependent GF signaling. Soluble and insoluble ECM components regulate VEGF activity in various ways. Several distinct VEGF genes produce multiple VEGF isoforms which exhibit variable binding domains for heparan sulfate (HS). Furthermore, every VEGF isoform plays a distinct role in vascular patterning and arterial development in the ECM [39, 40]. VEGF induces α1β1 and α2β1 integrin expression in microvascular endothelial cells, as well as endothelial cell migration and proliferation. Although the major role of VEGF is related to vascular tissue, several studies have shown VEGF to also be implicated in bone cell recruitment, activity, and survival [41]. VEGF binding to the cell surface or to ECM and various MMPs and plasminogen activators can generate diffusible, non-heparin-binding fragments. ECM component fibronectin can be modified in the presence of heparin or HSPGs which expose a binding site for VEGF [42–44]. The binding of VEGF to the ECM enhances endothelial cell proliferation, probably through an increase in mitogen-activated protein kinase (MAPK) activity [5, 45]. VEGFR-2 and VEGFR-1 tyrosine kinase receptors mediate VEGF activity [40]. VEGFR-1 has minimal kinase activity, but a high affinity for VEGF suggesting that it serves as a VEGF sink, preventing VEGF binding to the active VEGFR-2 receptor [40]. While VEGFR-2 mediates most of the endothelial growth and survival signals, VEGFR-1-mediated signaling plays an important role in certain pathological conditions such as inflammation and cancer. VEGF–VEGFR-2 binding on the cell surface leads to AKT activation and calcium release, whereas internalization of the receptor complex triggers ERK signaling in early endosomal compartments [46, 47]. Internalization and trafficking of VEGFR-2 can be influenced by many factors, including neuropilin-1 (NRP-1), syndecans, and hypothetically by β1-integrin [46, 47]. The activation of VEGFR-2

and its downstream targets leads to increased permeability, proliferation, migration, and survival of endothelial cells [40]. Matrix metalloproteinases (MMPs) have also implicated in the release of VEGF from ECM stores. Degradation of VEGF by MMPs, specifically MMP-1, -3, -7, -9, -16, -19, and MT1-MMP, regulates VEGF bioavailability. Proteolytic cleavage of insoluble VEGF and FGF by MMPs mobilizes these molecules, separating them from the ECM and allowing them to become soluble and spread. Consequently, they are therefore able to interact with specific cell membrane receptors and thus regulate their bioavailability [48].

TGF-βs family is crucial in development, wound healing, immune response, and tumor genesis [49]. The ECM helps impose tight regulation over the activation and activity of TGF-β, and there are multiple levels of posttranslational regulation. During biosynthesis, pro-TGF-β is initially associated with its pro-peptide, latency-associated peptide (LAP). This complex also binds to latent TGF-β-binding protein (LTBP) to form the large latent complex, which then binds to ECM proteins. MMPs and ADAMTSs help regulate TGF-β activity by cleaving ECM fibers and increasing its bioavailability. It has been demonstrated that MMPs-2, -9, -13, and MT1-MMP can proteolytically process the latent TGF-β, thus activating a highly important biological factor. TGF-β availability may also be indirectly affected by MMPs, by MT1-MMP, and MT3-MMP-regulated shedding of the TGF-β-binding, membrane-anchored, proteoglycan β-glycan [50]. In addition, several matrix proteases can activate TGF-β by cleaving latency peptides. Interestingly, there is also evidence suggesting that the mechanical stiffness of the ECM may lower the activation threshold of TGF-β. In this model, stiff ECM provides additional resistance to cell pulling and induces a conformational change in LAP facilitating release. During tissue repair, TGF-β stimulates fibroblasts and myofibroblasts, signaling through the SMAD pathway, to express numerous ECM-related genes including those encoding for collagens, TIMPs, and MMPs. In this way, TGF-β contributes to the deposition of newly synthesized ECM following tissue damage and remodeling of the ECM. Although TGF-β signaling is critical for successfully repairing damaged tissues, dysregulation of this pathway can lead to tissue fibrosis. Persistent inflammation, immune activation, and fibroblast stimulation via TGF-β can lead to excess deposition of ECM proteins and the generation of fibrotic tissue. TGF-β modulates nearly all stages of the immune response from early immune to later adaptive response and modulates immune cell activation, proliferation, and differentiation. Since active TGF-β participates in a number of important biological processes such as embryogenesis and wound healing, its deregulation has been correlated with numerous pathological states [50].

Insulin-like growth factor binding proteins (IGFBPs) are a family of proteins with six distinct subgroups (IGFBP-1 to -6), which bind to insulin-like growth factors (IGF-I and IGF-II) with high affinity and modulate the biological function of IGFs. The N-terminus is the primary binding site for IGF. The C-terminus is also required for IGF binding, as well as for binding to the extracellular matrix [51]. IGFBPs can stimulate the proliferation and migration of a number of cell populations and also play an important role in bone metabolism. Some studies have shown a correlation between the local concentration of IGF and new bone formation and

mineralization. Two mechanisms of IGF release have been identified: the ECM binding and the IGFBP proteolysis [52]. These two actions lower the affinity of IGFBPs for IGFs increasing the local concentration of bioactive IGF. IGFBP-2, -3, -5, and -6 interact with GAGs [53–55]. IGFBP-2 interacts with heparin-binding surface-pH-dependent and with GAGs; these process may be relevant where extracellular pH is low, like in site of wound healing. Interestingly, IGFBP-2 and -6 bind a broad range of GAGs whereas IGFBP-3 and -5 preferentially bind to heparin, heparan sulfate, dermatan sulfate, and minimally to chondroitin sulfates and hyaluronic acid [56]. These differences in GAG binding specificity confer tissue-specific actions upon the IGFBPs. GAG binding can also alter IGFBP interactions with other ligands, thereby modulating the IGF-independent actions of IGFBPs. For example, activation of plasminogen to plasmin is promoted by the interaction of IGFBP-5 and plasminogen activator inhibitor-1 (PAI-1), leading to ECM degradation and remodeling independently of IGF-I. This process is important in mammary gland involution. Further interaction with ECM proteins via IGFBP C-domains has been reported [57]. For example, IGFBP-3 binds fibrin/fibrinogen, fibronectin, vitronectin [58], and plasminogen, which then can influence both its IGF-dependent and IGF-independent actions. Also, fibronectin binds IGFBP-5 and inhibits its ability to promote IGF-dependent cell migration [59].

IGFBPs are well-known MMP substrates. MMPs cleave IGFBPs predominantly in the linker domain and are not dependent on IGF binding [52, 60]. Proteolysis provides a mechanism by which the concentration of freely bioavailable IGFs is increased, leading to subsequent activations of IGF1R. Specifically, the processing of IGFBP-3 and -5 by MMP-1,-2, -3,-7, and -19 and IGFBP-1 by MMP-3, -9, and -11 releases active forms of IGFs [50]. The dual effect of IGFs on MMP expression has also been observed: IGF-1 can upregulate MMP-2 synthesis via PI3-kinase/Akt/mTOR signaling while concomitantly transmitting a negative regulatory signal via the Raf/ERK pathway [50].

Protease Activity and Role of Proteolytic Enzyme in ECM

Remodeling enzymes, which are capable of modifying and degrading ECM proteins, are central to the interactions between host immune systems and ECMs. Migration of immune cells into areas of disease-induced tissue damage is facilitated by localized ECM breakdown. Additionally, the innate immune system initiates responses to infection; thanks to products of ECM degradation which can serve as early signals. In response to injury and infection, ECM remodeling enzymes contribute to modulation of inflammation and tissue repair processes. In this case, most of these remodeling enzymes are produced by immune cells, and in particular myeloid lineage immune cells [61].

There are a large number of molecules which play a role in protease activity and are also involved in proteolytic processes in the ECM, which can be divided into

three main groups: (1) serine proteases; (2) matrix metalloproteinases (MMPs), which are a large family of highly conserved, Zn-dependent endopeptidases; (3) bone morphogenetic protein 1 (BMP1), which is a member of the tolloid family of metalloproteinases.

These proteinases have been linked to cellular differentiation and pattern formation through a role in activating latent growth factors in the TGF-β superfamily.

Further, a disintegrin and metalloprotease proteins (ADAMs) are a family of transmembrane glycoproteins with diverse roles in cell–cell adhesion and proteolysis. MMPs/ADAMs are a family of zinc-dependent, ECM-degrading endopeptidases that share common functional domains, activation mechanisms, and, collectively, have the capability to degrade all types of ECM protein [61]. In addition to playing a central role in ECM macromolecule turnover, within tissue microenvironments MMPs/ADAMs can modify extracellular, soluble, or membrane-bound proteins and induce their rapid release or inactivation. MMPs, which were initially recognized for collagen remodeling, have now been shown to play a significant role in the control of immune responses. These enzymes can proteolytically activate or degrade a variety of non-matrix substrates, including signaling molecules such as growth factors, chemokines, and cytokines.

Moreover, the main proteolytic enzyme families, MMPs and ADAMs, are considered to be the predominant proteases in ECM pathophysiological regulation. Each individual MMP influences the properties of ECMs differently, generating distinct chemical, biomechanical, and morphological features in the ECM [61]. The effects produced by a specific MMP through selective ECM degradation uniquely influences cell behavior, including migration, morphology, gene expression profile, and activation of intracellular cascades. In addition to degrading ECM proteins, MMPs modulate a variety of biological factors and non-ECM molecules, directly influencing tissue homeostasis. Collagenolysis and elastolysis by MMPs occur during development, wound healing, and in major inflammatory diseases. MMP-2, MMP-7, MMP-9, MMP-12, and MT1-MMP have been suggested to be elastolytic. Controlled proteolytic cleavage of ECM protein-release growth factors, such as FGFs, contributes to localized cell proliferation and differentiation.

Studies have shown that certain cytokines and growth factors, including insulin growth factors (IGFs), epidermal growth factors (EGFs), vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), fibroblast growth factors (FGF), tumor necrosis factor-α (TNF-α), and interleukin-1b (IL-1b), can stimulate expression of MMPs and ADAMs [61].

Extracellular Vesicles in the ECM Structure/Organization

Extracellular vesicles (EVs) are lipid bilayer membrane-enclosed vesicles released by virtually all cells types as mediators for intercellular communication. They are highly heterogeneous in size (ranging from $\sim$50 nm to >1 μm, with the vast

majority <200 nm) and in molecular composition, carrying functional proteins, DNA, miRNA, ncRNA, and lipids that they transfer into the target cells. Based on recent studies, EVs can be considered to be one of the structural and functional components of the ECM that participate in matrix organization, in the regulation of cells within the ECM, and in determining the physical properties of soft connective tissues, bone, cartilage, and dentin. Studies have demonstrated that a variety of mechanisms regulate EVs-ECM communication: EVs act as key structural and functional components of the ECM and are able to transport and distribute the components of the ECM, in terms of both original structure and target tissue modulation and reorganization, and, furthermore, EVs modulate the matrix composition by means of the proteinases and signaling molecules which they carry. Extracellular vesicles are integral and functional components of the extracellular matrix [63]. More recently, it has been shown that the structural components of a decellularized heart have direct effects on the differentiation of stem/progenitor cells and better preserve the mechanical properties of the heart compared to synthetic scaffolds [64]. Minae An et al. found EVs with a large amount of microRNA (miRNA) and exosomal protein derived from decellularized cardiac ECM (ECM-EVs) [65]. A miRNA is a small noncoding RNA molecule containing about 22 nucleotides that mediates RNA silencing and posttranscriptional regulation of gene expression [66]. A large number of miRNAs have been reported to play a role in modulating cardiac regeneration; and a similar range of miRNAs is present in ECM-EVs from decellularized hearts. An et al. [65] demonstrated that ECM-derived miRNAs could participate in cardiac regeneration. In the context of the extracellular matrix, a great deal of further research into EV function and interaction will be crucial to understanding the range of biological effects and consequences of EVs and in revealing their full impact on countless potential applications for a wide range of diagnoses and therapies, including on tissue engineering.

Importance of ECM–Integrin Interactions

In addition to serving as a reservoir of growth factors and cytokines that modulate cell functions, the ECM interacts directly with cells and directs cell motility through integrins expressed on the surface of numerous cell types. The ECM itself provides a scaffold that cells utilize for their migration. While the ECM serves as a guide for some cell types, it can also serve as an obstacle, for example, to neutrophils that migrate to the site of infection. Integrins are a family of heterodimeric, transmembrane glycoproteins, are the most abundant cell surface receptors, and are expressed in all cell types except for erythrocytes. Integrins are composed of two type I transmembrane glycoproteins, the α and the β subunits, by non-covalent association. The extracellular domain interacts with ECM proteins such as laminins and collagens in basement membranes or with fibronectin in connective tissue

components, while the intracellular domain interacts with the cytoskeletal proteins like actin, affecting polarization and motility. For example, some integrins appear to only be linked to one type of ECM protein (fibronectin or laminin), whereas others may interact with a number of types (such as collagen, fibronectin, and laminin). It is also possible that a single integrin may bind to several distinct ligands and that an individual ligand may be recognized by more than one type of integrin. A significant number of cytoskeletal and signaling proteins have been reported to bind to β-cytoplasmic tails, and some have also been found to interact with specific α tails. Moreover, alternative mRNA splicing, in extracellular, as well as in intracellular, regions, produces a large number of variant forms of α and β subunits. Differently spliced isoforms of both α and β subunits can differ in their effect on signal transduction pathways, or in their specificity and affinity for ligands and interaction with cytoskeletons. The preferential selection of various splice variants in specific types of cells at precise differentiation stages leads to significant functional differences. For example, α7β1 integrin can be detected in proliferating and adult myofibers [67], and it is reported that α7A has a minor function in mature muscle fibers, but a relevant role in regenerating muscle fibers [68, 69]. Furthermore, integrin-mediated adhesion modulates signaling cascades in the control of cell survival, proliferation, differentiation, and motility. Migration of adhesive mesenchymal cells depends on ECM proteolysis and the interaction of integrins with ECM proteins. In addition, integrins are also involved in leukocyte interactions with the endothelial basement membrane and the transmigration of these cells to sites of inflammation. Some integrins even bind to receptors present on other cell types, such as intercellular adhesion molecules (ICAMs) or vascular cell adhesion molecules (VCAMs) which are expressed on leukocytes and endothelial cells. There is evidence suggesting that lymphocyte motility and retention in certain compartments is influenced by integrin–ECM interactions, in particular, in inflamed tissues with altered ECM composition and integrin expression, for example in the context of influenza infection. Another important role of integrins is in the activation of ECM-bound cytokines and growth factors. For example, the α4β6 integrin activates latent TGF-β regulating the spatial bioavailability of the growth factor. Integrin activation introduces another level of regulation of ECM-bound molecules. The effect of α4β6 activation of TGF-β is likely to be context dependent. An early study found that mice lacking α4β6 develop airway hyper-responsiveness due to infiltration of inflammatory cells into the lungs and skin. Activation of TGF-β by α4β6 may also contribute to fibrotic lung disease following influenza infection due to increased collagen deposition. Integrins contribute to development and tissue morphogenesis and also play a key role in tissue homeostasis and tissue repair. The presence of cytokines and growth factors in the ECM provides a means for host cells to rapidly respond to infection or injury as these molecules are released and/or activated. In this manner, these ECM-bound molecules may represent some of the earliest signals to the host immune system to further rapid responses.

Mechanical Signals Inside the ECM

The process of translating mechanical signals into a cellular response is called mechano-transduction [70]. Our cells actively sense and respond to a variety of mechanical signals. They experience numerous mechanical stimuli, like shear stresses from blood flow and stretching forces from tissues associated with muscular activity [71]. ECM stiffening in disease states (e.g., cancer and fibrosis), or due to aging processes, can adversely affect cell migration, differentiation, and proliferation [72, 73]. In this light, the ECM is more than just a passive network of ligands for cell attachment. It carries different types of mechanical signals, and it provides dimensionality. Focal adhesions (FAs) result from the complex interaction of hundreds of different trans-plasma membrane integrins and cytoplasmic proteins, and their exact composition varies in response to physical stimuli (mechano-transduction). The complexity of the composition and the dynamics of FA implicate intelligently designed, intricate, molecular interplay. FAs mediate the strength of cell adhesion, and cell migration, as well as mechano-sensing and signaling. Moreover, FAs transmit information in a bidirectional manner between the ECM and cytoplasm [74, 75]. At adhesion sites, integrins connect the ECM to the F-actin cytoskeleton and transduce the mechanical forces (generated by the actin retrograde flow and by myosin II) to the ECM through a group of mechano-sensitive FA proteins, termed "molecular clutch" [76]. Vinculin, which is prevalent at the distal ends of microfilament bundles on the cell membrane, binds a variety of FA proteins, thus mediating certain distinct cellular functions [77]. Since being identified as a component of focal adhesions and adherens junctions, vinculin has emerged as one of the principal components of the mechano-sensory machinery [78]. While it is clear that mechanical stimuli can have profound effects on cell behavior, the mechanisms that translate these forces into biochemical signaling remain largely unknown.

YAP and TAZ represent a signaling nexus which integrates mechanical and biochemical signals, influencing the stiffness of the ECM, adhesion ligand density, and cell–cell contacts. They are powerful regulators of cell proliferation and survival and play important roles in controlling organ growth, stem cell self-renewal, and cell differentiation [76, 79, 80].

The sensitivity of cells to forces and substrate stiffness has been recognized as a powerful tool in tissue engineering, where it can be harnessed to design biomaterials that optimally guide stem cells or resident cells toward generating functional replacement tissue. For instance, cell-derived matrices (CDM) are becoming an attractive alternative to conventional biological scaffolding platforms due to their unique ability to closely recapitulate the native extracellular matrix [81].

Morphogenesis

Morphogenesis is the developmental cascade of pattern formation, body plan establishment, and tissue differentiation which culminates in the adult form, and its principles are applicable to tissue engineering for regenerative medicine and surgery. Responding stem cells, morphogens, (morphogenetic protein signals), and other bioactive ECM components can be integrated into materials for functional tissue restoration.

Morphogenesis not only concerns the formation of embryonic tissues and organs but also maintenance, degeneration, and regeneration in adult tissues.

As mentioned before, ECMs play an important role in many cellular processes, such as growth, differentiation, and survival, and their precise characteristics can play a critical role during morphogenesis. The ECM affects cell morphogenesis and differentiation, providing support, tensile strength, and scaffolding for tissues and cells, as well as supplying biochemical signals (i.e., growth factors, chemokines, and cytokines). Cells remodel and reshape the ECM by degrading and reassembling it, thus playing an active role in sculpting their surrounding environment and directing their own phenotypes [82].

Integrins contribute to most, if not all, of the morphogenetic events that shape a complex, multicellular organism in development, in tissue formation, homeostasis, and repair [82]. For example, during vasculogenesis, endothelial cells migrate, proliferate, and form 3D tubular structures. This tubular morphogenesis requires integrin receptor signaling to regulate cell shape through changes in the cytoskeleton and cell–cell interactions that control the shape of the tubules [83]. Similarly, morphogenesis of branching organs, such as the salivary gland, lung, breast, and kidney, as well as prostate and pancreas, is dependent on the multiple downstream activities related to ECM and integrin receptor interactions [84].

Cell adhesions participate in these dynamic rearrangements and maintain tissue integrity throughout adult life. During collective cell movements that drive changes in tissue shape, cell–cell adhesions must be remodeled, broken down, or reinforced depending on the cellular behavior required. For example, cell adhesion to specific ECM proteins may modify gene expression and alter levels of adhesion molecules or other regulating proteins [85]. Alternatively, engagement or disengagement of one type of adhesion might modify the functional activities of another by effecting changes in membrane trafficking, cytoskeletal association and/or avidity or binding affinity [86]. For a given morphogenetic movement, the regulation of cadherin-based adhesions may be implicated in establishing cell polarity, mechanically coupling neighboring cells, and/or directing cell migration. These interactions involve the convergence of independently initiated cell signaling events, often involving downstream effectors that are common to both cadherin adhesions and integrin. Cadherin adhesions and integrin, which often share signaling effector molecules, are both transmembrane adhesion receptors, both link to common scaffolding and cytoskeletal elements and can both influence certain crucial downstream functions, such as cell growth, survival, and transcriptional

activity [87]. However, integrin and cadherin interaction may involve not only shared cytoskeletal linkages, cell–cell, or cell–ECM engagement, but can also potentially interact with adaptor proteins [i.e., tetraspanin or growth factor receptors like insulin growth factor I receptor (IGF1R)] which facilitate lateral association of integrins and cadherin. RhoGTPases are central to cell signaling pathways both upstream and downstream of cadherin and integrin adhesions [88, 89], making them prime candidates for mediating integration of adhesion-dependent signals [90].

Bone morphogenesis is induced by bone morphogenetic proteins (BMPs), which play a role in pattern formation, cell differentiation, maintenance, and regeneration of tissues. BMPs are pleiotropic and act on chemotaxis and mitosis and can differentiate progenitor cells and stem cells, which may also be programmed by BMPs. The name is slightly misleading as their range of action also extends beyond simple bone formation in that they play a role in the development of the teeth, heart, kidneys, eyes, skin, and brain. The ECM, the native scaffold, can be used to deliver morphogens such as BMPs for tissue engineering [91].

Morphogenesis of 3D Tissue Architecture in Vivo: Folds, Tubes, and Branches

Many morphogenetic processes begin with a flat or curved sheet of cells that eventually gives rise to complex topologies such as folds. Folds can be generated by a monolayer of cells, by stratified cell sheets, or by multiple interacting tissues. Consequently, cell–cell adhesions must play different roles depending on the cellular behaviors required. Although the precise mechanism remains unclear, E-cadherin, for example, is required for fold formation during embryogenesis [92]. It may be involved in transmitting mechanical cell–cell cues and could play a role in establishing appropriate cell polarity.

Tubulogenesis is generally initiated by tissue folding, usually generating tubes of larger diameters, and is completed by the formation of new adhesions to seal the tube [93]. Proper distribution and transmission of tension require precisely controlled adhesion between cells, in blood vessel tubulogenesis; for example, cell–cell adhesions participate in establishing polarity and may also act as mechano-sensors linking blood flow to vascular remodeling. Vascular endothelial (VE)-cadherin and apical–basal polarity are required for endothelial cells to form lumena [94, 95]. Polarity is established through interactions between VE-cadherin and cell polarity complex proteins Par3 and Pals1 [95]. Without proper localization of cell–cell junctions and establishment of cell polarity, lumen formation and, therefore, blood flow are prevented and blood flow is, itself, a major determinant of vascular morphogenesis, including sprouting and lumen elongation [96, 97]. Pressure and shear stress cause changes in endothelial cell behavior. These mechanical signals

may be transduced (via VE-cadherin), leading to cytoskeletal and junctional remodeling [98].

Branching morphogenesis requires the coordinated interplay of multiple cell types with the ECM. Organs morphology is highly varied, ranging from "lobular" in salivary glands to "tubular" in blood vessels [99]. The morphogenesis of a branched organ requires both formation of new branches and remodeling of existing branches. Branch formation in organs occurs through "budding" or "clefting." Budding is novel branching from the surface of an epithelium or from the side of a branch, whereas clefting splits a branch tip into, usually, two or three tips.

Branched organs contain multiple cell types plus their ECM. The core structure of all branched organs consists of tightly bound epithelial cells. The epithelium is surrounded by the basement membrane, a dense network of ECM glycoproteins and proteoglycans, beyond which there is a loosely organized mixture of mesenchymal cells. During branching, epithelial cells actively interact, both biochemically and biophysically, with the ECM and other cell types. Although mechanisms remain unclear, numerous growth factors and regulatory signaling pathways have been implicated, in branching morphogenesis. The formation of most branched organs requires one or more tyrosine kinase receptor (RTK) signaling pathway [40, 100–102]. RTK signaling modulators have been identified in the mesenchyme (which produces the ligands) and in the epithelium itself (where the corresponding RTK receptors are expressed) [40, 103–109].

Growth Factors and Morphogens for Tissue Engineering

In the complexity of the ECM, with the vast range of physical, biological, and chemical interactions, signaling pathways and networks all producing a host of specific responses to given stimuli, mimicking or recreating these processes through tissue engineering and regeneration techniques requires a number of different components. Vital among these are growth factors which, as described above, play fundamental roles in a variety of tissue and organ formation mechanisms and processes. The properties of the ECM itself, as a reservoir, have a pivotal role in the physiological delivery of GFs, crucial to the regenerative process [32]. While the use of GFs has had some clinical success, their potential as therapeutic agents has generally been hindered by certain limitations. Many growth factors are intrinsically unstable, with very brief functional activity [110], while the absence of suitable delivery methods excludes others. For instance, some GFs like BMP-2 and VEGF have minimal interaction in their native form with the surrounding ECM, which in clinical situations requiring repeated dosage due to rapid outward diffusion and fast proteolysis makes them unsuitable [111, 112]. Therefore, some form of GF engineering may be required in order for regenerative applications to overcome these limitations [32].

Similarly, given that morphogens are another essential regulatory mechanism in the creation of improved human tissue models, researchers over the past two

decades have used them to develop a variety of hydrogel compositions which can envelop 3D cell cultures, or modified, synthetic biomatrices, in order to better deliver both GFs and morphogens into the target tissue [113].

Growth Factor and Morphogen Delivery Through Engineered ECM

The major issue when a signaling delivery system has to be improved is how to best mimic naturally occurring, inductive signal sequestering which serves as a template for the design of synthetic molecules that can sequester GFs and morphogens [36]. "Biomimicry," which is the underlying mechanism of GF sequestration, was first used in studying the interaction between α2-M and TGF-β1, α2-M and PDGF-BB, and TGF-1β and TGF-1β receptor III [114–116] and can be used to identify GF sequestering moieties. Researchers have demonstrated that moieties engineered to mimic known proteins, or proteoglycans such as fibronectin and fibrinogen, exhibited GF or heparin-like sequestering [117]. In addition to soluble approaches, GFs can be sequestered at 2D and 3D interfaces in the ECM environment. Surfaces presenting proteoglycans and glycoproteins in a synthetic functionalized monolayer can sequester GFs and modulate GF-related cell response. As an example, it has been demonstrated that GFs can be sequestered by a self-assembled monolayer modified with a HEPpep peptide derived from the heparin-binding domain of FGF-2 and an Arg-Gly-Glu (RGD), derived from a sequence of fibronectin which allows integrin-mediated cell adhesion [118].

A 3D interface design, however, requires the optimum choice of an appropriate biomaterial which must retain GFs. Controlled tailoring of physical properties, like density, porosity, viscosity, and charge, is essential for the implementation of GF sequestration inside the scaffold [110]. Additionally, GF activity must be preserved when incorporated within the biomaterial, which can easily be done by designing formulations which mimic the ECM, such as hyaluronan, chitosan, collagen/gelatin, and fibrin-based scaffolds. Moreover, natural biomaterials are cell-growth-friendly and they avoid limitations such as pathogen transmission or ethical considerations which can arise using animal or human sources in a clinical situation. Furthermore, in a 3D matrix scenario, the proximity of the cells to the sequestering event and the source of the sequestered GF (cell secreted or supplemented into the growth medium) can influence the final cell response [36]. Hydrogels are excellent substitutes for native ECM because they can mimic its physical structure and biochemical functions. They are increasingly used for GF sequestering and to control release for a variety of purposes, such as to increase angiogenesis, and nerve and bone regeneration [119–121]. However, most organoids are currently formed by envelopment within Matrigel hydrogels, which are a poorly defined, heterogeneous mixture of soluble basement membrane proteins from mouse sarcomas. Some researchers have shown that chemically well-defined hydrogels could be used to

support organ morphogenesis, like hyaluronan–chitosan hydrogel blend which has been used with rudimentary cerebral organoids, or pure PEG hydrogels which can entrap a single mouse embryonic stem cell for neural tube morphogenesis [122–124].

Engineering Growth Factors and Morphogens for Interaction with Exogenous Biomaterials and for Delivery Through the Native ECM

A large number of strategies permit covalent attachment of GFs to biomaterials, among which the carbodiimide-mediated conjugation reaction has been used successfully, for example, in the cross-linking of VEGF-A/angiopoietin-1 onto collagen scaffolds which has been found to improve vascularization [125]. Instead of chemical coupling, GFs can be enzymatically conjugated to exogenous matrices and the incorporation of a substrate sequence in the scaffold allows site-specific control of the cross-linking location within the GF and minimizes non-specific interference with other sites of bioactivity [126]. Heparin-binding GFs can associate with GAGs, leading to the fusion of heparin-binding domains to non-heparin-binding factors. As an example, the heparin-binding (HB) domain of EGF has been added to IGF-1 creating the HB-IGF-1 formulation which has been demonstrated to interact with chondroitin sulfate GAGs within the cartilage matrix after intra-articular knee injection [127]. Furthermore, abundant collagen fibers are also a good target for GF binding as demonstrated by the fusion of collagen binding domain (CBD) and nerve growth factor-β (NGF-β) in order to improve nerve regeneration after nerve crush injury [128].

Light is beginning to be thrown on the extraordinary role of the ECM in the vast and complex mechanism underlying tissue and organ generation and in maintenance processes. In the present book we discussed critical aspects of the native ECM as well as application of innovative engineering methodology which would get an advancement towards tissue regeneration.

References

1. Katari R, Peloso A, Orlando G. Tissue engineering and regenerative medicine:semantic considerations for an evolving paradigm. Front Bioeng Biotechnol. 2015;12(2):57.
2. Hellman KB, Johnson PC, Bertram TA, Tawil B. Challenges in tissue engineering and regenerative medicine product commercialization: building an industry. Tissue Eng Part A. 2011;17(1–2):1–3.
3. Briquez PS, Hubbell JA, Martino MM. Extracellular matrix-inspired growth factor delivery systems for skin wound healing. Adv Wound Care (New Rochelle). 2015;4(8):479–89.

4. Patterson J, Siew R, Herring SW, Lin AS, Guldberg R, Stayton PS. Hyaluronic acid hydrogels with controlled degradation properties for oriented bone regeneration. Biomaterials. 2010;31(26):6772–81.
5. Hynes RO. The extracellular matrix: not just pretty fibrils. Science. 2009;326(5957):1216–9.
6. Chan BP, Leong KW. Scaffolding in tissue engineering: general approaches and tissue-specific considerations. Eur Spine J. 2008;17(Suppl 4):467–79.
7. Labouesse M. Role of the extracellular matrix in epithelial morphogenesis: a view from *C. elegans*. Organogenesis. 2012;8(2):65–70.
8. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123(Pt 24):4195–200.
9. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. Cold Spring Harb Perspect Biol. 2011;3(7).
10. Schaefer L. Extracellular matrix molecules: endogenous danger signals as new drug targets in kidney diseases. Curr Opin Pharmacol. 2010;10(2):185–90.
11. Iozzo RV, Schaefer L. Proteoglycan form and function: A comprehensive nomenclature of proteoglycans. Matrix Biol. 2015;42:11–55.
12. Iozzo RV, Zoeller JJ, Nyström A. Basement membrane proteoglycans: modulators Par Excellence of cancer growth and angiogenesis. Mol Cells. 2009;27(5):503–13.
13. Ori A, Wilkinson MC, Fernig DG. The heparanome and regulation of cell function: structures, functions and challenges. Front Biosci. 2008;1(13):4309–38.
14. Boyd DF, Thomas PG. Towards integrating extracellular matrix and immunological pathways. Cytokine. 2017;98:79–86.
15. Iozzo RV, San Antonio JD. Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. J Clin Invest. 2001;108(3):349–55.
16. Patel VN, Knox SM, Likar KM, Lathrop CA, Hossain R, Eftekhari S, Whitelock JM, Elkin M, Vlodavsky I, Hoffman MP. Heparanase cleavage of perlecan heparan sulfate modulates FGF10 activity during ex vivo submandibular gland branching morphogenesis. Development. 2007;134(23):4177–86.
17. Goldoni S, Iozzo RV. Tumor microenvironment: modulation by decorin and related molecules harboring leucine-rich tandem motifs. Int J Cancer. 2008;123(11):2473–9.
18. Schaefer L, Iozzo RV. Biological functions of the small leucine-rich proteoglycans: from genetics to signal transduction. J Biol Chem. 2008;283(31):21305–9.
19. Brinckerhoff CE. Matrix metalloproteinases in health and disease: sculpting the human body. 1st ed. (Republic of Singapore): World Scientific Publishing Co; 2017.
20. Kozel BA, Ciliberto CH, Mecham RP. Deposition of tropoelastin into the extracellular matrix requires a competent elastic fiber scaffold but not live cells. Matrix Biol. 2004;23 (1):23–34.
21. Gheduzzi D, Guerra D, Bochicchio B, Pepe A, Tamburro AM, Quaglino D, Mithieux S, Weiss AS, Pasquali Ronchetti I. Heparan sulphate interacts with tropoelastin, with some tropoelastin peptides and is present in human dermis elastic fibers. Matrix Biol. 2005;24 (1):15–25.
22. Trinh LA, Stainier DY. Fibronectin regulates epithelial organization during myocardial migration in zebrafish. Dev Cell. 2004;6(3):371–82.
23. Matsui T, Raya A, Callol-Massot C, Kawakami Y, Oishi I, Rodriguez-Esteban C, Izpisúa Belmonte JC. miles-apart-mediated regulation of cell-fibronectin interaction and myocardial migration in zebrafish. Nat Clin Pract Cardiovasc Med. 2007;4(Suppl 1):S77–82.
24. Taylor-Weiner H, Schwarzbauer JE, Engler AJ. Defined extracellular matrix components are necessary for definitive endoderm induction. Stem Cells. 2013;31(10):2084–94.
25. Takahashi S, Leiss M, Moser M, Ohashi T, Kitao T, Heckmann D, Pfeifer A, Kessler H, Takagi J, Erickson HP, Fässler R. The RGD motif in fibronectin is essential for development but dispensable for fibril assembly. J Cell Biol. 2007;178(1):167–78.
26. Pankov R, Yamada KM. Fibronectin at a glance. J Cell Sci. 2002;115(Pt 20):3861–3.
27. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. Annu Rev Cell Dev Biol. 2010;26:397–419.

28. Riederer I, Bonomo AC, Mouly V, Savino W. Laminin therapy for the promotion of muscle regeneration. FEBS Lett. 2015;589(22):3449–53.
29. Mosher DF, Adams JC. Adhesion-modulating/matricellular ECM protein families: a structural, functional and evolutionary appraisal. Matrix Biol. 2012;31(3):155–61.
30. Sweetwyne MT, Murphy-Ullrich JE. Thrombospondin1 in tissue repair and fibrosis: TGF-β-dependent and independent mechanisms. Matrix Biol. 2012;31(3):178–86.
31. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood. 2000;96(12):3772–8.
32. Mitchell AC, Briquez PS, Hubbell JA, Cochran JR. Engineering growth factors for regenerative medicine applications. Acta Biomater. 2016;30:1–12.
33. Hutchings H, Ortega N, Plouët J. Extracellular matrix-bound vascular endothelial growth factor promotes endothelial cell adhesion, migration, and survival through integrin ligation. FASEB J. 2003;17(11):1520–2.
34. Bissell MJ, Hines WC. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. Nat Med. 2011;17(3):320–9.
35. Wu J, Strawn TL, Luo M, Wang L, Li R, Ren M, Xia J, Zhang Z, Ma W, Luo T, Lawrence DA, Fay WP. Plasminogen activator inhibitor-1 inhibits angiogenic signaling by uncoupling vascular endothelial growth factor receptor-2-αVβ3 integrin cross talk. Arterioscler Thromb Vasc Biol. 2015;35(1):111–20.
36. Belair DG, Le NN, Murphy WL. Design of growth factor sequestering biomaterials. Chem Commun (Camb). 2014;50(99):15651–68.
37. Rundhaug JE. Matrix metalloproteinases and angiogenesis. J Cell Mol Med. 2005;9(2):267–85.
38. Davis GE, Senger DR. Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circ Res. 2005;97(11):1093–107.
39. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9(6):669–76.
40. Olsson R, Maxhuni A, Carlsson PO. Revascularization of transplanted pancreatic islets following culture with stimulators of angiogenesis. Transplantation. 2006;82(3):340–7.
41. Hsiong SX, Mooney DJ. Regeneration of vascularized bone. Periodontology. 2000;2006(41):109–22.
42. Wijelath ES, Rahman S, Namekata M, Murray J, Nishimura T, Mostafavi-Pour Z, Patel Y, Suda Y, Humphries MJ, Sobel M. Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism. Circ Res. 2006;99(8):853–60.
43. Mitsi M, Hong Z, Costello CE, Nugent MA. Heparin-mediated conformational changes in fibronectin expose vascular endothelial growth factor binding sites. Biochemistry. 2006;45(34):10319–28.
44. Mitsi M, Forsten-Williams K, Gopalakrishnan M, Nugent MA. A catalytic role of heparin within the extracellular matrix. J Biol Chem. 2008;283(50):34796–807.
45. Chen LL, Johansson JK, Hodges RR, Zoukhri D, Ghinelli E, Rios JD, Dartt DA. Differential effects of the EGF family of growth factors on protein secretion, MAPK activation, and intracellular calcium concentration in rat lacrimal gland. Exp Eye Res. 2005;80(3):379–89.
46. Eichmann A, Simons M. VEGF signaling inside vascular endothelial cells and beyond. Curr Opin Cell Biol. 2012;24(2):188–93.
47. Simons M. An inside view: VEGF receptor trafficking and signaling. Physiology (Bethesda). 2012;27(4):213–22.
48. Mettouchi A. The role of extracellular matrix in vascular branching morphogenesis. Cell Adh Migr. 2012;6(6):528–34.
49. Dong X, Zhao B, Iacob RE, Zhu J, Koksal AC, Lu C, Engen JR, Springer TA. Force interacts with macromolecular structure in activation of TGF-β. Nature. 2017;542(7639):55–9.

50. Levin M, Udi Y, Solomonov I, Sagi I. Next generation matrix metalloproteinase inhibitors—novel strategies bring new prospects. Biochim Biophys Acta. 2017;1864(11 Pt A):1927–1939.
51. Parker A, Rees C, Clarke J, Busby WH Jr, Clemmons DR. Binding of insulin-like growth factor (IGF)-binding protein-5 to smooth-muscle cell extracellular matrix is a major determinant of the cellular response to IGF-I. Mol Biol Cell. 1998;9(9):2383–92.
52. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev. 2002;23(6):824–54.
53. Booth BA, Boes M, Andress DL, Dake BL, Kiefer MC, Maack C, Linhardt RJ, Bar K, Caldwell EE, Weiler J, et al. IGFBP-3 and IGFBP-5 association with endothelial cells: role of C-terminal heparin binding domain. Growth Regul. 1995;5(1):1–17.
54. Kuang Z, Yao S, Keizer DW, Wang CC, Bach LA, Forbes BE, Wallace JC, Norton RS. Structure, dynamics and heparin binding of the C-terminal domain of insulin-like growth factor-binding protein-2 (IGFBP-2). J Mol Biol. 2006;364(4):690–704.
55. Forbes BE, Hartfield PJ, McNeil KA, Surinya KH, Milner SJ, Cosgrove LJ, Wallace JC. Characteristics of binding of insulin-like growth factor (IGF)-I and IGF-II analogues to the type 1 IGF receptor determined by BIAcore analysis. Eur J Biochem. 2002;269(3):961–8.
56. Fowlkes JL, Serra DM. Characterization of glycosaminoglycan-binding domains present in insulin-like growth factor-binding protein-3. J Biol Chem. 1996;271(25):14676–9.
57. Bach LA, Headey SJ, Norton RS. IGF-binding proteins–the pieces are falling into place. Trends Endocrinol Metab. 2005;16(5):228–34.
58. Kricker JA, Hyde CE, Van Lonkhuyzen DR, Hollier BG, Shooter GK, Leavesley DI, Herington AC, Upton Z. Mechanistic investigations into interactions between IGF-I and IGFBPs and their impact on facilitating cell migration on vitronectin. Growth Factors. 2010;28(5):359–69.
59. Xu J, Liao K. Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. J Biol Chem. 2004;279 (34):35914–22.
60. Bunn RC, Fowlkes JL. Insulin-like growth factor binding protein proteolysis. Trends Endocrinol Metab. 2003;14(4):176–81. Review. PubMed PMID: 12714278.
61. Boyd DF, Thomas PG. Towards integrating extracellular matrix and immunological pathways. Cytokine. 2017;98:79–86.
62. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. J Cell Physiol. 2007;211(1):19–26.
63. Rilla K, Mustonen AM, Arasu UT, Härkönen K, Matilainen J, Nieminen P. Extracellular vesicles are integral and functional components of the extracellular matrix. Matrix Biol. 2017.
64. Perea-Gil I, Prat-Vidal C, Bayes-Genis A. In vivo experience with natural scaffolds for myocardial infarction: the times they are a-changin'. Stem Cell Res. 2015;6(6):248.
65. An M, Kwon K, Park J, Ryu DR, Shin JA, Lee Kang J, Choi JH, Park EM, Lee KE, Woo M, Kim M. Extracellular matrix-derived extracellular vesicles promote cardiomyocyte growth and electrical activity in engineered cardiac atria. Biomaterials. 2017;146:49–59.
66. Berardocco M, Radeghieri A, Busatto S, Gallorini M, Raggi C, Gissi C, D'Agnano I, Bergese P, Felsani A, Berardi AC. RNA-seq reveals distinctive RNA profiles of small extracellular vesicles from different human liver cancer cell lines. Oncotarget. 2017;8 (47):82920–39.
67. Belkin AM, Zhidkova NI, Balzac F, Altruda F, Tomatis D, Maier A, Tarone G, Koteliansky VE, Burridge K. Beta 1D integrin displaces the beta 1A isoform in striated muscles: localization at junctional structures and signaling potential in nonmuscle cells. J Cell Biol. 1996;132(1–2):211–26.
68. Kääriäinen M, Nissinen L, Kaufman S, Sonnenberg A, Järvinen M, Heino J, Kalimo H. Expression of alpha7beta1 integrin splicing variants during skeletal muscle regeneration. Am J Pathol. 2002;161(3):1023–31.

69. Tarone G, Hirsch E, Brancaccio M, De Acetis M, Barberis L, Balzac F, Retta SF, Botta C, Altruda F, Silengo L. Integrin function and regulation in development. Int J Dev Biol. 2000;44(6):725–31. Review. Erratum in: Int J Dev Biol 2001 Sep;45(5–6): following 770.
70. Jansen KA, Donato DM, Balcioglu HE, Schmidt T, Danen EH, Koenderink GH. A guide to mechanobiology: where biology and physics meet. Biochim Biophys Acta. 2015;1853(11 Pt B):3043–52.
71. Jaalouk DE, Lammerding J. Mechanotransduction gone awry. Nat Rev Mol Cell Biol. 2009;10(1):63–73.
72. Lu P, Weaver VM, Werb Z. The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol. 2012;196(4):395–406.
73. Snedeker JG, Gautieri A. The role of collagen crosslinks in ageing and diabetes—the good, the bad, and the ugly. Muscles Ligaments Tendons J. 2014;4(3):303–8.
74. Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science. 2001;294(5547):1708–12.
75. Winograd-Katz SE, Fässler R, Geiger B, Legate KR. The integrin adhesome: from genes and proteins to human disease. Nat Rev Mol Cell Biol. 2014;15(4):273–88.
76. Sun Z, Guo SS, Fässler R. Integrin-mediated mechanotransduction. J Cell Biol. 2016;215 (4):445–56.
77. Geiger B, Tokuyasu KT, Dutton AH, Singer SJ. Vinculin, an intracellular protein localized at specialized sites where microfilament bundles terminate at cell membranes. Proc Natl Acad Sci USA. 1980;77(7):4127–31.
78. Atherton P, Stutchbury B, Jethwa D, Ballestrem C. Mechanosensitive components of integrin adhesions: role of vinculin. Exp Cell Res. 2016;343(1):21–7.
79. Dupont S. Role of YAP/TAZ in cell-matrix adhesion-mediated signalling and mechanotransduction. Exp Cell Res. 2016;343(1):42–53.
80. Elbediwy A, Vincent-Mistiaen ZI, Thompson BJ. YAP and TAZ in epithelial stem cells: a sensor for cell polarity, mechanical forces and tissue damage. BioEssays. 2016;38(7):644–53.
81. Kim IG, Gil CH, Seo J, Park SJ, Subbiah R, Jung TH, Kim JS, Jeong YH, Chung HM, Lee JH, Lee MR, Moon SH, Park K. Mechanotransduction of human pluripotent stem cells cultivated on tunable cell-derived extracellular matrix. Biomaterials. 2018;150:100–11.
82. Clause KC, Barker TH. Extracellular matrix signaling in morphogenesis and repair. Curr Opin Biotechnol. 2013;24(5):830–3.
83. Lubarsky B, Krasnow MA. Tube morphogenesis: making and shaping biological tubes. Cell. 2003;112(1):19–28.
84. Hoffman MP, Kidder BL, Steinberg ZL, Lakhani S, Ho S, Kleinman HK, Larsen M. Gene expression profiles of mouse submandibular gland development: FGFR1 regulates branching morphogenesis in vitro through BMP- and FGF-dependent mechanisms. Development. 2002;129(24):5767–78.
85. Nam JM, Onodera Y, Bissell MJ, Park CC. Breast cancer cells in three-dimensional culture display an enhanced radioresponse after coordinate targeting of integrin alpha5beta1 and fibronectin. Cancer Res. 2010;70(13):5238–48.
86. Avizienyte E, Wyke AW, Jones RJ, McLean GW, Westhoff MA, Brunton VG, Frame MC. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. Nat Cell Biol. 2002;4(8):632–8.
87. Weber GF, Bjerke MA, DeSimone DW. A mechanoresponsive cadherin-keratin complex directs polarized protrusive behavior and collective cell migration. Dev Cell. 2012;22 (1):104–15.
88. Huveneers S, Danen EH. Adhesion signaling—crosstalk between integrins, Src and Rho. J Cell Sci. 2009;122(Pt 8):1059–69.
89. Watanabe T, Sato K, Kaibuchi K. Cadherin-mediated intercellular adhesion and signaling cascades involving small GTPases. Cold Spring Harb Perspect Biol. 2009;1(3):a003020.
90. Van Aelst L, Symons M. Role of Rho family GTPases in epithelial morphogenesis. Genes Dev. 2002;16(9):1032–54.

91. Reddi AH. Cartilage morphogenetic proteins: role in joint development, homoeostasis, and regeneration. Ann Rheum Dis. 2003;62(Suppl 2):ii73–8.
92. Bondow BJ, Faber ML, Wojta KJ, Walker EM, Battle MA. E-cadherin is required for intestinal morphogenesis in the mouse. Dev Biol. 2012;371(1):1–12.
93. Iruela-Arispe ML, Beitel GJ. Tubulogenesis. Development. 2013;140(14):2851–5.
94. Lampugnani MG. Endothelial adherens junctions and the actin cytoskeleton: an 'infinity net'? J Biol. 2010;9(3):16.
95. Brinkmann BF, Steinbacher T, Hartmann C, Kummer D, Pajonczyk D, Mirzapourshafiyi F, Nakayama M, Weide T, Gerke V, Ebnet K. VE-cadherin interacts with cell polarity protein Pals1 to regulate vascular lumen formation. Mol Biol Cell. 2016;27(18):2811–21.
96. Gebala V, Collins R, Geudens I, Phng LK, Gerhardt H. Blood flow drives lumen formation by inverse membrane blebbing during angiogenesis in vivo. Nat Cell Biol. 2016;18(4):443–50.
97. Galie PA, Nguyen DH, Choi CK, Cohen DM, Janmey PA, Chen CS. Fluid shear stress threshold regulates angiogenic sprouting. Proc Natl Acad Sci USA. 2014;111(22):7968–73.
98. Barry AK, Wang N, Leckband DE. Local VE-cadherin mechanotransduction triggers long-ranged remodeling of endothelial monolayers. J Cell Sci. 2015;128(7):1341–51.
99. Wang S, Sekiguchi R, Daley WP, Yamada KM. Patterned cell and matrix dynamics in branching morphogenesis. J Cell Biol. 2017;216(3):559–70.
100. Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature. 1994;367(6461):380–3.
101. Luetteke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC. Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. Development. 1999;126(12):2739–50.
102. De Moerlooze L, Spencer-Dene B, Revest JM, Hajihosseini M, Rosewell I, Dickson C. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development. 2000;127(3):483–92.
103. Hennighausen L, Robinson GW. Information networks in the mammary gland. Nat Rev Mol Cell Biol. 2005;6(9):715–25.
104. Costantini F, Shakya R. GDNF/Ret signaling and the development of the kidney. BioEssays. 2006;28(2):117–27.
105. Djouad F, Delorme B, Maurice M, Bony C, Apparailly F, Louis-Plence P, Canovas F, Charbord P, Noël D, Jorgensen C. Microenvironmental changes during differentiation of mesenchymal stem cells towards chondrocytes. Arthritis Res Ther. 2007;9(2):R33.
106. Lu P, Werb Z. Patterning mechanisms of branched organs. Science. 2008;322(5907):1506–9.
107. Affolter M, Zeller R, Caussinus E. Tissue remodelling through branching morphogenesis. Nat Rev Mol Cell Biol. 2009;10(12):831–42.
108. Costantini F, Kopan R. Patterning a complex organ: branching morphogenesis and nephron segmentation in kidney development. Dev Cell. 2010;18(5):698–712.
109. Blake J, Rosenblum ND. Renal branching morphogenesis: morphogenetic and signaling mechanisms. Semin Cell Dev Biol. 2014;36:2–12.
110. Lee K, Silva EA, Mooney DJ. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. J R Soc Interface. 2011;8(55):153–70.
111. Ferrara N. Binding to the extracellular matrix and proteolytic processing: two key mechanisms regulating vascular endothelial growth factor action. Mol Biol Cell. 2010;21(5):687–90.
112. Hankenson KD, Gagne K, Shaughnessy M. Extracellular signaling molecules to promote fracture healing and bone regeneration. Adv Drug Deliv Rev. 2015;1(94):3–12.
113. Marti-Figueroa CR, Ashton RS. The case for applying tissue engineering methodologies to instruct human organoid morphogenesis. Acta Biomater. 2017;54:35–44.

114. Webb DJ, Roadcap DW, Dhakephalkar A, Gonias SL. A 16-amino acid peptide from human alpha2-macroglobulin binds transforming growth factor-beta and platelet-derived growth factor-BB. Protein Sci. 2000;9(10):1986–92.
115. Ezquerro IJ, Lasarte JJ, Dotor J, Castilla-Cortázar I, Bustos M, Peñuelas I, Blanco G, Rodríguez C, Lechuga Mdel C, Greenwel P, Rojkind M, Prieto J, Borrás-Cuesta F. A synthetic peptide from transforming growth factor beta type III receptor inhibits liver fibrogenesis in rats with carbon tetrachloride liver injury. Cytokine. 2003;22(1–2):12–20. Erratum in: Cytokine. 2006;33(2):119.
116. Serratì S, Margheri F, Pucci M, Cantelmo AR, Cammarota R, Dotor J, Borràs-Cuesta F, Fibbi G, Albini A, Del Rosso M. TGFbeta1 antagonistic peptides inhibit TGFbeta1-dependent angiogenesis. Biochem Pharmacol. 2009;77(5):813–25.
117. Martino MM, Briquez PS, Ranga A, Lutolf MP, Hubbell JA. Heparin-binding domain of fibrin(ogen) binds growth factors and promotes tissue repair when incorporated within a synthetic matrix. Proc Natl Acad Sci USA. 2013;110(12):4563–8.
118. Terada T, Mizobata M, Kawakami S, Yabe Y, Yamashita F, Hashida M. Basic fibroblast growth factor-binding peptide as a novel targeting ligand of drug carrier to tumor cells. J Drug Target. 2006;14(8):536–45.
119. Modaresifar K, Hadjizadeh A, Niknejad H. Design and fabrication of GelMA/chitosan nanoparticles composite hydrogel for angiogenic growth factor delivery. Artif Cells Nanomed Biotechnol. 2017;24:1–10.
120. Berkovitch Y, Cohen T, Peled E, Schmidhammer R, Florian H, Teuschl A, Wolbank S, Yelin D, Redl H, Seliktar D. Hydrogel composition and laser micro-patterning to regulate sciatic nerve regeneration. J Tissue Eng Regen Med. 2017.
121. Paduano F, Marrelli M, Alom N, Amer M, White LJ, Shakesheff KM, Tatullo M. Decellularized bone extracellular matrix and human dental pulp stem cells as a construct for bone regeneration. J Biomater Sci Polym Ed. 2017;28(8):730–48.
122. Hughes CS, Postovit LM, Lajoie GA. Matrigel: a complex protein mixture required for optimal growth of cell culture. Proteomics. 2010;10(9):1886–90.
123. Lindborg BA, Brekke JH, Vegoe AL, Ulrich CB, Haider KT, Subramaniam S, Venhuizen SL, Eide CR, Orchard PJ, Chen W, Wang Q, Pelaez F, Scott CM, Kokkoli E, Keirstead SA, Dutton JR, Tolar J, O'Brien TD. Rapid induction of cerebral organoids from human induced pluripotent stem cells using a chemically defined hydrogel and defined cell culture medium. Stem Cells Transl Med. 2016;5(7):970–9.
124. Ranga A, Girgin M, Meinhardt A, Eberle D, Caiazzo M, Tanaka EM, Lutolf MP. Neural tube morphogenesis in synthetic 3D microenvironments. Proc Natl Acad Sci USA. 2016;113 (44):E6831–9.
125. Boccardo S, Gaudiello E, Melly L, Cerino G, Ricci D, Martin I, Eckstein F, Banfi A, Marsano A. Engineered mesenchymal cell-based patches as controlled VEGF delivery systems to induce extrinsic angiogenesis. Acta Biomater. 2016;15(42):127–35.
126. Lorentz KM, Yang L, Frey P, Hubbell JA. Engineered insulin-like growth factor-1 for improved smooth muscle regeneration. Biomaterials. 2012;33(2):494–503.
127. Miller RE, Grodzinsky AJ, Cummings K, Plaas AH, Cole AA, Lee RT, Patwari P. Intraarticular injection of heparin-binding insulin-like growth factor 1 sustains delivery of insulin-like growth factor 1 to cartilage through binding to chondroitin sulfate. Arthritis Rheum. 2010;62(12):3686–94.
128. Sun W, Lin H, Chen B, Zhao W, Zhao Y, Xiao Z, Dai J. Collagen scaffolds loaded with collagen-binding NGF-beta accelerate ulcer healing. J Biomed Mater Res A. 2010;92(3): 887–95.

Chapter 2
ECM Hydrogels for Regenerative Medicine

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Abstract The ECM is a highly complex mix of structural and functional proteins and other biomolecules. These molecules are secreted by the cells resident in every tissue in the body but can also influence their behavior through a process of "dynamic reciprocity." As a result, there has been significant interest in utilizing ECM as a biologic scaffold material in tissue repair and replacement. Numerous preclinical and clinical studies have demonstrated the efficacy of ECM biomaterials, and more than 4 million patients have now been treated with these scaffold materials. The discovery that these materials could be formed into hydrogels promised to further expand their clinical utility by offering minimally invasive delivery and the ability to fill irregularly shaped defects. This chapter will briefly outline the history and characterization of ECM biomaterials and their evolution from single sheet to multisheet, powder, and ultimately hydrogel form. The first studies describing the production of early-generation ECM hydrogels used well-characterized porcine small intestinal submucosa and urinary bladder matrix, and these materials will be discussed in the context of the general methods used to produce and characterize ECM hydrogels. A detailed consideration of the many second-generation hydrogels which have since been produced from a wide range of tissues will then be discussed

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in the context of tissue specificity. The hydrogels discussed in this chapter have been evaluated in vitro or in small scale in vivo animal studies. More substantial evaluation is required before these materials can be considered ready for clinical application, but these hydrogels provide the possibility for minimally invasive delivery, treatment of irregularly shaped defects in anatomic sites that prove challenging for invasive surgical procedures, and may provide an ECM formation that delivers immediate bioactivity as a consequence of its distinctive biomolecular composition.

Keywords Adipose · Brain · Cartilage · Central nervous system
Cornea · Decellularization · Demineralized bone matrix · Dermis
Extracellular matrix · Hydrogel · Intervertebral disk · Ligament
Liver · Myocardium · Nucleus pulposus · Pericardium · Regenerative medicine
Skeletal muscle · Skin · Spinal cord · Small intestinal submucosa
Tendon · Tissue engineering · Tissue specificity · Urinary bladder matrix
Vocal fold

Historical Development of ECM-Derived Materials

Once considered to be a passive bystander in tissue morphology, the extracellular matrix (ECM) is now understood to instruct cell behavior through "dynamic reciprocity" [13, 59]. Specifically, cell surface receptors transduce signals from the microenvironmental niche to the cytoskeleton, the nuclear matrix, and the chromatin, thereby affecting gene expression, and then signal back to the niche.

Mechanical dissection and tissue decellularization have been widely used to generate biologic scaffold materials from the native ECM. These ECM-derived materials release natural factors upon degradation that alter the default immune response to injury, and recruit endogenous cells for the formation of site-specific functional tissue (constructive remodeling). The safety and efficacy of ECM scaffolds have been demonstrated in numerous preclinical animal studies and clinical human studies for a wide variety of tissues, including musculoskeletal, cardiovascular, and urogenital. ECM-derived materials represent an attractive tissue engineering solution because they are FDA approved, "off the shelf," and obviate the need to add exogenous growth factors or cells, representing nontrivial regulatory hurdles for clinical translation [71]. Moreover, the use of ECM-derived materials bypasses the issues of cell sourcing and engineering immune acceptance since the cells populating the scaffold are the patient's own cells [60]. Currently, more than 4 million patients have benefited from the use of ECM scaffolds.

Biochemical Content

The ECM consists of the structural and functional proteins secreted by the cells resident in each tissue and organ and is a primary component of the cell's microenvironmental niche. ECM is comprised primarily of fibrillar collagen but also contains glycoproteins (laminin, fibronectin), proteoglycans, glycosaminoglycans (GAGs), and growth factors. The 3D arrangement and biochemical composition of these proteins are specific to tissue type [59]. Ideally, an ECM-derived material would preserve the components of native tissue ECM. However, the choice of decellularization agents, techniques, and post-processing terminal sterilization steps to produce ECM-derived materials will unavoidably affect the ECM biochemical properties. The balance lies in achieving decellularization and sterility criteria while best preserving ECM composition and structure [28]. Thin tissues such as urinary bladder matrix (UBM) and small intestinal submucosa (SIS) may be effectively decellularized with mechanical scraping, agitation in a dilute acidic solution (0.1% peracetic acid), and subsequent rinsing with deionized water. UBM and SIS contain growth factor and GAG levels similar to native ECM after decellularization [3, 88]. Brown et al. [18] reported the urinary bladder matrix contained an intact basement membrane after decellularization.

In contrast, denser tissues such as porcine dermis require a harsher decellularization protocol, usually involving a combination of detergents, organic solvents, and enzymatic solutions. Reing et al. [67] tested the effect of different processing steps on the properties of porcine dermal matrix. While a trypsin/Triton X-100 protocol moderately affected ECM properties, the addition of a sodium dodecyl sulfate (SDS) processing step more severely affected ECM mechanical strength, growth factor, and GAG content, and the ability of the ECM to support cell growth in vitro. The results may not be surprising considering Triton X-100 is a relatively gentle nonionic detergent and trypsin is an enzyme that selectively cleaves cell adherent proteins to detach cells from the tissue surface. In contrast, SDS is a more harsh ionic detergent that can disrupt protein covalent bonding.

When processed appropriately, ECM-derived materials have many advantages compared to their synthetic counterparts. ECM-derived materials recapitulate the rich natural diversity of proteins, unlike matrices that are manufactured from individual molecular components of the extracellular matrix (e.g., laminin or collagen gels). Furthermore, the GAGs in the ECM-derived material present growth factors to cell surface receptors in biologically relevant ratios and 3D conformations [3]. It is well known that growth factors have an important impact on regulating cell behavior [83]. Finally, non-chemically cross-linked ECM-derived materials degrade and release biochemical signals at a similar rate to native tissue ECM.

Bioinductive Properties

ECM-derived materials act as an inductive template for the formation of site-specific functional tissue through the following identified mechanisms: the release of sequestered growth factors and cytokines [3] and the release of cryptic peptide motifs which possess diverse biological activities including antimicrobial activity [16, 73], chemoattraction of endogenous stem/progenitor cells [2, 68], and modulation of the host immune response toward an M2/Th2 phenotype [6]. Interestingly, it appears that degradation of the ECM-derived material is essential for the bioinductive properties of ECM. For example, ECM degradation products show bactericidal activity, while intact ECM does not show the same antimicrobial effects. Furthermore, if the scaffold is chemically cross-linked such that it cannot be degraded, the immune response will be directed toward an M1/Th1 phenotype and a fibrous capsule will form as opposed to constructive remodeling.

Antimicrobial Properties

ECM-derived materials have displayed consistent resistance to bacterial infection in clinical applications and even demonstrated resistance to deliberate bacterial infection in preclinical studies [4, 10, 16, 43, 79]. It has been shown that naturally occurring gene-encoded antimicrobial peptides (AMPs)/polypeptides such as defensins [39], cecropins [58], and magainins [12] are found in vertebrates and invertebrates. These peptides, present in native tissue, act as a potent first line of defense in the innate immune response to pathogens [21].

At least 18 AMPs have been identified in porcine tissues which are the primary source of SIS and UBM [15]. Sarikaya et al. [73] showed the antimicrobial peptide activity of ECM was retained after processing. UBM and SIS degradation products demonstrated a strong antibacterial effect against gram-negative *Escherichia coli* and gram-positive *Staphylococcus aureus* and completely inhibited the growth of both bacterial strains after 13 h. Similarly, Brennan et al. [16] tested multiple fractions of UBM and liver ECM degradation products on their ability to inhibit *S. aureus* and *E. coli* growth. The results suggested multiple antibacterial molecules are present in ECM degradation products and the profile of these molecules differs between ECMs derived from different tissues.

Chemoattraction

Badylak et al. [7] used a mouse model to show that a population of bone marrow cells contributes to constructive tissue remodeling. Briefly, mice deficient for the β-galactosidase gene were lethally irradiated and received both a transplantation of β-galactosidase^{+} bone marrow cells, and one of 4 scaffold implants. The mice received ECM-derived materials (SIS or urinary bladder submucosa) or control

scaffolds (collagen or degradable synthetic material PLGA). All scaffolds showed the presence of β-galactosidase$^+$ mononuclear cell infiltration at 7 days. At 56 days, the ECM-derived material was completely degraded and replaced with highly organized tissue, with β-galactosidase$^+$ epithelial cells and fibroblasts. In contrast, the collagen control and PLGA showed chronic inflammatory reaction around the implants with little to no β-galactosidase$^+$ epithelial cells and fibroblasts present within each scaffold. This study identified that bone marrow cells were capable of being recruited and could later differentiate into endothelial and fibroblast cells.

Reing et al. [67] demonstrated that enzymatically digested UBM in vitro possessed chemotactic and mitogenic activities for human multipotent progenitor cells with a fivefold increase in migration over control digest buffer and 67% increase in 3-day proliferation over negative control. The digested UBM also inhibited chemotaxis and proliferation of differentiated endothelial cells.

Chemoattraction by enzymatically digested UBM was illustrated in vivo [1] by recruitment of a population of multipotent cells (e.g., Sca1$^+$, Sox2$^+$, Rex1$^+$) in a mammalian model of digit amputation. A single matricryptic peptide with positive chemotactic potential was subsequently identified [2]. Briefly, ECM degradation products were serially fractionated by different biochemical properties (i.e., ionic charge, size, and hydrophobicity) and tested for chemotactic activity in a transwell assay. The fraction with the greatest chemotactic potential was isolated for sequence analysis by mass spectroscopy, which identified a single peptide from the alpha subunit of the collagen III molecule. A synthesized version of the peptide showed positive chemotactic activity toward several types of stem, progenitor, and differentiated cells in vitro. The peptide was also chemotactic in vivo for the same cell types in a mouse model of digit amputation [2].

Macrophage Polarization

ECM-derived materials have been shown to facilitate the functional reconstruction of many tissue types including cardiac, esophageal, dermal, and musculotendinous structures, among others [48]. Mechanisms underlying the clinical success of ECM-derived materials are still not fully understood. However, it is logical to assume the innate immune cells (e.g., neutrophils and macrophages), the first cells to infiltrate the site of the implanted biomaterial, play a key role in the host response and subsequent tissue remodeling outcome.

ECM-derived materials polarize macrophages away from the classically activated pro-inflammatory "M1" macrophages and toward the alternatively activated immunomodulatory or "M2" macrophages [6]. M1 and M2 represent the extremes of the continuum for macrophage phenotypic polarization, with markedly different physiologic roles. M1 macrophages secrete copious amounts of nitric oxide, reactive oxygen species, and pro-inflammatory cytokines such as interleukin (IL)-12, IL-6, and tumor necrosis factor alpha. In contrast, M2 macrophages secrete large amounts of IL-10, TGF-β, and arginase and scavenge debris, promote angiogenesis, and recruit cells involved in constructive tissue remodeling.

The host remodeling response to SIS and its chemically cross-linked form has been characterized using autologous graft tissue as a control [8]. SIS elicited a predominantly "M2" profile ($CD163^+$) when implanted into a rat model, with long-term constructive remodeling evidenced by vascularization, new skeletal muscle fiber formation, and organized connective tissue at 16 weeks. Conversely, the chemically cross-linked SIS elicited a predominantly "M1" profile ($CD80^+$, $CCR7^+$) with chronic inflammation at 16 weeks evidenced by fibrosis and scarring around the implant, presence of multinucleated giant cells, and the associated classic foreign body response. The autologous tissue graft elicited a mixed M1/M2 profile at 16 weeks with moderately well-organized scar tissue, but no detectable new muscle fibers. A subsequent study showed that the presence of a cellular component, even an autologous one, polarized macrophages to an M1 profile [20]. Furthermore, the M2:M1 ratio for 14 commercially available ECM-derived materials was shown to correlate with in vivo constructive remodeling histologic scores at 14 and 35 days post-implantation, an indication of the broad applicability of this concept [19].

Types of ECM-Derived Materials

The first ECM scaffolds were intact sheets (60 μm), reflecting "skilled decellularization" [3]. The fragile tissue layer of interest had to be carefully separated from other layers and withstand the scraping and decellularization process without tearing. These initial sheets were typically stored as lyophilized sheets and hydrated before use [5]. ECM sheets are commonly used for wound repair (e.g., partial and full thickness wounds, ulcers, second-degree burns, trauma wounds). A common misconception in the use of ECM-derived materials is the requirement of the material to replace volumetric tissue loss and provide mechanical strength at the wound site. Instead, the ECM sheets are intended to serve as a bioinductive template that degrades as the tissue is restored.

Single-layer ECM sheets may be insufficient for regenerative medicine applications with load-bearing requirements (e.g., esophagus repair, laryngeal reconstruction, abdominal wall reconstruction, hernia repair, pelvic floor). ECM-derived materials were vacuum compressed to form multilayer devices (4–8 layers) to increase the strength of the material. Collagen fiber orientation has been used advantageously in sheet stacking to improve the strength of the material device. For example, a 10-layer ECM device (Restore®, DePuy Orthopedics) was stacked with collagen fibers at 72° angles every 2 layers resulting in an isotropic construct that matched the strength of the tendon tissue it was intended to replace [5]. In addition, vacuum pressing allowed for the creation of a variety of 3D shapes for certain applications (e.g., tube for trachea, funnel for esophageal anastomosis) [9].

ECM-derived materials can be powdered to apply topically to the wound site and fill irregular defects (e.g., digit amputation) [40]. The particulates derived from powdering are of the order of 50–200 um in diameter and retain the surface

topology of the native matrix [5, 72]. The ECM powder may also be suspended in a pharmacologically approved carrier (e.g., saline, water). High concentrations of ECM powder suspension (e.g., 500 mg/mL) can form a putty with complex rheological behavior which has been applied to fill tissue defects [22].

ECM Hydrogel Formation

The discovery that extracellular matrix-derived materials could be formed into hydrogels was a notable advance that further expanded the clinical utility of ECM-derived materials [38]. ECM hydrogels retained the inherent bioactivity of the matrix and provided nanofibrous structural support to guide cellular behavior [45]. Additionally, key advantages compared to sheets include minimally invasive delivery, i.e., injection as a viscous pre-gel fluid via catheter, followed by gelation in situ and the filling of irregularly sized defects. Compared to a suspension of ECM powder, the ECM hydrogel could be injected with a more homogenous concentration and with greater ease [40].

Hydrogels derived from ECM-derived materials occupy a specific niche as a diverse class of biomaterials. "Hydrogels" are defined as highly hydrated polymer materials (>30% water by weight) [34] composed of synthetic or natural polymer chains with cross-links to maintain the structural integrity of a solid [50]. Hydrogels from naturally derived materials containing one or more molecules of the extracellular matrix (e.g., collagen, agarose, alginate, chitosan, fibrin, gelatin, and hyaluronic acid) are often called "ECM mimics" and have been extensively characterized [34]. The limitation of reducing the complex cellular microenvironment to one or more ECM proteins prompted the development of more complex hydrogels: in particular Matrigel (reconstituted basement membrane extract) from Engelbreth–Holm–Swarm tumor, and gels from decellularized tissue. When the present chapter cites "ECM hydrogels," the term is applied specifically to ECM hydrogels derived from decellularized mammalian tissue.

The first ECM hydrogels were composed of porcine small intestinal submucosa (SIS) (Voytik-Harbin [87] or porcine urinary bladder matrix (UBM) [38]. SIS and UBM are two of the most well-characterized ECM-derived materials. The first studies of SIS and UBM hydrogels determined whether the materials retained bioactivity and the ability to promote constructive remodeling in non-heterologous tissues; properties characteristic of the 2D sheet forms of SIS and UBM [38]. The first studies also demonstrated feasibility of ECM hydrogel production and described test methods for characterization of these materials and thus established a precedent for the production of the next-generation ECM hydrogels for homologous tissue applications.

Methods for ECM Hydrogel Production

Gelation of the first SIS and UBM hydrogels was based on well-established protocols for collagen gelation. Native collagen is a main constituent of the ECM hydrogel, and its gelation characteristics have been extensively characterized [42, 46, 47, 62]. Although ECM hydrogel preparation has been described with respect to collagen biochemistry and fibrillogenesis, it is important to note that ECM hydrogel formation is undoubtedly influenced by the presence of other ECM components [42]. While bulk mechanical properties of the hydrogel can be described, further work is required to understand microscale differences that affect collagen polymerization and are manifested in distinct viscoelastic properties.

The pepsin enzyme is used in both the SIS and UBM digestion protocols. While pepsin, derived from the gastric juices, is not a physiologically relevant method for ECM degradation, biochemists have been using pepsin since 1972 to solubilize a substantial portion (up to 99%) of the acid-insoluble collagen [33, 57]. It has been shown that pepsin selectively cleaves telopeptides, the protein domains external to the triple helix-body of the tropocollagen molecule [42]. These telopeptides form the intramolecular bonds between the collagen fibril aggregates [33, 70]. The unraveling of the collagen fibril aggregates by pepsin digestion produces almost entirely monomeric segments [70]. Visible changes are observed as the pepsin in dilute acid solubilizes the ECM powder and the suspension transforms into an opaque, viscous solution ("solubilized ECM"). The solubilized ECM is ready for gelation when the liquid is homogenous, with no visible particles [45].

Another general method of protein solubilization which has been applied to ECM materials is homogenization, either by pestle and mortar or high-speed shear mixer. This is carried out in the presence of a high salt buffer and acts to physically break down the ECM particles and disrupt the collagen fiber structure [23, 25, 37, 64, 65, 84, 85]. Prior to this physical processing, the material is often treated with dispase, a specific protease which cleaves fibronectin, collagen IV, and collagen I [23, 64, 65, 84, 85]. The treatment is used primarily to decellularize the tissue but will also begin to digest the ECM protein structure. After dispase treatment and homogenization, the ECM is then typically further processed via one or more urea extraction steps [23, 37, 64, 65, 84, 85]. Urea in high concentrations acts to disrupt the non-covalent bonding which is essential to protein folding and structure and thereby denatures many proteins and increases their solubility. Centrifugation can be used to remove any remaining insoluble ECM components leaving a viscous, cloudy solution similar to that obtained by pepsin digestion.

The solubilized ECM is neutralized to physiologic pH and salt concentration and is referred to as "pre-gel solution." Gelation is chiefly determined by the polymerization of fibrillar collagen as the primary ECM constituent. When the temperature is raised to physiologic conditions (37 °C), solubilized collagen can self-assemble in an entropy-driven process. It is energetically favorable for collagen monomers to lose surface water and bind to aggregates in order to bury hydrophobic residues within the fibril [42, 62]. Self-assembly follows four

characteristic growth phases: a nucleation phase, a lag phase (collagen monomers form metastable nuclei), a rapid growth phase (fibrils mediate from the nuclei), and plateau phase [42]. At the end of this process, the solubilized ECM is now an "ECM hydrogel."

ECM Hydrogel Characterization

The unique biological, biochemical, mechanical, and topological properties of SIS and UBM hydrogels have been well characterized using the techniques described below.

Biocompatibility

At minimum, a biocompatible hydrogel should not cause detrimental effects on cells interacting with the material and should exist within the body without eliciting a pro-inflammatory response [53]. Beyond the minimum requirements of biocompatibility, the ECM hydrogel should induce or maintain tissue-specific differentiation of cells to promote an appropriate host response.

Cell response to SIS hydrogels has been characterized in vitro and compared to intact SIS and common tissue culture substrates including tissue culture plastic, pure collagen gel, and Matrigel™ [87]. Specifically, cell shape, morphology, and proliferation were characterized for four cell types: squamous epithelial cells (pulmonary artery), fibroblasts (Swiss 3T3), glandular epithelial cells (adenocarcinoma), and smooth muscle-like cells (urinary bladder). All cells were able to adhere, survive, proliferate, and invade the SIS gel. SIS gels promoted similar cell shapes as the intact SIS. The SIS gel also promoted more cell–cell contact than collagen and tissue culture plastic, with cells spreading on the surface of the gel and invading to form three-dimensional multilayers. This cell growth pattern was markedly different compared to the other substrates where Matrigel™ induced the formation of aggregates with limited to no cell proliferation for each of the cell types, tissue culture plastic induced formation of cell monolayers, and collagen gels displayed mixed 2D and 3D morphologies and restricted matrix penetration.

In another study, SIS hydrogel was characterized as a tissue culture substrate for cardiac cells and compared to Matrigel™ [30]. Neonatal rat cardiac cells were combined with SIS gel and Matrigel™ on a porous, elastomeric scaffold. The SIS gel and Matrigel™ both permitted cell contraction on days 3–4, which continued for 10 days. Compared to cardiac cells cultured in Matrigel™, those cultured in SIS gels displayed significantly higher mean contraction rate (more closely mimicking native rat myocardium), expression of cardiac-specific marker Troponin-T, and a more homogenous distribution within the construct. Matrigel™ exhibited greater cell adhesion compared to SIS gel, likely attributed to the high laminin content of the former. Overall, the superior performance of SIS gel for tissue-specific cell

differentiation and function is likely attributable to the biochemical composition of the SIS gel more closely mimicking that of native myocardium, i.e., high content of collagen I and III.

UBM gels were also characterized for their ability to support growth of rat aorta-derived smooth muscle cells. Similarly to SIS gels, the smooth muscle cells were able to adhere, proliferate, form multilayers, and contract. After 10 days, UBM gels promoted significantly greater cell numbers than collagen gels, but lesser numbers than intact UBM. The authors proposed the difference between intact UBM and the UBM gel could be attributed to the loss of certain bioactive peptides during the hydrogel formation process [38]. UBM gels also have been shown to support the growth of NIH 3T3 fibroblasts, with almost 100% cell viability after 7 days and gel infiltration [90].

Biochemical Composition

Studies of UBM and SIS gels provided confirmation that the hydrogels possessed similar biochemical compositions to the intact UBM and SIS scaffolds in terms of collagen and GAG content [17, 38, 90]. Intact SIS contained 90% collagen, primarily collagen I, with minor amounts of collagen III, IV, V, and VI. Intact UBM had a similar composition to intact SIS, but with greater amounts of collagen III and the additional presence of collagen VII [5]. SIS hydrogels were shown to contain at least three types of collagen (predominantly collagen I and collagen III with minor amounts of collagen IV), and sulfated GAGs [17]. Gel electrophoresis of the UBM gel displayed similar bands to purified collagen I, with additional bands present for other ECM proteins [38].

Recognizing the biochemical complexity of ECM hydrogels, mass spectroscopy is beginning to be used to determine the proteomic profile of pepsin-solubilized ECM [14, 76]. Published studies to date have used reversed-phase high-performance liquid chromatography interfaced with tandem mass spectroscopy (LC-MS/MS) and compared the peptide fragments generated with a protein data bank (Swiss Protein data bank). SIS and UBM hydrogels have yet to be characterized by LC-MS/MS.

Gelation Kinetics and Mechanical Properties

The delivery of solubilized ECM via catheter or syringe to irregularly shaped anatomic sites provides increased clinical utility of ECM-derived materials [5]. The kinetics of gelation and the viscoelastic properties of the gel can be designed to be similar to those of the tissue being repaired. For instance, desired mechanical properties important for cell behavior can be achieved by changing the ECM concentration [90]. The biochemical composition of the ECM (e.g., collagen, glycoproteins, GAGs, growth factors) is specific to tissue type, and microscale differences in intermolecular bonding/microstructure are manifested in distinct

viscoelastic properties of the gels. Furthermore, the viscoelastic properties are affected by any modifications to the gel, including changes in the decellularization treatment and the addition of exogenous cells or biologics.

The turbidimetric gelation kinetics and rheological behavior of UBM gels have been well characterized [38, 90]. Gelation kinetics for UBM showed a sigmoidal shape, similar to that achieved with collagen type I gels. The lag phase and time required to reach half the final turbidity were greater in the UBM gels than the collagen type I gels [38]. This difference between the ECM gel and the purified collagen gel is presumably due to the presence of GAGs and other molecules such as fibronectin which may modulate self-assembly. The role of GAGs in the gelation kinetics of ECM-derived gels was investigated by time-lapse confocal reflection microscopy and spectrophotometry of purified components of SIS hydrogels [17]. A decrease in final turbidimetric absorbance and changes in gelation kinetics, including increased lag phase, occurred when collagen type I gels were mixed with different GAGs [17].

ECM hydrogels are thermosensitive viscoelastic materials that display mechanical properties similar to a viscous liquid at 10 °C and mechanical properties intermediate between that of a solid and liquid at 37 °C. The mechanical response of ECM hydrogels can be described as a combination of the elastic (energy storing) and viscous (energy dissipating) components and can be measured by rheology. The elastic response of ECM hydrogels may be described by Hooke's law, i.e., strain is proportional to applied stress, and the sample quickly returns to its original shape when the stress is removed. The viscous response of ECM hydrogels may be described by Newton's law, i.e., the strain rate is proportional to applied stress, and the sample will continue to strain with time. Viscoelastic ECM hydrogels display intermediate behavior that is time-dependent: The hydrogel is able to instantaneously strain, but further deformation of the polymer chains is time-dependent.

Rheological measurements for UBM gels have been reported [38, 90]. Solubilized ECM was loaded onto a parallel-plate rheometer at 10 °C, and deformation in response to constant shear stress was measured to determine viscosity ("pre-gel creep test"). At 10 °C, UBM gels exhibited low viscosity [38]; the temperature was rapidly increased from 10 to 37 °C to measure the gelation kinetics and viscoelastic properties of the forming ECM hydrogel ("time sweep test"). An oscillatory strain at fixed frequency (ω) of 1 rad/s was applied to the UBM hydrogel, and the stress response characterized. At time zero, UBM hydrogels exhibited low storage (G′) and loss (G″) moduli. As temperature was raised, G′ and G″ followed a sigmoidal shape over time and reached a plateau in $\sim$12 min, indicating that gelation was complete. Solid-like behavior was confirmed since the storage modulus (G′) was greater than the loss modulus (G″) by approximately a factor of 10 after gelation [38].

Freytes et al. [38] undertook a frequency sweep analysis of UBM hydrogels which indicated "shear thinning" behavior, i.e., the complex viscosity decreased with increasing shear rate. This may be a useful property for ECM hydrogels which are to be directly injected or delivered via catheter. Recently, Wolf et al. [90]

undertook post gelation creep analysis of UBM in comparison with a hydrogel derived from dermal extracellular matrix. Both hydrogels displayed a viscoelastic strain profile characterized by a rapid initial strain increase with creep ringing and a subsequent slower increase over time.

Gel Topology

Upon gelation, SIS, and UBM hydrogels form porous, nanofibrous structures, which provide cells with a dynamic 3D environment. In contrast to other forms of ECM-derived materials (sheet and powder), the ECM hydrogels lose the native 3D structure. However, the complex nanoscale mesh of ECM fibers guides cellular behavior via contact guidance and fibril-bound proteins that interact with cell membrane-bound integrin receptors.

Visualization of the SIS and UBM gel topology with scanning electron microscopy (SEM) showed that both hydrogels formed loosely organized, interwoven networks of fibers [38]. D'Amore et al. [31] developed a MATLAB algorithm that enables high-throughput, automated analysis of SEM images to more accurately assess the characteristics of the overall fiber network. Using the image-based algorithm, UBM hydrogel characteristics were assessed [90]. The UBM hydrogels had a fiber diameter of 0.074 ± 0.004 μm and pore size of 0.112 ± 0.005 μm, which is within biological criteria for hydrogel design. Fiber alignment was randomly organized (isotropic) as characterized by fiber alignment close to 0% with respect to any axis. The described metrics were concentration-independent for UBM hydrogels, but ECM concentration has been shown to be an important covariate of fiber diameter and porosity for other ECM hydrogels, such as dermal matrix hydrogels [90].

Tissue-Specific Hydrogels

Recent studies of ECM-derived biomaterials in various forms have demonstrated significant tissue specificity in achieving complex biological functions [27, 63, 78, 86]. As a result, having established that SIS and UBM could be processed into hydrogels, the next step for researchers was to demonstrate hydrogel formation from a wide range of tissues and establish the efficacy of these materials in tissue repair. Published studies to date which have contributed to this process will be discussed in detail in the following sections, grouped according to their target tissue. The mechanical properties, fiber structures, and gelation kinetics of many of these novel ECM hydrogels have been characterized using techniques similar to those described in Section "ECM Hydrogel Characterization," albeit with tissue-specific testing conditions and parameters. However, as discussed earlier, the primary purpose of an ECM hydrogel is to act as a bioinductive template for new tissue formation, and as a result, the focus of this chapter will be on the biological

performance of the novel gels rather than their physicochemical characteristics. It is only a matter of a few years since the first publication detailing the production of an ECM-derived hydrogel. As a result, data published to date in this field have typically arisen from in vitro experiments and small-scale proof-of-principle studies in vivo, rather than from fully developed preclinical or clinical evaluations.

Heart

Among the most frequently targeted tissues is cardiac muscle, primarily with the aim of restoring heart function after myocardial infarction (MI), which is the leading cause of death in most industrialized countries (Rosamond et al. [69]. Hydrogels from heart tissue have been developed, characterized, and applied to tissue repair in a number of studies published by Christman and colleagues [44, 45, 75–77, 80, 81]. A protocol was developed for the production of decellularized matrix from porcine myocardium via a detergent-based technique using sodium dodecyl sulfate (SDS) and Triton X-100 [80]. Pepsin digestion of this matrix resulted in a solution which underwent thermoresponsive gelation both in vitro and in vivo after catheter-based delivery, thus suggesting that the material had good clinical handling properties. As with many other ECM materials, the myocardial matrix was shown to be composed predominantly of collagen, but also to contain a significant amount of GAGs. The most important result of this initial study, however, was that both smooth muscle cells and endothelial cells migrated toward the matrix in vitro and in vivo to a greater degree than toward collagen or fetal bovine serum (FBS). The cell-free material may therefore be sufficient to recruit appropriate cell types to defect sites and to ultimately form appropriate new tissue. Further development of myocardial matrix was shown in a subsequent study which demonstrated that the fiber size and structure, mechanical properties, and gelation kinetics could be modulated by alterations of the pH, temperature, ionic strength, and concentration used for gelation [45]. Modification of the hydrogel in this fashion could allow the handling properties and cell response to be tailored to specific applications.

Most recently, the treatment efficacy of the material has been evaluated in both rat and porcine preclinical models of MI [77, 81]. In the rat model, myocardial matrix was injected into the infarcted area 1 week after MI and led to significantly increased numbers of cardiomyocytes and proliferative cells 1 month after injection when compared to saline-injected controls [81]. Cardiac function was also significantly increased at this time point as measured by parameters such as the ejection fraction, end-diastolic dimension, and end-systolic volume. In a later study in the porcine model [77], myocardial matrix was delivered into the affected tissue 2 weeks after the induction of MI. Treatment contributed to an increased muscle volume in the affected area when compared to saline-injected controls 3 months post-MI, and significant increases in cardiac function were again observed. Taken together the results of these studies suggest great promise for this material in

post-MI cardiac regeneration. This promise is further supported by positive results obtained during examination of the safety and immune response to myocardial matrix. In response to injection into healthy rat hearts, no significant increase in infiltrating inflammatory cell numbers was seen relative to saline-injected controls. Finally, in vitro hemocompatibility studies showed no effect of myocardial matrix on platelet activation and clotting.

A recent study sought to evaluate the production of a similar material from human, rather than porcine myocardium, in order to eliminate the use of xenogenic material [44]. The efficacy of the two materials was compared in vitro, and it was shown in principle that the human myocardial matrix could undergo in vivo gelation. In order to effectively decellularize the human myocardium, the incubation time in SDS had to be significantly extended relative to the porcine tissue process and additional DNA and lipid removal steps had to be included. This was attributed to the accumulation of additional lipids and fibrous tissue in the myocardial matrix of the human tissue, which was harvested, relatively speaking, from older donors. As a result of this additional processing, the sulfated GAG content of the human-derived material was significantly lower than that of the porcine material, suggesting the complex matrix composition and architecture may be less well maintained. Both rat aortic smooth muscle cells and human coronary artery endothelial cells proliferated more rapidly over 5 days on human myocardial matrix than porcine. However, less than half of the human donors tested gave rise to matrix solutions which would undergo gelation, and very significant donor-to-donor variability was observed. As a result, the ability to translate this approach to large-scale clinical application may be limited.

An alternative material has been developed by the same researchers, but derived from the pericardium rather than cardiac muscle. A similar approach was taken to evaluate the potential of this material, with both porcine and human pericardium decellularized using SDS and solubilized with pepsin [76]. Again, these materials were composed primarily of collagen, though with significant components of GAG and other proteins. In vitro chemotaxis assays demonstrated that, as with myocardial matrix, both endothelial and smooth muscle cells migrated toward pericardial matrix, with porcine-derived material significantly outperforming human in this analysis. The in vivo results of injecting these matrices into healthy rat myocardium also showed significant infiltration by both cell types, and the formation of arterioles, with the human ECM-derived gel outperforming porcine. Finally, positive staining for c-kit indicated the presence of stem cells in both materials, suggesting their ability to direct stem cell homing.

The utility of the pericardial matrix in a preclinical MI rat model was evaluated [75]. In this study, an additional function of the matrix was assessed, in the form of its ability to sequester and then slowly release basic fibroblast growth factor (bFGF) in an effort to boost cardiac regeneration. The in vitro release of bFGF from pericardial matrix was significantly delayed relative to collagen. This delay was attributed to the ability of GAGs, and more specifically heparin in the matrix to bind the growth factor, though the specific presence of heparin was not demonstrated at this stage. Greater retention of bFGF in the infarcted tissue in vivo was shown when

the protein was delivered in matrix than in collagen or saline, and this resulted in significant increases in arteriole density. Most importantly, the newly formed vasculature was anastomosed with that preexisting in the host, allowing it to function to restore oxygen supply to the ischemic tissue. The next stage for the development of pericardial matrix will be to demonstrate that the observed improvements in neo-vessel formation result in recovery of cardiac function, as observed for myocardial matrix.

In addition to the heart-derived materials described thus far, porcine bone marrow has also been considered as a source tissue for hydrogel biomaterials to treat MI [66]. A series of nuclease, ethylenediaminetetraacetic acid (EDTA), vortex and centrifuge steps were used to produce a bone marrow ECM extract which contained a number of growth factors important in angiogenesis and cardiac repair. Coating non-tissue culture-treated polystyrene (non-TCPS) with this extract was found to significantly increase endothelial cell adherence relative to fibronectin. Fibronectin is an effective single component substrate coating containing many RGD peptide sequences which can interact with integrins on the cell surface. The ability of the bone marrow ECM-derived material to support greater cell attachment is therefore a highly significant result. For three-dimensional hydrogel studies, the bone marrow extract was reinforced with methylcellulose in order to improve material handling and retention at the injection site in vivo. When this reinforced material was delivered into a rat MI model, the infarct area was rapidly reduced relative to untreated controls, with associated increases in cardiac function. Furthermore, 3 weeks post-intervention there was a significant reduction in the numbers of apoptotic cells and macrophages present, alongside increases in stem cell and blood vessel numbers. This bone marrow-derived ECM material may therefore be a useful supplement to other biomaterials utilized for cardiac repair post-MI.

As one of the most heavily targeted tissues for the development of ECM-derived hydrogel biomaterials, it is perhaps unsurprising that proposed therapies in the area of post-MI cardiac repair are also among the most well-developed. All three distinct materials, developed from different source tissues, have shown, in principle at least, that they can contribute to the generation of functional repair tissue in vivo.

Fat

Like cardiac tissue, fat has also been widely studied as a target for repair by extracellular matrix biomaterials [23, 65, 85, 92]. If a successful therapy could be developed, then it could be used in a large number of procedures following trauma and tumor resection which require the reconstruction of significant volumes of adipose tissue. The use of ECM hydrogels to support adipogenesis was first explored in a pair of studies by Brey and coworkers [23, 84]. In the first of these studies, a combination of dispase digestion, high salt homogenization, and urea extraction were used to produce rat adipose tissue extracts which underwent

thermo- or pH-responsive gelation [84]. When seeded with pre-adipocytes in vitro adipose-derived gels supported significantly greater adipogenic differentiation than Matrigel™ based on histological staining for lipid loading. These results were echoed in vivo when the matrix was implanted around a rat epigastric pedicle bundle. Hydrogels formed by changes in temperature or pH were implanted but only those formed by pH changes supported increased adipogenesis relative to Matrigel™ at time points greater than 6 weeks post-implantation.

An alternative rat dermis-derived hydrogel, produced by the same protocol, has also been assessed for its ability to support adipogenesis [23]. As was found for adipose-derived materials, the dermis-derived gels supported significantly more fat tissue formation in vivo than Matrigel™. In vitro experiments, however, showed no difference between the two in this case. The results of in vitro evaluation in these two studies suggested that in order to promote adipogenesis, an ECM hydrogel must be sourced from fat tissue. However, gels from both sources considered here were highly effective substrates for fat tissue formation in vivo. This highlights the uncertainty inherent in forming conclusions about tissue specificity based on a single model and more particularly in relying exclusively on in vitro studies.

Porcine fat tissue extracts have also been prepared using various combinations of high salt homogenization, dispase treatment, urea extraction, and pepsin digestion. By combining a number of such extracts, a thermoresponsive gel-forming material known as adipose-derived matrix (ADM) was produced [65]. A number of relevant growth factors such as FGF-2 and TGF-β1 were quantified in ADM, which prompted no significant inflammatory cytokine production by in vitro macrophage cultures. Adipose-derived stem cells (ASCs) were differentiated on ADM in the absence of adipogenic medium supplements with greater than 90% efficiency. Furthermore, when ADM, with or without ASCs, was implanted subcutaneously in rats, it resulted in significantly more adipose tissue generation than Matrigel™. This was the case even when the latter was supplemented with FGF-2 in an effort to increase adipogenesis. The ADM containing groups also both showed significantly fewer activated immune cells, echoing the in vitro macrophage results.

More recently, a thermoresponsive matrix has also been generated from human lipoaspirate by a combination of surfactant and enzymatic treatments, with pepsin digestion to fully solubilize the protein mixture [92]. This material underwent gelation upon subcutaneous injection in rats and supported proliferation of patient-matched ASCs in vitro. However, the growth rate on this novel matrix was not significantly enhanced relative to collagen or TCPS. It is interesting to note that the ECM-derived hydrogel was not able to exert a mitogenic effect on relevant stem cells, but studies of cell differentiation and matrix production may be necessary to allow comparison with studies of ECM-derived materials from other species.

The results of the studies discussed herein indicate that extracts from both dermal and subcutaneous adipose tissues may provide useful scaffolds for the generation of fat tissue in a range of reconstructive surgical procedures. Nonetheless, it may be instructive to determine whether the lack of benefit seen with human lipoaspirate was due to the source species or the extraction protocol. With a readily available tissue such as subcutaneous fat, there is a clear case for

autologous or allogeneic, rather than xenogeneic tissue as the ideal source for scaffold material as it carries less risk of disease transmission or adverse immune response.

Skin

In an attempt to generate biomaterials for wound healing and skin repair, hydrogels have been derived from rat dermis by a combination of dispase treatment, high salt homogenization, and urea extraction to produce tissue extracts to undergo thermo- or pH-responsive gelation [64, 85]. In the first of these studies, it was shown that both endothelial cells and fibroblasts could proliferate on these dermis-derived biomaterials and that endothelial cells formed tubule networks as an indicator of the potential for blood vessel formation [85]. However, it is noteworthy that fibroblasts placed into the gels as spheroids were unable to migrate into the substrate as they did when cultured in Matrigel™.

The second study examined the effects of glutaraldehyde cross-linking on the dermis-derived hydrogels [64]. Cross-linking was shown to significantly increase the elastic moduli and yield stresses of the hydrogels and to significantly slow enzymatic degradation in vitro and also after subcutaneous implantation in the rat. Cross-linked gels exhibited minimal cytotoxicity provided they were adequately washed prior to exposure to cells or tissue. Furthermore, glutaraldehyde cross-linking led to significantly increased blood vessel density in the tissue formed after implantation, suggesting that it has the potential to improve the performance of these materials in wound healing.

Dermis-derived hydrogels show promise for application in wound healing as they permit proliferation of relevant cell types and can support new blood vessel formation both in vitro and in vivo. Nevertheless, it remains to be established that fibroblasts can migrate into and deposit ECM proteins within these materials. This is a necessary step if the appropriate structural matrix is to be deposited around the neovasculature formed in these scaffolds.

Liver

Liver is another tissue in which the potential for ECM-derived hydrogels to support tissue formation has been established in principle [52, 78]. Initially, a porcine liver-derived gel was produced using a combined surfactant and enzyme-based approach to decellularize the tissue, followed by pepsin digestion to solubilize the protein mixture [78]. It was shown that primary human hepatocytes grown in a sandwich culture between two layers of liver-derived matrix expressed relevant genes and proteins as highly as those grown between layers of Matrigel™, the gold standard substrate. Subsequently, promising hydrogel materials were generated from rat liver

using Triton X-100 and ammonium hydroxide for decellularization and pepsin-based solubilization of the ECM proteins [52]. During in vitro culture experiments, primary rat hepatocytes showed greater cell adhesion and viability on liver-derived substrate coatings than on collagen I or Matrigel™. Viability and hepatocyte functions of albumin secretion and urea synthesis were all significantly enhanced when cells were grown within three-dimensional ECM gels relative to collagen controls. More promising from a potential clinical standpoint is that ASCs were also shown to more highly express hepatocyte genes such as albumin and hepatocyte growth factor when grown on the ECM-derived hydrogels. This material was also utilized in an early demonstration of in vivo treatment efficacy. Primary hepatocytes were delivered subcutaneously into mice in either collagen or liver-derived ECM pre-gel solutions which underwent gelation post-injection. The results of this investigation echoed those of the in vitro experiments, with hepatocyte marker expression and function being significantly enhanced in the tissue-specific material. This latter study in particular suggests great potential for liver-derived hydrogels being applied to liver regeneration, but orthotopic, functional in vivo studies are required before any definitive conclusions can be drawn.

Skeletal Muscle

The use of hydrogels for the repair and regeneration of damaged skeletal muscle has been investigated using two distinct materials in two distinct models [32, 91]. Firstly, a hydrogel material was developed from porcine skeletal muscle and evaluated in a rat hindlimb ischemia model [32]. The source tissue was decellularized by the action of SDS, with pepsin digestion used for ECM solubilization. The solution resulting from this process was found to significantly enhance proliferation of both rat smooth muscle cells and C2C12 myoblasts relative to pepsin-digested collagen as an in vitro culture supplement. The results of in vivo experiments in which the muscle-derived hydrogel was injected intramuscularly into ischemic tissue were also positive. Relative to collagen gels, those derived from skeletal muscle matrix resulted in significant increases in arteriole density, endothelial cell numbers, and proliferating muscle cell numbers at all time points over a 2-week period post-injection.

The second type of hydrogel considered for skeletal muscle repair was developed from porcine dermis by a combined enzyme, surfactant, and peracetic acid treatment, followed by pepsin digestion [91]. The performance of this material was compared to that of a gel derived from UBM both in vitro and in a partial-thickness rat abdominal wall defect. In vitro results clearly suggested that dermal ECM was a superior scaffold material for this application. The C2C12 myoblast cell line more efficiently fused to form myotubes on this substrate, and it was found to be significantly more resistant to fibroblast-mediated contraction. The same resistance to contraction was also found after in vivo implantation, and this is important in ensuring scar-free wound healing. However, UBM hydrogels contained

significantly more cells expressing muscle markers 5-week post-implantation. It is interesting to note that, as for studies examining adipogenesis in ECM-derived hydrogels, tissue specificity was observed in some, but not all, evaluations of muscle repair. Longer-term studies of functional tissue formation would be needed to definitively establish the ability of these materials to repair skeletal muscle. Nevertheless, the results presented thus far demonstrate potential for both dermis- and muscle-derived ECM hydrogels in promoting recovery after skeletal muscle injury.

Central Nervous System

A more recent development in the application of ECM-derived hydrogels to tissue repair is their use to regenerate tissue in the central nervous system (CNS). The first study to address this used UBM due to its ready availability and history of use [93]. As per previous studies, porcine UBM was produced by peracetic acid treatment and pepsin digestion was then used to solubilize the material for the formation of hydrogels. Injection of these hydrogels into healthy rat brain tissue was found to generate no adverse response. It did not trigger significant activation of microglia, astrocytosis, or neurodegeneration. Further analysis was conducted in a rat model of mechanically induced traumatic brain injury. In this setting, the gels were found to significantly reduce lesion volume relative to PBS injected controls 3-week post-intervention. More importantly, significant functional recovery of improved motor function was seen in the treatment group relative to controls. This improvement was mediated by the ability of UBM hydrogel to prevent myelin disruption after injury. Although cognitive recovery was not observed, these results suggest fundamental promise in the application of ECM-derived hydrogels in the repair of CNS tissue.

A subsequent pair of studies from Badylak and coworkers has sought to build on this potential by first developing hydrogels from the ECM of the CNS itself and subsequently comparing their performance to that of UBM [29, 54]. The hydrogels were produced from the ECM of porcine brain and spinal cord by a combined surfactant, enzyme, and peracetic acid treatment, followed by solubilization with pepsin [54]. Rheological measurements and SEM imaging showed significant structural and mechanical differences between the CNS-derived hydrogels and those produced from UBM. Nevertheless, when the materials were added as pre-gel solutions to the culture medium of murine neuroblastoma cells, all three increased the proportion of cells exhibiting neurite extensions in a dose-dependent manner. No significant differences between materials were observed based on this measure, suggesting that all three could promote a neuronal phenotype. However, only brain ECM gels induced an increase in mean neurite length, indicating that tissue repair by ECM hydrogels exhibits at least some degree of tissue specificity.

This concept was explored further in a subsequent study examining the effects of the three matrices on two types of adult human stem cells in vitro [29]. Once again,

the pre-gel solutions were tested as culture medium additives to assess the effects of ECM-derived soluble peptides. None of the three materials adversely affected the viability of either neural stem cells (NSCs) or perivascular stem cells at concentrations of up to 100 μg mL^{-1}. Furthermore, all three were able to exert positive chemotactic effects on both cell types relative to FBS controls, though both the presence and size of these effects were dose- and source tissue-dependent. UBM pre-gel solutions were also able to significantly increase the proliferation rate of both stem cell types at all concentrations, while the CNS-derived solutions exhibited more complex, dose-dependent effects. However, both CNS-derived solutions were able to direct neuronal differentiation of NSCs with equivalent efficiency to the positive control differentiation medium. The observed expression of βIII-Tubulin and neurite extension suggested that the CNS-derived materials promote a less proliferative, more differentiated, and functional phenotype and could drive tissue repair by endogenous stem cells. The conclusions of this investigation suggest that in the case of CNS repair, a match between the source and target tissues may be important for cell maturation and tissue formation.

Cartilage

The first ECM-derived material considered for cartilage repair was an extract from human lipoaspirate, produced by homogenization, followed by SDS and nuclease treatment [25]. A number of relevant growth factors were identified in this tissue extract, including transforming growth factor-β1 (TGF-β1), an important mediator of chondrogenic differentiation. Pellet cultures of this material in combination with ASCs showed that the cells retained high viability across a culture period of more than 6 weeks. The inclusion of the adipose-derived material in the pellets, with or without the addition of TGF-β1, led to significant increases in total protein, collagen, and sulfated GAG content of cultures indicating greater new matrix synthesis. Important cartilage markers such as SOX9, aggrecan, and collagen types II and X were expressed by cells in all ECM-containing pellets, though more strongly in those cultured in the presence of TGF-β1. This suggests further that the matrix being synthesized in these constructs had an appropriate, cartilage-like composition. Although in vivo studies are necessary for progression, the fact that this material could direct both stem cell differentiation and cartilage-like tissue formation in vitro is nonetheless promising. It may be instructive to consider whether the positive results obtained here are specific to the use of ASCs in conjunction with a hydrogel of the same tissue origin. For a number of practical or biological reasons, it may be desirable to design therapies using cells from other sources, such as chondrocytes or mesenchymal stem cells. In these cases, it would be necessary to assess whether the benefits observed here were retained.

A material derived from cartilage itself has also been considered for use in cartilage repair, though its evaluation is at an earlier stage than that for the adipose-derived hydrogel [51]. Porcine articular cartilage was decellularized by the use of

hypotonic buffer, SDS, and nuclease and subsequently solubilized by the action of pepsin. This study extended the "classical" ECM-derived hydrogel approach by including lyophilization of the pre-gel solution to form a powder which was subsequently soluble in PBS. Gel formation by subcutaneous injection of reconstituted material into rats was shown in principle, as was the capability of the ECM-derived material to bind bovine serum albumin as a model protein and subsequently release it in vivo over 2–3 weeks. This ability can presumably be attributed to the presence of GAGs such as heparin in the material, but the exact mechanism has yet to be conclusively deduced. A dose-dependent inflammatory response was observed for the cartilage-derived matrix when implanted subcutaneously in rats, but the proportions of activated immune cells within implant sites were low in all cases. This study demonstrated the fundamental safety of the approach. Future work will be required to show that endogenous and/or exogenous cells interacting with the cartilage-derived material can proliferate and deposit appropriate matrix.

Tendon and Ligament

As was the case for cartilage, the first study aiming to demonstrate ECM-derived hydrogel mediated tendon/ligament repair relied on the use of an already well-characterized ECM material, namely porcine SIS [36]. This study used a large animal (goat) model of anterior cruciate ligament (ACL) repair involving the suturing of a surgically transected ligament with no tissue removal. In an effort to augment repair, the sutured ligament was wrapped with a bilayered sheet of SIS and fibrin sponge, and an SIS-derived pre-gel solution, produced by pepsin digestion, was injected into the defect area. In ECM treated groups, the ACL cross-sectional area was restored to equivalence with the sham-operated controls 12-week post-intervention, which was several times the area of ligaments sutured without augmentation. Furthermore, significant recovery of mechanical strength was seen relative to suture-only tissue, though these ligaments still only withstood around half of the load supported by the sham-operated tissues. It is unclear what proportion of the benefit derived in this study is attributable to the gel, rather than the bilayered sheet scaffold. Additionally, it will be necessary to address more challenging models before it becomes clear what promise SIS-derived hydrogels may hold for the repair of damaged tendons and ligaments.

A subsequent study established that human tendon tissue could also be processed to form hydrogels [35]. Decellularization using SDS and EDTA was followed by pepsin digestion, resulting in a thermoresponsive gelling solution composed primarily of collagen type I. Pre-gel solutions were delivered subcutaneously into rats to demonstrate in vivo gel formation and the host cell response. Histological staining of explanted gels revealed that they were infiltrated by macrophages and other immune cells around the periphery. Fibroblasts were also seen throughout the hydrogels, elongated and aligned along the material fiber structure, indicating the ability of the scaffold to direct cell behavior. Additionally, it was

shown that ASCs could remain viable during in vitro culture either on the surface of preformed gels or after encapsulation within them. This demonstrates the potential of this material to be used in cell-seeded form in addition to the off-the-shelf, cell-free alternative.

A third and final study sought to demonstrate that this human tendon-derived hydrogel material could contribute to improved functional recovery after tendon injury [49]. Defects 5 mm in length were created in the Achilles tendons of rats, and the injection of tendon derived pre-gel solutions compared to contralateral controls of injected saline. Histological analysis suggested that tendon-derived gel was able to promote accelerated wound healing, with greater amounts of collagen seen in gel-treated tendons 2-week post-injection than those injected with saline. At 4 weeks, this difference was still apparent, with a good degree of alignment seen in the matrix deposited in gel-treated tendons; however, by 8-week post-injection, there was no discernable difference. Functional mechanical evaluation of the healing tendons revealed a similar trend, with gel treatment leading to significantly increased ultimate failure load at the 4-week time point, with no differences seen at 2 or 8 weeks.

As with many other tissues, ECM-derived materials show promise for application in tendon and ligament repair and regeneration. They appear to be well tolerated in vivo and may be able to accelerate defect healing. However, more extensive studies are required to determine whether long-term tissue repair is significantly enhanced by the presence of these hydrogels.

Intervertebral Disk

The nucleus pulposus (NP) of the intervertebral disk (IVD) exists in a naturally gel-like state and does not therefore require the extensive processing used to generate hydrogels from other tissues. This has been exploited by Simionescu and colleagues who have developed decellularized porcine NP as a scaffold for the reconstitution of the IVD [55, 56]. They showed that both the mechanical properties and relative concentrations of protein and GAG constituents of the decellularized NP were unchanged relative to the native tissue [55]. When ASCs were seeded on the surface of decellularized NP, they remained highly viable over the culture period of 7 days and were able to migrate into the scaffold. This latter result suggests that decellularized NP scaffolds could support the infiltration of appropriate host cell types for tissue repair after cell-free implantation.

In a second study by the same authors, decellularized NP was further studied to elucidate the effects of the material on ASC differentiation and tissue repair in vivo [56]. When ASCs were cultured on decellularized NP scaffolds, they expressed a number of markers of the NP cell phenotype at significantly higher levels than on TCPS. This study was conducted in basal growth medium in the absence of soluble cues, indicating some inherent capacity for the material to direct appropriate stem cell differentiation. The cells remained predominantly viable across 2 weeks,

contracted the hydrogel scaffold, and deposited a matrix containing collagen and GAGs, leading to significant increases in scaffold mechanical properties. Histological staining of scaffolds after subcutaneous implantation in rats indicated that 4-week post-implantation the material was infiltrated by immune cells and contained newly formed vasculature and newly deposited collagen. These results established in principle that the material was well tolerated and the next step would be to extend the in vivo analysis to include longer-term demonstrations of appropriate functional tissue formation.

Others

As described earlier, skeletal muscle-derived ECM hydrogels have been applied in the reconstruction and regeneration of damaged muscle tissue [32], but their potential for corneal repair has also been examined [37]. In a proof-of-principle study, high salt homogenization and urea extraction were used to process human muscle tissue and form coatings for plastic cell culture substrates. Cells from primary human limbal epithelial explants were used in this study which aimed to develop novel substrates for human limbal epithelial stem cell transplantation and corneal repair. The growth rate of cells on the skeletal muscle extract was equivalent to that on amniotic membrane, the gold standard substrate for this application. Moreover, this material significantly outperformed Matrigel^{TM} and a corneal stromal extract. Cells grown on the ECM-derived gel also expressed the limbal epithelial stem cell marker ABCG2, which did not occur on the amniotic membrane. These results must now be extended to thicker gels which can be surgically manipulated and function as biomaterials, rather than quasi-two-dimensional coatings. This muscle tissue extract may offer a new and improved substrate for treating limbal stem cell deficiency which also has the potential to alleviate the issues of availability and variability encountered in the use of amniotic membrane.

As a mineralized tissue, bone presents a unique challenge from the point of view of decellularization and subsequent solubilization. Harsh demineralization and lipid extraction steps are required to expose the underlying protein structure for further processing. The demineralized bone matrix (DBM) which results from this initial process is widely used clinically and generally considered to be an acellular biomaterial. However, in the only study to date to address the aim of generating a hydrogel from mineralized bone tissue it was found that an additional decellularization step was necessary to ensure efficient cell lysis and debris removal [74]. Biochemical characterization showed that both the ECM and DBM gels were composed predominantly of collagen. Turbidimetric and rheological analysis demonstrated further that the bone-derived hydrogels had similar gelation kinetics and mechanical properties to collagen gels. However, when the three materials were utilized as culture substrates for murine primary calvarial cells only the ECM hydrogel promoted cell proliferation, leading to significantly higher cell numbers than on the other substrates. This represents a preliminary study, but nevertheless

this bone ECM hydrogel has potential as a carrier and supplement for existing bone graft materials such as DBM and allogeneic bone.

The vocal fold (VF) is another tissue, where SIS hydrogel has been evaluated. In an in vivo proof-of-principle study, SIS gel showed promise, primarily as a carrier for mesenchymal stem cells rather than as a therapy in its own right [26]. This publication details the production of an SIS hydrogel by mechanical dissociation and saline washing of the small intestine, followed by pepsin digestion to solubilize the resulting material. As in an earlier study targeting cartilage repair (see Section "Cartilage"), hydrogels formed from this solubilized material were lyophilized and milled to form a PBS soluble powder. A rabbit model of VF scar formation and regeneration was used to evaluate injectable therapies composed of rabbit MSCs, SIS gel, or both components together. The combined therapy led to increased MSC retention and engraftment at the injection site, with fluorescently labeled cells visible 3-week post-injection by in vivo imaging. Significantly, more cells were observed histologically in defects treated with SIS at both 3- and 8-week time points, although by this method cells were visible in all MSC-treated groups. The combined therapy also promoted increased accumulation of sulfated GAGs and significantly decreased collagen deposition relative to all other groups, indicating the propensity for more effective regeneration and reduced scar formation. Finally, these compositional changes were reflected in the assessment of functional regeneration of the tissue, as combined treatment led to significantly increased vibratory capacity relative to other groups. Longer-term large animal studies are needed to further establish the potential of combined SIS-gel and MSCs as a therapy to prevent vocal fold scarring, but this study is nevertheless an effective proof of principle.

Hybrid Hydrogels

A recent evolution in the use of hydrogels derived from decellularized tissue has been the combination of these materials with other natural or synthetic hydrogels, to provide mechanical reinforcement and/or enhanced biological activity [41, 82]. Other approaches have utilized particulate ECM materials as additives to synthetic hydrogels [24, 61] or applied tissue derived hydrogels as coatings for solid biomaterials [89], but these will not be considered further in this chapter.

The first application of this next-generation concept occurred in liver repair and utilized extracts from liver cross-linked to the natural ECM components collagen type I and hyaluronic acid (HyA) [82]. Liver tissue was decellularized using Triton X-100 and ammonium hydroxide; non-decellularized liver was used as a comparator to deduce any adverse effects of the residual cellular and genetic component. Both materials were solubilized by the action of pepsin and combined with collagen or HyA and the cross-linker prior to gel formation. In sandwich culture of primary human hepatocytes, the addition of liver tissue extracts was found to result in significantly increased cell numbers. This effect was especially pronounced at

later time points in the 4-week experiment. Decellularized liver extract had a greater effect than that from non-decellularized tissue, which can be attributed to the higher concentrations of both structural ECM constituents and growth factors. Although it is promising that these materials can boost primary cell proliferation, the data in this study show no significant effect on hepatocyte functions such as albumin and urea secretion despite the increase in cell numbers. To fully establish their potential, it must be shown that they can aid functional recovery in damaged tissue.

The second example of this hybrid approach again utilized chemical cross-linking to conjugate ECM-derived peptides to supplementary polymers, based on poly(ethylene glycol) (PEG), and intended to offer enhanced mechanical properties and delayed degradation. These materials were used to reinforce a porcine myocardial matrix generated by SDS-based decellularization [41]. Incorporation of PEG-based polymers in the hydrogel network provided significant mechanical reinforcement alongside structural changes which were dependent on the incorporation method and the polymer molecular weight. Experiments with a murine fibroblast cell line showed that cells seeded on the surface of preformed hydrogels could migrate into the gels and proliferate on the surfaces regardless of the presence of the PEG component. This is despite the fact that PEG is an inert synthetic polymer that generally does not interact with cells as it contains no binding sites for cell surface receptors such as integrins and cadherins. It was also shown that cells encapsulated within gels could proliferate rapidly across all conditions, with no inhibitory effect due to the synthetic component. The fibroblast cell line used has broad relevance to a range of tissues and the cross-linking methods could be used with a range of tissue extracts, containing a significant collagen component and similar functional groups. This technique could therefore potentially be used to overcome current limitations and tailor the mechanical and degradation properties of naturally derived hydrogel biomaterials.

Future Directions

The lessons learned from the manufacturing and application of ECM-derived scaffolds must be applied to ECM hydrogels in order to permit clinical use of these materials. Since the ECM of each tissue or organ is produced by the unique combination of cell types resident within it, it is intuitive that a substrate composed of a tissue-specific ECM may be favorable for regeneration and repair. However, in some cases the need to use a tissue-specific matrix may be questioned as more readily available SIS and UBM may have similar effects. A recent comparison of a tissue-specific muscle ECM and SIS demonstrated constructive remodeling in a rodent muscle defect model; there was no apparent advantage to using the tissue-specific ECM despite differences in structure and composition [91]. The use of non-homologous ECM has many advantages: SIS and UBM are produced by established standardized protocols, tissue is more accessible, and xenogeneic tissue is available in large volumes. Furthermore, SIS ECM has been thoroughly

evaluated both in vitro and in vivo and is used clinically [11]. In addition, SIS and UBM hydrogels have been well characterized in terms of biocompatibility, biochemical composition, gelation kinetics, viscoelastic properties, and gel topology.

The choice to use a tissue-specific ECM hydrogel must therefore be driven by significantly increased efficacy or additional functionality in specific applications. The small-scale proof-of-principle studies undertaken to date do not sufficiently demonstrate tissue specificity in order to justify the additional time and cost involved in developing clinical grade products for therapeutic application. In all cases, longer-term preclinical studies are required to fully inform this decision-making process. It is important that researchers are able to determine the biochemical and structural factors driving cell responses and tissue formation in these novel ECM hydrogels. In particular, it is important to consider the effect of the tissue source (species and anatomical location where appropriate) and the macrophage polarization response to tissue-specific ECM hydrogels as part of the global inflammatory and immune response. These factors have been studied for ECM materials in powder and sheet form but not yet for hydrogels, which have altered three-dimensional architecture and subtle differences in biochemical composition.

The knowledge derived from these further characterization studies and preclinical trials may allow the development of optimized homologous and non-homologous ECM hydrogels for a wide range of applications. These novel materials may then provide a new hope for the treatment of defects with complex architecture.

References

1. Agrawal V, Johnson SA, Reing J, Zhang L, Tottey S, Wang G, Hirschi KK, Braunhut S, Gudas LJ, Badylak SF. Epimorphic regeneration approach to tissue replacement in adult mammals. Proceedings of the National Academy of Sciences of the USA. 2010;107(8):3351–5. https://doi.org/10.1073/pnas.0905851106.
2. Agrawal V, Tottey S, Johnson SA, Freund JM, Siu BF, Badylak SF. Recruitment of progenitor cells by an extracellular matrix cryptic peptide in a mouse model of digit amputation. Tissue Eng Part A. 2011;17(19–20):2435–43. https://doi.org/10.1089/ten.TEA.2011.0036.
3. Badylak SE. The extracellular matrix as a scaffold for tissue reconstruction. Semin Cell Dev Biol. 2002;13(5):377–83. https://doi.org/10.1016/S1084-9521(02)00094-0.
4. Badylak SF, Coffey AC, Lantz GC, Tacker WA, Geddes LA. Comparison of the resistance to infection of intestinal submucosa arterial autografts versus polytetrafluoroethylene arterial prostheses in a dog model. J Vasc Surg. 1994;19(3):465–72.
5. Badylak SF, Freytes DO, Gilbert TW. Extracellular matrix as a biological scaffold material: structure and function. Acta Biomater. 2009;5(1):1–13. https://doi.org/10.1016/j.actbio.2008.09.013.
6. Badylak SF, Gilbert TW. Immune response to biologic scaffold materials. Sem Immunol. 2008;20(2):109–16. https://doi.org/10.1016/j.smim.2007.11.003.

7. Badylak SF, Park K, Peppas N, McCabe G, Yoder M. Marrow-derived cells populate scaffolds composed of xenogeneic extracellular matrix. Exp Hematol. 2001;29(11):1310–8.
8. Badylak SF, Valentin JE, Ravindra AK, McCabe GP, Stewart-Akers AM. Macrophage phenotype as a determinant of biologic scaffold remodeling. Tissue Eng Part A. 2008;14 (11):1835–42. https://doi.org/10.1089/ten.tea.2007.0264.
9. Badylak SF, Vorp DA, Spievack AR, Simmons-Byrd A, Hanke J, Freytes DO, Thapa A, Gilbert TW, Nieponice A. Esophageal reconstruction with ECM and muscle tissue in a dog model. J Surg Res. 2005;128(1):87–97.
10. Badylak SF, Wu CC, Bible M, McPherson E. Host protection against deliberate bacterial contamination of an extracellular matrix bioscaffold versus Dacron mesh in a dog model of orthopedic soft tissue repair. J Biomed Mater Res. 2003;67(1):648–54.
11. Benders KE, van Weeren PR, Badylak SF, Saris DB, Dhert WJ, Malda J. Extracellular matrix scaffolds for cartilage and bone regeneration. Trends Biotechnol. 2013;31(3):169–76. https://doi.org/10.1016/j.tibtech.2012.12.004.
12. Berkowitz BA, Bevins CL, Zasloff MA. Magainins: a new family of membrane-active host defense peptides. Biochem Pharmacol. 1990;39(4):625–9.
13. Bissell MJ, Hall HG, Parry G. How does the extracellular matrix direct gene expression? J Theor Biol. 1982;99(1):31–68.
14. Booth AJ, Hadley R, Cornett AM, Dreffs AA, Matthes SA, Tsui JL, Weiss K, Horowitz JC, Fiore VF, Barker TH, Moore BB, Martinez FJ, Niklason LE, White ES. Acellular normal and fibrotic human lung matrices as a culture system for in vitro investigation. Am J Respir Crit Care Med. 2012;186(9):866–76. https://doi.org/10.1164/rccm.201204-0754OC.
15. Brennan BJ, Brown AB, Kolis SJ, Rutman O, Gooden C, Davies BE. Effect of R667, a novel emphysema agent, on the pharmacokinetics of midazolam in healthy men. J Clin Pharmacol. 2006;46(2):222–8. https://doi.org/10.1177/0091270005283836.
16. Brennan EP, Reing J, Chew D, Myers-Irvin JM, Young EJ, Badylak SF. Antibacterial activity within degradation products of biological scaffolds composed of extracellular matrix. Tissue Eng. 2006;12(10):2949–55. https://doi.org/10.1089/ten.2006.12.2949.
17. Brightman AO, Rajwa BP, Sturgis JE, McCallister ME, Robinson JP, Voytik-Harbin SL. Time-lapse confocal reflection microscopy of collagen fibrillogenesis and extracellular matrix assembly in vitro. Biopolymers. 2000;54(3):222–34. https://doi.org/10.1002/1097-0282(200009)54:3<222:aid-bip80>3.0.co;2-k.
18. Brown B, Lindberg K, Reing J, Stolz DB, Badylak SF. The basement membrane component of biologic scaffolds derived from extracellular matrix. Tissue Eng. 2006;12(3):519–26.
19. Brown BN, Londono R, Tottey S, Zhang L, Kukla KA, Wolf MT, Daly KA, Reing JE, Badylak SF. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. Acta Biomater. 2012;8(3):978–87. https://doi.org/10.1016/j.actbio.2011.11.031.
20. Brown BN, Valentin JE, Stewart-Akers AM, McCabe GP, Badylak SF. Macrophage phenotype and remodeling outcomes in response to biologic scaffolds with and without a cellular component. Biomaterials. 2009;30(8):1482–91. https://doi.org/10.1016/j.biomaterials.2008.11.040.
21. Bulet P, Stocklin R, Menin L. Anti-microbial peptides: from invertebrates to vertebrates. Immunol Rev. 2004;198:169–84.
22. Chen TM, Wang HJ. Cranioplasty using allogeneic perforated demineralized bone matrix with autogenous bone paste. Ann Plast Surg. 2002;49(3):272–7. https://doi.org/10.1097/01.SAP.0000015488.91165.B8.
23. Cheng M-H, Uriel S, Moya ML, Francis-Sedlak M, Wang R, Huang J-J, Chang S-Y, Brey EM. Dermis-derived hydrogels support adipogenesis in vivo. J Biomed Mater Res. 2010;92A(3):852–8. https://doi.org/10.1002/jbm.a.32410.
24. Cheung HK, Han TTY, Marecak DM, Watkins JF, Amsden BG, Flynn LE. Composite hydrogel scaffolds incorporating decellularized adipose tissue for soft tissue engineering with adipose-derived stem cells. Biomaterials. 2014;35(6):1914–23.

25. Choi JS, Kim BS, Kim JD, Choi YC, Lee HY, Cho YW. In vitro cartilage tissue engineering using adipose-derived extracellular matrix scaffolds seeded with adipose-derived stem cells. Tissue Eng Part A. 2012;18(1–2):80–92. https://doi.org/10.1089/ten.tea.2011.0103.
26. Choi JW, Park JK, Chang JW, Kim DY, Kim MS, Shin YS, Kim CH. Small intestine submucosa and mesenchymal stem cells composite gel for scarless vocal fold regeneration. Biomaterials. 2014;35(18):4911–8. https://doi.org/10.1016/j.biomaterials.2014.03.008.
27. Cortiella J, Niles J, Cantu A, Brettler A, Pham A, Vargas G, Winston S, Wang J, Walls S, Nichols JE. Influence of acellular natural lung matrix on murine embryonic stem cell differentiation and tissue formation. Tissue Eng. 2010;16(8):2565–80. https://doi.org/10.1089/ten.tea.2009.0730.
28. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials. 2011;32(12):3233–43. https://doi.org/10.1016/j.biomaterials.2011.01.057.
29. Crapo PM, Tottey S, Slivka PF, Badylak SF. Effects of biologic scaffolds on human stem cells and implications for CNS tissue engineering. Tissue Eng. 2014;20(1–2):313–23. https://doi.org/10.1089/ten.TEA.2013.0186.
30. Crapo PM, Wang Y. Small intestinal submucosa gel as a potential scaffolding material for cardiac tissue engineering. Acta Biomater. 2010;6(6):2091–6. https://doi.org/10.1016/j.actbio.2009.10.048.
31. D'Amore A, Stella JA, Wagner WR, Sacks MS. Characterization of the complete fiber network topology of planar fibrous tissues and scaffolds. Biomaterials. 2010;31(20):5345–54. https://doi.org/10.1016/j.biomaterials.2010.03.052.
32. DeQuach JA, Lin JE, Cam C, Hu D, Salvatore MA, Sheikh F, Christman KL. Injectable skeletal muscle matrix hydrogel promotes neovascularization and muscle cell infiltration in a hindlimb ischemia model. Eur Cells Mater. 2012;23:400–12.
33. Drake MP, Davison PF, Bump S, Schmitt FO. Action of proteolytic enzymes on tropocollagen and insoluble collagen. Biochemistry. 1966;5(1):301–12.
34. Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials. 2003;24(24):4337–51.
35. Farnebo SJ, Woon CY, Schmitt T, Joubert LM, Kim M, Pham H, Chang J. Design and characterization of an injectable tendon hydrogel: a scaffold for guided tissue regeneration in the musculoskeletal system. Tissue Eng. 2013. https://doi.org/10.1089/ten.TEA.2013.0207.
36. Fisher MB, Liang R, Jung HJ, Kim KE, Zamarra G, Almarza AJ, McMahon PJ, Woo SLY. Potential of healing a transected anterior cruciate ligament with genetically modified extracellular matrix bioscaffolds in a goat model. Knee Surg Sports Traumatol Arthrosc. 2012;20(7):1357–65. https://doi.org/10.1007/s00167-011-1800-x.
37. Francis D, Abberton K, Thompson E, Daniell M. Myogel supports the *ex vivo* amplification of corneal epithelial cells. Exp Eye Res. 2009;88(3):339–46. https://doi.org/10.1016/j.exer.2008.06.016.
38. Freytes DO, Martin J, Velankar SS, Lee AS, Badylak SF. Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. Biomaterials. 2008;29(11):1630–7. https://doi.org/10.1016/j.biomaterials.2007.12.014.
39. Ganz T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol. 2003;3(9):710–20. https://doi.org/10.1038/nri1180.
40. Gilbert TW, Stolz DB, Biancaniello F, Simmons-Byrd A, Badylak SF. Production and characterization of ECM powder: implications for tissue engineering applications. Biomaterials. 2005;26(12):1431–5.
41. Grover GN, Rao N, Christman KL. Myocardial matrix-polyethylene glycol hybrid hydrogels for tissue engineering. Nanotechnology. 2014;25(1). https://doi.org/10.1088/0957-4484/25/1/014011.
42. Hulmes DJS. Collagen diversity, synthesis, and assembly. In: Fratzl P, editors. Collagen: structure and mechanics. Berlin: Springer; 2008.

43. Jernigan TW, Croce MA, Cagiannos C, Shell DH, Handorf CR, Fabian TC. Small intestinal submucosa for vascular reconstruction in the presence of gastrointestinal contamination. Ann Surg. 2004;239(5):733–8.
44. Johnson TD, Dequach JA, Gaetani R, Ungerleider J, Elhag D, Nigam V, Behfar A, Christman KL. Human versus porcine tissue sourcing for an injectable myocardial matrix hydrogel. Biomater Sci. 2014;2(5):735–44. https://doi.org/10.1039/c3bm60283d.
45. Johnson TD, Lin SY, Christman KL. Tailoring material properties of a nanofibrous extracellular matrix derived hydrogel. Nanotechnology. 2011;22(49):494015. https://doi.org/10.1088/0957-4484/22/49/494015.
46. Kadler KE, Hill A, Canty-Laird EG. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. Curr Opin Cell Biol. 2008;20(5):495–501. https://doi.org/10.1016/j.ceb.2008.06.008.
47. Kadler KE, Holmes DF, Trotter JA, Chapman JA. Collagen fibril formation. Biochem J. 1996;316(Pt 1):1–11.
48. Keane TJ, Londono R, Turner NJ, Badylak SF. Consequences of ineffective decellularization of biologic scaffolds on the host response. Biomaterials. 2012;33(6):1771–81. https://doi.org/10.1016/j.biomaterials.2011.10.054.
49. Kim MY, Farnebo S, Woon CYL, Schmitt T, Pham H, Chang J. Augmentation of tendon healing with an injectable tendon hydrogel in a rat achilles tendon model. Plast Reconstr Surg. 2014;133(5):645e–53e. https://doi.org/10.1097/PRS.0000000000000106.
50. Kopecek J. Hydrogel biomaterials: a smart future? Biomaterials. 2007;28(34):5185–92. https://doi.org/10.1016/j.biomaterials.2007.07.044.
51. Kwon JS, Yoon SM, Shim SW, Park JH, Min KJ, Oh HJ, Kim JH, Kim YJ, Yoon JJ, Choi BH, Kim MS. Injectable extracellular matrix hydrogel developed using porcine articular cartilage. Int J Pharm. 2013;454(1):183–91. https://doi.org/10.1016/j.ijpharm.2013.06.023.
52. Lee JS, Shin J, Park HM, Kim YG, Kim BG, Oh JW, Cho SW. Liver extracellular matrix providing dual functions of two-dimensional substrate coating and three-dimensional injectable hydrogel platform for liver tissue engineering. Biomacromol. 2013;15(1):206–18. https://doi.org/10.1021/bm4015039.
53. Lee KY, Mooney DJ. Hydrogels for tissue engineering. Chem Rev. 2001;101(7):1869–79.
54. Medberry CJ, Crapo PM, Siu BF, Carruthers CA, Wolf MT, Nagarkar SP, Agrawal V, Jones KE, Kelly J, Johnson SA, Velankar SS, Watkins SC, Modo M, Badylak SF. Hydrogels derived from central nervous system extracellular matrix. Biomaterials. 2013;34(4):1033–40. https://doi.org/10.1016/j.biomaterials.2012.10.062.
55. Mercuri JJ, Gill SS, Simionescu DT. Novel tissue-derived biomimetic scaffold for regenerating the human nucleus pulposus. J Biomed Mater Res. 2011;96A(2):422–35. https://doi.org/10.1002/jbm.a.33001.
56. Mercuri JJ, Patnaik S, Dion G, Gill SS, Liao J, Simionescu DT. Regenerative potential of decellularized porcine nucleus pulposus hydrogel scaffolds: stem cell differentiation, matrix remodeling, and biocompatibility studies. Tissue Eng. 2013;19(7–8):952–66. https://doi.org/10.1089/ten.TEA.2012.0088.
57. Miller EJ. Structural studies on cartilage collagen employing limited cleavage and solubilization with pepsin. Biochemistry. 1972;11(26):4903–9.
58. Moore AJ, Beazley WD, Bibby MC, Devine DA. Antimicrobial activity of cecropins. J Antimicrob Chemother. 1996;37(6):1077–89.
59. Nelson CM, Bissell MJ. Of extracellular matrix, scaffolds, and signaling: tissue architecture regulates development, homeostasis, and cancer. Annu Rev Cell Dev Biol. 2006;22:287–309. https://doi.org/10.1146/annurev.cellbio.22.010305.104315.
60. Nerem R. The challenge of imitating nature. Principles of tissue engineering. 3rd ed. Burlington: Elsevier; 2007.
61. Ni P, Ding Q, Fan M, Liao J, Qian Z, Luo J, Li X, Luo F, Yang Z, Wei Y. Injectable thermosensitive PEG-PCL-PEG hydrogel/acellular bone matrix composite for bone regeneration in cranial defects. Biomaterials. 2014;35(1):236–48. https://doi.org/10.1016/j.biomaterials.2013.10.016.

62. Parkinson J, Kadler KE, Brass A. Simple physical model of collagen fibrillogenesis based on diffusion limited aggregation. J Mol Biol. 1995;247(4):823–31. https://doi.org/10.1006/jmbi.1994.0182.
63. Petersen TH, Calle EA, Zhao LP, Lee EJ, Gui LQ, Raredon MB, Gavrilov K, Yi T, Zhuang ZW, Breuer C, Herzog E, Niklason LE. Tissue-engineered lungs for in vivo implantation. Science. 2010;329(5991):538–41. https://doi.org/10.1126/science.1189345.
64. Pilipchuk SP, Vaicik MK, Larson JC, Gazyakan E, Cheng M-H, Brey EM. Influence of crosslinking on the stiffness and degradation of dermis-derived hydrogels. J Biomed Mater Res. 2013;101(10):2883–95. https://doi.org/10.1002/jbm.a.34602.
65. Poon CJ, Pereira E. Cotta MV, Sinha S, Palmer JA, Woods AA, Morrison WA, Abberton KM. Preparation of an adipogenic hydrogel from subcutaneous adipose tissue. Acta Biomaterialia. 2013;9(3):5609–20. https://doi.org/10.1016/j.actbio.2012.11.003.
66. Ravi S, Caves JM, Martinez AW, Xiao J, Wen J, Haller CA, Davis ME, Chaikof EL. Effect of bone marrow-derived extracellular matrix on cardiac function after ischemic injury. Biomaterials. 2012;33(31):7736–45. https://doi.org/10.1016/j.biomaterials.2012.07.010.
67. Reing JE, Brown BN, Daly KA, Freund JM, Gilbert TW, Hsiong SX, Huber A, Kullas KE, Tottey S, Wolf MT, Badylak SF. The effects of processing methods upon mechanical and biologic properties of porcine dermal extracellular matrix scaffolds. Biomaterials. 2010;31(33):8626–33. https://doi.org/10.1016/j.biomaterials.2010.07.083.
68. Reing JE, Zhang L, Myers-Irvin J, Cordero KE, Freytes DO, Heber-Katz E, Bedelbaeva K, McIntosh D, Dewilde A, Braunhut SJ, Badylak SF. Degradation products of extracellular matrix affect cell migration and proliferation. Tissue Eng. 2009;15(3):605–14. https://doi.org/10.1089/ten.tea.2007.0425.
69. Rosamond W, Flegal K, Friday G, Furie K, Go A, Greenlund K, Haase N, Ho M, Howard V, Kissela B, Kittner S, Lloyd-Jones D, McDermott M, Meigs J, Moy C, Nichol G, O'Donnell CJ, Roger V, Rumsfeld J, Sorlie P, Steinberger J, Thom T, Wasserthiel-Smoller S, Hong Y, American Heart Association Statistics C, Stroke Statistics S. Heart disease and stroke statistics—2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation. 2007;115(5):e69–e171. https://doi.org/10.1161/circulationaha.106.179918.
70. Rubin AL, Drake MP, Davison PF, Pfahl D, Speakman PT, Schmitt FO. Effects of pepsin treatment on the interaction properties of tropocollagen macromolecules. Biochemistry. 1965;4(2):181–90. https://doi.org/10.1021/bi00878a001.
71. Russell A, Bertram T. Moving into the clinic. Principles of tissue engineering. 3rd ed. Burlington: Elsevier; 2007.
72. Sampath TK, Reddi AH. Importance of geometry of the extracellular matrix in endochondral bone differentiation. J Cell Biol. 1984;98(6):2192–7.
73. Sarikaya A, Record R, Wu CC, Tullius B, Badylak S, Ladisch M. Antimicrobial activity associated with extracellular matrices. Tissue Eng. 2002;8(1):63–71. https://doi.org/10.1089/107632702753503063.
74. Sawkins MJ, Bowen W, Dhadda P, Markides H, Sidney LE, Taylor AJ, Rose FRAJ, Badylak SF, Shakesheff KM, White LJ. Hydrogels derived from demineralized and decellularized bone extracellular matrix. Acta Biomater. 2013;9(8):7865–73. https://doi.org/10.1016/j.actbio.2013.04.029.
75. Seif-Naraghi SB, Horn D, Schup-Magoffin PJ, Christman KL. Injectable extracellular matrix derived hydrogel provides a platform for enhanced retention and delivery of a heparin-binding growth factor. Acta Biomater. 2012;8(10):3695–703. https://doi.org/10.1016/j.actbio.2012.06.030.
76. Seif-Naraghi SB, Salvatore MA, Schup-Magoffin PJ, Hu DP, Christman KL. Design and characterization of an injectable pericardial matrix gel: a potentially autologous scaffold for cardiac tissue engineering. Tissue Eng. 2010;16(6):2017–27. https://doi.org/10.1089/ten.TEA.2009.0768.

77. Seif-Naraghi SB, Singelyn JM, Salvatore MA, Osborn KG, Wang JJ, Sampat U, Kwan OL, Strachan GM, Wong J, Schup-Magoffin PJ, Braden RL, Bartels K, DeQuach JA, Preul M, Kinsey AM, DeMaria AN, Dib N, Christman KL. Safety and efficacy of an injectable extracellular matrix hydrogel for treating myocardial infarction. Sci Transl Med. 2013;5(173).
78. Sellaro TL, Ranade A, Faulk DM, McCabe GP, Dorko K, Badylak SF, Strom SC. Maintenance of human hepatocyte function *in vitro* by liver-derived extracellular matrix gels. Tissue Eng. 2010;16:1075–82. https://doi.org/10.1089/ten.TEA.2008.0587.
79. Shell DH, Croce MA, Cagiannos C, Jernigan TW, Edwards N, Fabian TC. Comparison of small-intestinal submucosa and expanded polytetrafluoroethylene as a vascular conduit in the presence of gram-positive contamination. Ann Surg. 2005;241(6):995–1001.
80. Singelyn JM, DeQuach JA, Seif-Naraghi SB, Littlefield RB, Schup-Magoffin PJ, Christman KL. Naturally derived myocardial matrix as an injectable scaffold for cardiac tissue engineering. Biomaterials. 2009;30(29):5409–16. https://doi.org/10.1016/j.biomaterials.2009.06.045.
81. Singelyn JM, Sundaramurthy P, Johnson TD, Schup-Magoffin PJ, Hu DP, Faulk DM, Wang J, Mayle KM, Bartels K, Salvatore M, Kinsey AM, DeMaria AN, Dib N, Christman KL. Catheter-deliverable hydrogel derived from decellularized ventricular extracellular matrix increases endogenous cardiomyocytes and preserves cardiac function post-myocardial infarction. J Am Coll Cardiol. 2012;59(8):751–63. https://doi.org/10.1016/j.jacc.2011.10.888.
82. Skardal A, Smith L, Bharadwaj S, Atala A, Soker S, Zhang Y. Tissue specific synthetic ECM hydrogels for 3-D in vitro maintenance of hepatocyte function. Biomaterials. 2012;33(18):4565–75. https://doi.org/10.1016/j.biomaterials.2012.03.034.
83. Streuli C. Extracellular matrix remodelling and cellular differentiation. Curr Opin Cell Biol. 1999;11(5):634–40.
84. Uriel S, Huang J-J, Moya ML, Francis ME, Wang R, S-y Chang, Cheng M-H, Brey EM. The role of adipose protein derived hydrogels in adipogenesis. Biomaterials. 2008;29(27):3712–9. https://doi.org/10.1016/j.biomaterials.2008.05.028.
85. Uriel S, Labay E, Francis-Sedlak M, Moya ML, Weichselbaum RR, Ervin N, Cankova Z, Brey EM. Extraction and assembly of tissue-derived gels for cell culture and tissue engineering. Tissue Eng. 2009;15(3):309–21. https://doi.org/10.1089/ten.tec.2008.0309.
86. Uygun BE, Soto-Gutierrez A, Yagi H, Izamis M-L, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K. Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med. 2010;16(7):814–20. https://doi.org/10.1038/nm.2170.
87. Voytik-Harbin SL, Brightman AO. Small intestinal submucosa: a tissue derived extracellular matrix that promotes tissue-specific growth and differentiation of cells in vitro. Tissue Eng. 1998;4:157–74.
88. Voytik-Harbin SL, Brightman AO, Kraine MR, Waisner B, Badylak SF. Identification of extractable growth factors from small intestinal submucosa. J Cell Biochem. 1997;67(4):478–91.
89. Wolf MT, Carruthers CA, Dearth CL, Crapo PM, Huber A, Burnsed OA, Londono R, Johnson SA, Daly KA, Stahl EC, Freund JM, Medberry CJ, Carey LE, Nieponice A, Amoroso NJ, Badylak SF. Polypropylene surgical mesh coated with extracellular matrix mitigates the host foreign body response. J Biomed Mater Res. 2014;102(1):234–46. https://doi.org/10.1002/jbm.a.34671.
90. Wolf MT, Daly KA, Brennan-Pierce EP, Johnson SA, Carruthers CA, D'Amore A, Nagarkar SP, Velankar SS, Badylak SF. A hydrogel derived from decellularized dermal extracellular matrix. Biomaterials. 2012;33(29):7028–38. https://doi.org/10.1016/j.biomaterials.2012.06.051.

91. Wolf MT, Daly KA, Reing JE, Badylak SF. Biologic scaffold composed of skeletal muscle extracellular matrix. Biomaterials. 2012;33(10):2916–25. https://doi.org/10.1016/j.biomaterials.2011.12.055.
92. Young DA, Ibrahim DO, Hu D, Christman KL. Injectable hydrogel scaffold from decellularized human lipoaspirate. Acta Biomater. 2011;7(3):1040–9. https://doi.org/10.1016/j.actbio.2010.09.035.
93. Zhang L, Zhang F, Weng Z, Brown BN, Yan H, Ma XM, Vosler PS, Badylak SF, Dixon CE, Cui XT, Chen J. Effect of an inductive hydrogel composed of urinary bladder matrix upon functional recovery following traumatic brain injury. Tissue Eng. 2013;19(17–18):1909–18. https://doi.org/10.1089/ten.tea.2012.0622.

Chapter 3
Biologically Relevant Laminins in Regenerative Medicine

Anna Domogatskaya and Sergey Rodin

Abstract Need for biologically relevant coatings in regenerative medicine. Recent advances in stem cell-based regenerative medicine allow to expand human cells in almost unlimited amounts. However, expanded cells become devoid of natural niche cues and, therefore, may become prone to risks of function loss and malignant transformation. In order to maintain the expanded cells in a safe and functional way, one has to imitate in vitro the natural niche, comprising biologically relevant extracellular matrix molecules. For majority of cell types, including neurons, insulin-producing β-cells and vascular endothelial cells, biologically relevant laminins are essential part of natural niche. **Laminins: 16 tissue-specific isoforms that mediate cell maintenance and behavior**. Laminins (LM) are large, cross-shaped molecules that convey extracellular matrix cues via cell receptors to cell signaling systems and thus affect cell maintenance and behavior. Laminins can mediate behavior of associated cells, such as survival, adhesion, migration, proliferation, phenotype maintenance or differentiation. Each of known 16 laminin isoforms has unique biological function; mutations in laminin-encoding genes often result in severe pathologies or lethality. Despite molecular structure similarity and evolutionary homology, certain laminin isoforms may exert antagonistic effects on cell behavioral patterns. Importantly, biologically relevant laminins act in synergy with specific growth factors and cell–cell contact molecules, such as E-cadherin. Lack of either may result in malfunctioning cell culture systems. Lack of biologically relevant laminins may result in cell apoptosis, phenotype loss, or malignant transformation. Survival pathways for majority of mammalian cells depend on niche-specific extracellular matrix anchorage. Lack of such anchorage or irrelevant anchorage triggers apoptotic pathways and results in anoikis (apoptosis, caused by

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lack of relevant anchorage). In the absence of biologically relevant matrix cues, cells may undergo apoptosis or activate malignant pathways of anchorage-independent anti-apoptotic signaling. Majority of mammalian cell types depend on interaction with biologically relevant laminins. Biologically relevant laminins benefit quality cell culture in vitro. In vitro, cell culture systems based on use of niche-specific laminins are described for human and mouse embryonic stem cells, hematopoietic stem cells, neurons and Schwann cells, insulin-producing β-cells, and other cell types. We shall discuss success criteria and possible pitfalls for generating laminin-based safe, robust, and efficient technologies for culturing other cell types needed to treat various diseases.

Keywords Laminin · Embryonic stem cell · Stem cell · Extracellular matrix Regenerative medicine · Neuron · Pancreatic islets · Insulin-producing beta cell Stem cell technologies · Proliferation · Survival · Apoptosis · Differentiation Adhesion · Safety · Cell culture · Signaling · Integrin

Introduction

Since traditional approach of organ transplantation in medicine is limited due to shortage of donor material, cell therapies technologies based on possibility to expand donor or autologous cells in vitro are rapidly developing. Recent advances in stem cell technologies allow to expand the pool of human stem cells to almost unlimited amounts, which would ease the need for donor material. Ethical concerns regarding human embryonic stem cell derivation are finally resolved by technologies that allow to isolate a single blastomere without harming the embryo and expand it in vitro into a human embryonic stem cell line [67]. Cell banks can be generated to serve a source of immunohistocompatible stem cell lines. Use of induced pluripotent stem cells (iPS) that imitate properties of embryonic stem cells is another attractive alternative, since it allows autologous cells use [67, 68]. Third alternative is to expand the tissue-specific adult cell lines, stem cells or differentiated, and it is successfully implemented in medicine. In future perspective, cell therapies approach seems to be more practical alternative compared to traditional organ transplantation; however, besides obvious benefits, it also bears certain risks. Mammalian cells, when isolated from tissues, become devoid of their natural niche cues, become prone to loss of phenotype and function, and, worst of all, may undergo malignant transformation [7]. Many human embryonic stem (ES) cell lines develop chromosomal abnormalities after long-term culturing in vitro, some of which are cancer-related [3, 52].

In order to maintain long-term healthy functionality of the expanded cells, it is essential to imitate the natural environment, comprising (1) growth factors, (2) cell–cell contacts, and (3) extracellular matrix contacts, similar to the ones present in vivo. Laminins are the essential components of basement membranes, providing niche-specific cues to majority of mammalian cell types, such as

embryonic stem cells, hematopoietic stem cells, peripheral neurons, Schwann cells, insulin-producing pancreatic β-cells, vascular endothelial cells, keratinocytes, muscle cells [15]. Laminins can mediate behavior of associated cells in many different ways, such as: survival, adhesion, migration or stable anchorage, proliferation or quiescence, phenotype stability or dedifferentiation, undifferentiated status in stem cells, or differentiation. Moreover, laminins are essential for morphogenesis during embryonic/prenatal development and may enable selective restriction for cell permeability between compartments and cell-guiding function in regeneration and development. There is evidence that niche-specific laminins act in concert with specific growth factors and cell–cell contacts to affect cell behavior.

In mammals, at least 16 laminin isoforms are known up to date, and every isoform has a specific tissue and developmental stage-specific function [15, 16]. Laminins are large cross-shaped heterotrimeric proteins, comprising one α, one β, and one γ chain. Molecular weight of a laminin molecule varies from 400 to 1000 kDa. Laminins convey signals from surrounding extracellular matrix to the adherent cells via binding to laminin-specific cell receptors, e.g., integrins, and are essential part of natural mechanotransduction system [25].

Laminins first appear early in evolution, already in hydra, and are essential for ability of hydra to regenerate [70, 72, 97]. In primitive species, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, every laminin isoform is essential; lack of any laminin chain results in early lethality [28, 32, 46, 85, 92]. In zebrafish, mouse and human laminin chains mutations often result in either embryonic/postnatal lethality or diseases. Notably, knockout phenotypes are totally different for any of α chains, or β chains, or γ chains [15, 16, 51]. This indicates that biological role of every laminin isoforms is unique. It is shown that certain laminins isoforms, despite visible similarity in structure and amino acid sequence, exert distinct, and sometimes even opposite influences on certain cell types.

In early mammalian embryos, laminins are the first extracellular matrix molecules to appear: already at 2–4-cell stage, β- and γ-laminin chains are detected, and at 8-cell stage the whole laminin trimer appears in extracellular space [10, 17, 38]. At the stage of blastula, at least two laminin isoforms appear: α5 laminins are expressed between pluripotent cells of inner cell mass and, probably, supporting their pluripotency and proliferation, while α1 laminins, provided by trophectoderm, cause polarization of pluripotent cells and subsequent differentiation into ectoderm, endoderm, and mesoderm [34, 39–41]. At the later stages of embryonic development, tissue-specific and developmental stage-specific laminins drive morphogenesis; lack of specific laminins often cannot be compensated by similar laminin isoforms and results in diseases or sometimes lethality (reviewed in [15, 51]). For example, in kidney development all the laminin α chains are expressed in a niche-specific manner; in development of glomerular basement membrane (GBM) early LM-111 is replaced by intermediate LM-511 and finally by LM-521. Inability to transit from LM-511 to LM-521 results in renal failures (Pierson syndrome) [50, 76].

Use of niche-specific laminins for expanding functional cells in vitro is an emerging trend in regenerative medicine-related research. Long-term (up to 150 doublings) and comprehensive (including in-depth analysis of random mutations

and chromosomal abnormalities) studies were performed for mouse and human embryonic stem cells [14, 67, 68]; short-term studies demonstrated obvious benefits of biologically relevant laminins for in vitro cultures of adult stem cells and differentiated cells, such as neurons, insulin-producing β-cells, and even whole pancreatic insulin-producing islets [53, 64, 82, 86]. However, for many mammalian cell types that could be used in regenerative medicine, knowledge of biologically relevant laminins in vivo and in vitro is scarce. Therefore, we shall discuss not only existing laminin-based technologies, but rather the approaches, success criteria, and possible pitfalls in implementing concept of biologically relevant laminins use into generation of new cell culture systems. In conclusion, we shall discuss the existing challenges in laminin research, regarding regenerative medicine needs, and potential possibilities to exploit more sophisticated functions of laminins in technologies that are yet to be invented.

Cell Niche and Extracellular Matrix

Expanding Cells in Vitro: Future of Regenerative Medicine

Whole organ transplantation is a traditional approach in medicine. Techniques of organ transplantations are well-established and enable high survival rates. For example, one-year survival rate in liver transplantations is 85% and five-year survival rate is 70% [12]. However, there is a drastic shortage in donor material appropriate for use in medicine [12]. Abilities of whole human organs or tissues to regenerate are limited; therefore, the very approach of organ transplantation does not allow to resolve the needs of all patients.

Regenerative medicine is a rapidly developing field, aiming to be able to expand cells and/or tissues in sufficient amounts to serve every patient in need [58]. Technologies based on use of human embryonic stem cells (hESC) or induced pluripotent cells (iPS cells) give promise to provide unlimited amounts of human cells for medical use. Major ethical problems, concerning human embryonic cell derivation from human embryos, have been recently resolved. Now, human embryonic stem cell lines can be established from single-cell biopsies that do not jeopardize the human embryo survival [35, 67]. It also becomes possible to create GMP-compliant HLA-matched human ES cell banks to provide patients with immunocompatible cells. Possibility to expand cells and assemble them into functional organs in vitro seems a far more promising approach in a long run, compared to traditional organ transplantation. Technologies that allow expansion of mature cell cultures for transplantation are also used in medicine.

However, mammalian cells, both stem cells and mature differentiated cells, when deprived of the natural niche cues, have high risk to undergo transformation, such as loss of function, loss of phenotype, and even malignant transformations [7]. The risk increases with number of cell culture passages. Human vascular endothelial cells, when cultured in vitro, lose phenotype within less than 15 doublings [6].

Human embryonic stem cells, when cultured in vitro for long passages, may undergo not only random mutagenesis, but also non-random gains of chromosomes or large chromosome fragments, similar to what happens during malignant transformation [3, 52]. Though such cells may have typical phenotypic features and high proliferation rate, they can be dangerous in transplantation.

Prolonged cell culture in vitro, unlike organ transplantation, requires use of culture media, specific supplements, growth factors, and other molecular substances, often undefined chemical compositions. One should consider risks of introducing pathogens to the cultured cells. For example, bacterial collagenase preparations used for clinical derivation of insulin-producing pancreatic islets aimed for transplantation were subject to bovine spongiform encephalopathy contamination risk; therefore, measures were taken to exclude it [80]. Human embryonic stem cells, when cultured in the presence of animal-derived substances, have been shown to acquire immunogenicity [47]. Therefore, FDA and EU regulatory agencies suggest that all the compounds in contact with the cells aimed for medical use would better be chemically defined and xeno-free [26, 27, 84].

Human laminins are chemically defined, xeno-free, non-immunogenic, and biologically relevant molecules [67, 68]. Specific laminins, as essential part of natural niches for majority of mammalian cell types, such as insulin-producing β-cells, vascular endothelial cells, or embryonic stem cells, can contribute to generation of safe and robust in vitro culture systems that would imitate the natural cell niches and maintain healthy phenotypes.

Three Pillars of Cell Niche: Growth Factors, Cell–Cell Contacts, and Niche-Specific Extracellular Matrix

Cell behavior is guided by signals that cell receives from its environment. Influenced by environmental cues, a cell may survive or undergo apoptosis, migrate in scattered or directed fashion, form a stable anchorage, proliferate or remain quiescent, maintain its phenotype, differentiate, dedifferentiate, or lose phenotype. Understanding the nature of cues received by cell from its microenvironment is essential to design a solution to safe and robust regenerative medicine protocols. Mimicking natural niches by providing a set of appropriate molecular cues to mammalian cells in vitro could allow to expand them in healthy and functional way [67]. On the contrary, unnatural molecular cues may provoke loss of function and phenotype and even genetic mutations [7].

Usually, a cell receives three types of cues from its microenvironment: (1) growth factors, hormones, and other soluble molecules that activate cell receptors by binding them, (2) cell–cell contacts, being essential for cells like embryonic stem cells or insulin-producing pancreatic β-cells, and (3) niche-specific extracellular matrix. The latter modulates cell signaling not merely by binding to the cell receptors, but also by mechanotransduction [25].

Effect of extracellular matrix on cell behavior is often underestimated. Extracellular matrix is usually considered a neutral, passive substrate with mere function of keeping cultured cells well adherent. However, multiple studies demonstrated that different extracellular matrix coatings can exhibit strikingly distinct effects on cultured cells. We have demonstrated that use of different laminin isoforms on same culture of embryonic stem cells with the same medium, passaging, and other culture procedures can facilitate such different events as: (1) rapid detachment and following apoptosis, (2) rapid differentiation into quiescent cells, (3) continuous proliferation in undifferentiated state for over 150 doublings [14]. Not only the binding and dissociating constants of laminin/receptor interactions, but also mechanical properties of extracellular milieu incorporating the laminins such as the matrix stiffness [2, 13, 18] can affect cell behavior.

Laminins: Sixteen Niche-Specific Extracellular Matrix Molecules with Unique Biological Function

Idea of extracellular matrix being a neutral, passive substance—sort of biological adhesive for 2D and gel for 3D cell cultures—arises from early studies when niche-specific extracellular matrix molecules were not available for wide scientific community. Laminin-111 (i.e., often referred to as just "laminin") and Matrigel (a tumor-derived gel containing mostly laminin-111 and collagen IV) were widely available, but since they are not natural substrata for majority of mammalian cell types, they could not exert strong positive influence on majority of niche-sensitive mammalian cells. However, in early 90s it became clear that several laminin isoforms exist and, as further experiments showed, tissue-specific laminins facilitated cell effects such as proliferation, adhesion, migration, differentiation.

Evidently, laminins have tissue-specific and even niche-specific expression patterns and unique biological functions. Laminins, serving as a natural bridge between extracellular matrix and cell surface receptors, are the very molecules that provide the niche-specific cues to majority of mammalian cell types, including epithelial and endothelial cells. In combination with niche-specific growth factors and cell–cell adhesion molecules, niche-specific laminins can provide a close imitation of natural environment for many specific mammalian cell types in vitro.

Laminins: Molecular Aspects and Cell Signaling

In this chapter, we shall briefly review common knowledge about the laminin family: (1) molecular structure of laminins, (2) functional interactions with other molecules, such as cell receptors and other extracellular matrix molecules, (3) effect

of proteolytical processing on laminin function, (4) in vivo biological functions of laminins, and knockout phenotypes/mutations.

Then, we shall discuss influence of laminin–cell receptors interaction on cell signaling pathways and, consequently, cell behavior patterns: (1) principles of cell signaling, mediated by laminin interaction with cell receptors, (2) *co-signaling*, which is synergetic effect of biologically relevant laminin and growth factor interaction with appropriate receptors, (3) cell behavior that requires biologically relevant laminin signaling.

Laminins: Chains and Trimers

Laminins are large, heterotrimeric molecules that comprise one α, one β, and one γ chain. Size of laminin trimer varies from 400 to 1000 kDa. Laminin trimers have either cross-like, or Y-like, or rodlike shape (see Fig. 3.1). Short arms of laminins (N-terminal parts of α, β, and γ chains) can bind other laminins short arms and other extracellular matrix proteins. Long arm consists of (1) α, β, and γ chains intertwined together into a trimeric spiral via coil-coiled domains and (2) C-terminal fragment of α chain, comprising of five globular LG domains (LG1-LG5) that interact with cell receptors.

Five α (α1-α5), four β (β1-β4), and three γ (γ1-γ3) chains are known in mammals; α5, β1, β2, and γ1 are expressed ubiquitously, and other chains are more tissue-restricted [4]. Up to date, 16 trimeric laminin isoforms present in vivo have been characterized in mammals; however, theoretically 60 combinations are possible. Modern nomenclature describes laminin isoforms according to their chain composition [4]. For example, ubiquitous laminin-511 (LM-511) consists of α5, β1, and γ1 chains.

It is shown by Macdonald et al. [43] that not all the combinations of α, β, and γ chains can assemble into trimeric forms. Sixteen laminin trimers have been identified in vivo and two more have been predicted by Macdonald et al. [43].

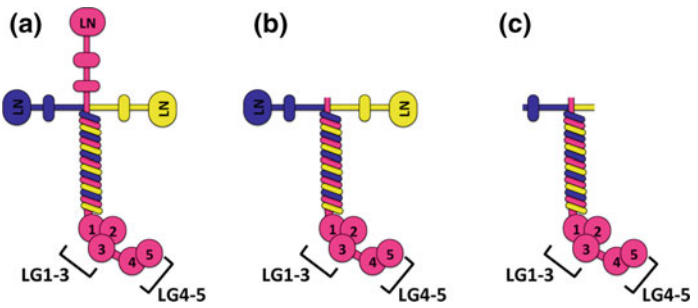

Fig. 3.1 Schematic representation of typical laminin trimer forms. **a** Cross-shaped. **b** Y-shaped. **c** Rod-shaped

Associations of γ1 with β1 or β2 form trimers with all the five α chains. The α1, α2, α3B, α5 trimers are cross-shaped, while the α3A, α4 trimers are Y-shaped or rod-shaped.

Extensive body of data has been generated on single laminin chain expression and distribution in various mammalian tissues, and yet very little is known about specific trimers localizations in vivo. However, information on single laminin chains expression patterns and knockout animal models can give us some insights into in vivo function of certain laminin trimers.

α1 laminins. Vast majority of laminin studies in twentieth century involved either LM-111 (called "laminin") or Matrigel, a tumor-derived gel comprising LM-111. Lack of α1 results in early embryonic lethality, presumably, due to inner cell mass polarization failure [49, 71]. However, expression pattern of LM-111 in mature mammalian organisms is restricted (compared to ubiquitous LM-511/521) and biological function clearly known for embryonic events, such as blastocyst inner cell mass polarization [39, 41, 49] and early kidney glomerular basement membrane development [50, 76].

α2 laminins play important roles in differentiation and maintenance of muscular and neural cells; mutations in LAMA2 (gene encoding α2 laminin) result in muscular dystrophy and neural system disorders [8, 31, 78, 90]. α2 laminins are the major laminins in muscle [61, 69] and play an important role in smooth muscle myogenesis [66]. α2 laminins are an important part of neural system and are essential for thymocytes maturation [29, 44]. α2 laminins are expressed in testis [37] and deficiency of those results in infertility [23].

α3 laminins are specific for epithelial basement membranes, for example underlying skin epidermis and intestinal epithelia. Two splice variants of LAMA3 gene exist: α3A, the short chain, and α3B, the long chain.

α4 laminins, Y-shaped, are supposed to be "not very important" since no lethal or early severe phenotype is observed. However, mutations in LAMA4 cause microcirculation development disorders [81], cardiomyopathy [36, 87], chronic kidney disorders [1], neurodisorders [62, 86], and impairment of leukocyte recruitment [33]. It appears that α4 laminins attenuate fine guidance of cells, such as tip cells Dll4/Notch signaling in angiogenesis [77], or high-precision spatial matching of active zones of motor axon terminus with junctional folds on muscle end plate within developing neuromuscular junctions [62].

α5 laminins, cross-shaped molecules capable of self-polymerization, are ubiquitously expressed. They first appear in earliest embryonic development stages; for example, they are expressed by blastocyst inner cell mass to provide themselves vital cues; α5 laminins are essential part of vascular basement membranes, skin, hair follicle niches, and many others. Detailed review of knockout phenotypes and list of references are available in [15, 16]. Lack of α5 laminins causes late-stage embryonic lethality in mice [48] and cannot be compensated by other isoforms.

β1 laminins are ubiquitously expressed. They are essential for organism maintenance from the earliest stages of embryonic development to complete maturation of mammalian organism. β1 laminins are present in almost every organ

comprising basement membranes. Lack of β1 results in early embryonic lethality, already on the stage of blastocyst [49].

β2 laminins are also expressed in many organs and yet are more restricted in expression patterns. Though β2 laminin chain is closely evolutionary related to β1, some biological roles of β2-laminins are quite opposite of analogous β1 isoforms. β1 and β2 isoforms may coexist within same tissue, but share different functional niches. For example, surface of motor axon and of muscle fiber is coated with β1-laminins except for the neuromuscular junctions, which specifically contains β2, but not β1-laminins. As β1-laminins support Schwann cell spreading upon axon surface, β2-laminins restrict Schwann cell migration into the neuromuscular junction so that neurotransmitter, released by motor axon end plate, could reach receptors on the muscle end plate [59, 60]. Mature β2 isoform LM-521 replaces embryonic β1 isoform LM-511 within kidney glomerular basement in order to provide durable, stress-resistant GBM. If it fails, LM-511 is not able to resist stress and renal failure occurs [56]. β2-laminins mediate Ca ++ signaling within neuromuscular junction by binding Ca++ channels, while β1-laminins do not [54].

β3 laminins are mostly epithelial-specific. Laminin β3 chain can only associate with γ2 [43] and both of them can only associate with α3 to form LM-332. This is the only known up-to-date rod-shaped laminin isoform. LM-332 is specific for epithelial basement membranes and driving epithelial cells stable anchoring or migration. LM-332 plays an important role in wound healing. Mutations in any of LM-332 chains (LAMA3, LAMB3, or LAMC3) result in severe, often lethal phenotype of junctional epidermolysis bullosa, associated with skin blistering.

β4 laminin chain, encoded by LAMB4 gene, exists in human, but not in mouse. Number of laminin β chains differs in vertebrates. For instance, zebrafish has six β chains. Function in β4 in human is unknown, and mouse knockout model cannot be established, since no LAMB4 exists in mouse [15]. It has not described any trimers comprising β4 chain yet.

γ1 laminins are ubiquitously expressed. They are essential for organism maintenance from the earliest stages of embryonic development to complete maturation of mammalian organism and are major laminins in almost every basement membrane, except for γ2-rich epithelial basement membranes. Lack of γ1 results in early embryonic lethality, already at the stage of blastocyst [75]. Laminin γ1 in cooperation with either β1 or β2 can form trimers with any of α chains, thus giving rise to LM-111, 121, 211, 221, 311, 321, 411, 421, 511, and 521.

γ2 laminin chain associates with α3 and β3 to form epithelial-specific LM-332.

γ3 laminins have restricted expression pattern in tissues. LM-213 is present in testis basement membrane [37]; LM-423 and LM-523 are present in central nervous system and eye retinal structures [42]. γ3 laminins may exist in mammalian tissues in non-basement membrane forms. LAMC3 (gene encoding γ3) was first not considered to have important biological role, since knockout model in mouse lacked obvious defects in phenotype [11] except for retinal defects [63]. However, it has been recently shown that mutations in human LAMC3 affect brain structure and function [5].

Macdonald et al. predicted that two additional isoforms LM-312 and LM-422 may exist since coil-coiled domains of the named chains can associate as trimers [43].

Molecular Interactions of Laminins

Unique function of laminins is ability to bind both extracellular matrix molecules and cell receptors and thus transmit signals from extracellular matrix to the cells. The knowledge concerning these molecular interactions is vast, and we address our readers to specific articles devoted to molecular interactions of laminins for more details, like [83], [57, 79, 93]. Hereby, we give very short and generalized overview of laminins interactions.

Interactions with Cell Receptors and Co-Signaling

Laminins interact with cell receptors mostly via C-terminal LG domains of α chains. Integrin receptors such as α3β1, α6β1, α6β4, and α7β1 are well-known receptors to bind laminins. Integrin receptors are known to bind LG1-3 domains, while dystroglycan and syndecans are known to bind LG45 domains. Shift from LG45 binding via integrin α3β1 to LG1-3 via integrin α6β4 affects epithelial cell behavior changing from migration to stable anchoring via hemidesmosomes formation [45]. Different laminins have been shown to interact with same integrin receptors, however, with different affinities (for complete review, see (Yurchenco [93]).

Laminin signaling is more complex as mere activation of certain integrin receptors by binding them. For instance, laminins that bind same integrin receptor may exhibit different effects on the cells, while two different laminins acting via two different integrin receptors may induce same cell phenotypes [64]. Below we shall discuss effects of laminin co-signaling, receptor clustering, and other complex laminin–cell interaction patterns.

Co-signaling in mammary epithelial cells, Xu et al. have demonstrated [91] that only synergy between (1) LM-111 binding the integrin receptors and (2) prolactin binding prolactin receptor allows to activate a STAT5-driven signaling pathway that leads to β-casein secretion by mammary epithelial cells (essential for lactation). In vivo, mammary endothelial cells respond to prolactin stimulation during nursing period by expression of β-casein, the milk protein. However, when the cells are cultured upon LM-111 in vitro and are stimulated by prolactin, no β-casein expression occurs. Thus, the cells fail to respond to naturally stimulating growth factor. However, when LM-111 polarity is inverted, that is, LM-111 and prolactin stimulation occur from the same basal side of the cell, the STAT5 pathway is activated and β-casein is produced. It has been hypothesized that LM-111

binding the mammary epithelial receptors on the basal side of the cell attracts the prolactin receptor to the same side, as it occurs in vivo. If prolactin is provided from the same basal side, it meets the prolactin receptor and signaling pathway occurs [91].

Co-signaling in vascular endothelial cells. Co-signaling from LM-411 and VEGF to vascular endothelial tip cell allows to switch on the Notch/Dll pathway, thus restricting the occurrence of vessel branching [77]. In the absence of LM-411, the mechanism fails, and pathological vessel branching occurs and results in pathologies.

Clustering calcium channels in motor nerve terminals. Laminin β2 binds and clusters together voltage-gated calcium channels within synaptic cleft, which is essential for neurotransmitter release from motor nerve terminals [54]. Neuromuscular junctions in mice lacking laminin β2 suffer abnormalities: synaptic cleft being blocked by invading Schwann cell processes and reduced number of active zones in nerve terminals [55, 60]. Apparently, β1-laminins present in the synaptic cleft cannot compensate for the missing β2 isoforms.

Extracellular Matrix Interactions

Self-assembly and interaction with other laminin isoforms. Laminins are capable of self-assembly via N-terminal short arms. N-terminal parts of non-truncated α, β, and γ chains can interact with each other. Cross-shaped laminins, like LM-111, can form 3D gels, for which they require calcium ions [94]. It is important to note that Y-shaped laminins, like LM-311/321 or 411/421, as such cannot form 3D gels without help of cross-shaped laminins or basement membranes molecules. Possible combinations of interaction between N-terminal short arms of α1, α2, α5, β1, β2, β3, γ1, and γ3 laminin chains and respective K_d values are described in [57].

Other extracellular matrix molecules. Laminins constitute basement membranes together with collagen IV. Collagen IV and laminins can self-assemble; however, those two intertwining networks are not connected directly, but via smaller connector molecules, such as nidogens [95, 96].

Mechanotransduction

Biological function of laminins relies on ability to interact with cell receptors and extracellular matrix scaffold simultaneously. It is shown that cell function, such as differentiation or activation, depends on surrounding extracellular scaffold mechanical properties (stiffness or elasticity modulus) [13, 18–20]. Laminins are capable of outside-in signaling.

Important implications for biotechnology are: (1) laminin peptides or single domains, such as whole or partial LG domains, often lack an ability to couple

extracellular scaffold to cell receptors, and (2) it is important to consider the mechanoelasticity message the laminin transmits to the cultured cells. Plastic culture dish surface is abnormally stiff, compared to majority of mammalian tissues except for bone, while 3D gels like Matrigel are more relevant in respect of mechanical properties.

Proteolytically Degraded Forms of Laminins

Proteolytically degraded forms of laminin isoforms used in research are often named same as their full-size natural trimmers. However, the degraded forms may lack functionally important domains responsible either for interaction with cell receptors or with extracellular matrix molecules. Wondumi et al. [88] have demonstrated structural difference between complete trimeric laminin molecules comprising all the functional domains that are produced in cultured mammalian cells and laminins that are produced by non-specific proteolytical degradation of tissues, for example human placenta. The proteolytically degraded laminins that are lacking some functional domains cannot be considered as chemically defined, because the degradation is batch-to-batch difference and cannot be controlled. It is also important to note that laminin single chains, domains, peptides, and peptide-based hydrogels may affect the cultured cells in totally different way in comparison with biologically relevant and intact trimeric molecules. For practical applications in cell biology, it is always important to know if all the functional domains are present in the laminin molecule used.

Possible Antagonistic Functions of Related Laminins

Certain laminin isoforms are highly homological and are expressed in same tissues. One may assume that they would exert similar influence on the cells, which are in contact with them. Indeed, it sometimes such as laminins impose similar effects on the cells; for example, LM-511 and LM-521 support embryonic stem cells self-renewal in vitro [67, 68]. However, sometimes closely related laminin isoforms exert antagonistic impact on cells.

Example 1: β1- versus β2-laminins within neuromuscular junction. Laminin β1 isoforms, such as LM-211, 411, and 511, are enveloping surface of muscular fibers and motor nerves that innervate them, in exception for neuromuscular junction: the very spot where signal is transmitted from nerve terminal to the muscle. The neuromuscular junction contains β2-analogues of the same laminins: LM-221, 421, and 521. In LAMB2-/knockout mice, the β1 isoforms LM-211, 411, and 511 appear within synaptic cleft, in order to compensate for the absence of β2-laminins. The Schwann cells are repelled by β2-laminin LM-521 and thus prevented from spreading processes into synaptic cleft in healthy mice.

On contrary, the Schwann cells are attracted by β1-laminins to invade and block the synaptic cleft in LAMB2-/knockout mice that results in severe neural system disorders [55, 60].

Example 2: Processed versus unprocessed 3 chain in LM-332 in cell adhesion. LM-332 affects epithelial cells behavior differently depending on whether α3 chain is processed or not. Unprocessed α3 chain binds integrin α3β1 and facilitates cell migration, wherein cells form temporary contacts with the LM-332 surface. However, processed α3 LM-332 binds integrin α6β4 and forms hemidesmosomes that enable stable anchorage of the cells.

Biologically Relevant Laminins for In Vitro Cell and Organoid Cultures

In this part, we shall present several examples where biologically relevant laminins are used for establishing functional cell cultures in vitro.

Human and Mouse Embryonic Stem Cells

Laminins are the first extracellular matrix molecules emerging in embryonic development. Laminin chains are first detected at two-cell stage and full-size trimer at eight-cell stage of embryonic development. Blastocyst consists of trophectoderm that will give rise to extraembryonic tissues and inner cell mass (ICM) that will give origin to all three embryonic germ layers: ectoderm, mesoderm, and endoderm. Embryonic stem cells, derived from inner cell mass of blastocyst, have unlimited proliferative capacity and potential to differentiate into any mammalian cell type, from neurons to insulin-producing β-cells.

Some stem cells, due to their high proliferation potential, are especially prone to malignant transformations [3, 52], which are a major concern in regenerative medicine. Therefore, it is most essential (1) to imitate a healthy, natural niche of inner cell mass: natural origin of embryonic stem cells and (2) ensure that chromosomal abnormalities and mutations do not occur after long-term passaging.

Two major laminin types are detected in blastocysts. α5-laminins are present between cells of the inner cell mass (ICM), so that every ICM cell in vivo is in contact with α5-laminins, while α1-laminins form outer basement membrane [34]. α1-laminins are essential for the very first event of ICM cells differentiation: polarization following differentiation toward ectoderm, mesoderm, and endoderm [39, 41, 71]. However, would α5-laminins support the pluripotency state of embryonic stem cells in vitro?

Mouse embryonic stem cells that were cultured on α1-, α3-, α4- and α5-laminins, respectively, in the absence of differentiation inhibitors underwent four different scenarios [14]. Cells, cultured on LM-111, differentiated within 2 weeks, and proliferation abruptly reduced (which is consistent with natural role of LM-111 [40]). Cells cultured on LM-411 suffered lack of adhesion contacts, detached and died. Cells cultured on LM-332 and LM-511 proliferated for over 150 doublings with approximately same rate, while continuously expressing the pluripotency markers: Oct-4, Sox2, and Nanog. However, the functional test—ability to form three germ layers and give rise to whole functional organism—revealed the drastic difference between LM-511 and LM-332. As latter gave rise to very weakly chimeric and/or sick chimeric animals which failed to undergo germ line transfer, the former (LM-511) gave rise to healthy animals with strong chimerism [14] that in turn gave rise to germ line transfer, healthy animals (Domogatskaya, Rodin, Tryggvason: unpublished manuscript).

Due to unlimited proliferative capacity and ability to differentiate into practically all adult cell types, human embryonic stem (ES) cells may be a valuable source of cells for regenerative medicine. Nevertheless, only a few clinical trials have been approved for human ES therapies. One of the major reasons of that was lack of xeno-free (animal substance-free) and chemically defined environments that support self-renewal of human pluripotent stem (PS) cells (human ES and induced pluripotent stem cells). Using α5-laminins, we have been able to develop such human cell culture systems. Indeed, LM-511 and especially LM-521 allow robust proliferation of human PS cells for more than 6 months in culture. The number of cultured cells has been multiplied more than 10^{20} times, which is enough to develop enough cells to treat hundreds of people. During the culturing, the cells express stable levels of markers of pluripotency Oct-4, Nanog, Sox2, SSEA-4, etc. After six months in culture, human PS cells can be differentiated into all three germ lineages of the human embryo both in in vivo and in vitro assays. In-depth genotyping assay has revealed that the cells cultured on LM-521 acquire little number of genetic abnormalities and can be used in regenerative medicine. Importantly, LM-521 allows passaging of human PS cells in single-cell suspensions, which facilitates automation of the culturing.

Individualized human PS cells die from anoikis that is a form of apoptosis caused by unnatural milieu. That feature of human PS cells complicates manipulations with their genomes, which can be used in regenerative medicine, e.g., for correction of hereditary monogenic diseases. Since relevant integrin-mediated extracellular matrix signaling and cadherin-mediated cell–cell signaling prevent anoikis, we sought to identify molecular cues that are sufficient to mimic natural environment of human PS cells. E-cadherin, which is abundantly expressed on human ES cell membranes, and LM-521 taken at 1:9 w/w ratio and used as cell culture substratum have been able to prevent anoikis and permit survival of individualized human PS cells. LM-521/E-cadherin substratum also allows derivation of new hES cell lines from single blastomeres acquired through a single-cell biopsy

from an eight-cell in vitro fertilization (IVF) embryo. This enables development of cells without a need to destroy the parental embryo and confirms that LM-521 and E-cadherin can mimic human ES cell niche.

Bone Marrow-Derived Hematopoietic Stem Cells

Many early in vitro studies with hematopoietic stem cells have been performed on Engelbreth-Holm-Swarm sarcoma-derived LM-111; however, it is not a natural laminin for this type of cells. LM-511 and LM-411 are expressed in human and bone marrow, and LM-211 expression is weak and restricted to arterioles and LM-111 not expressed at all. β1-laminins are present in adult bone marrow, but not β2-laminins [21, 73].

In vitro, human CD34+ cells adhere strongly to LM-511/-521, but not to LM-211 or LM-111. LM-511 exerts mitogenic activity in human hematopoietic progenitor cells. LM-511/-521, unlike LM-111, are strongly adhesive for multipotent hematopoietic FDCP cells. LM-521/-511, unlike other isoforms, enable robust adhesion for variety of hematopoietic lineages [22, 65, 73].

Insulin-Producing Pancreatic Islets

Insulin-producing pancreatic islets (islets of Langerhans) are small endocrine organoids formed by several endocrine cell populations, including insulin-producing β-cells. In order to regulate glucose blood levels, islet β-cells have to function properly, and their function depends on receiving proper cues from their niche. β-cells in vivo are in direct contact with underlying vascular basement membrane, containing specific laminin isoforms (reviewed in [15]). As we have previously demonstrated, the basement membrane contains α4- and α5-laminins, but not α1-, α2-, or α3-laminins [53]. In vitro, experiments have shown that β-cells depend on contact with those natural laminins in order to produce insulin. Notably, β-cells are not capable of producing any laminins themselves; they depend on vascular endothelial cells producing the laminins [15, 53].

We have demonstrated that α5-laminins have unique effect on islet culture in vitro. Isolated islets, which are used for transplantation purposes and diabetes research, have extremely high affinity toward natural α5-laminins, but not α1-laminin (LM-111), which is not part of natural islet niche. The adhesion force between the islet cells and natural α5-laminins is so strong that islets attain flat shape and yet remain cohesive. Since contact with α5-laminins is part of natural β-cell niche, it allows islet survival, islet cell proliferation, and insulin secretion by β-cells after 3–4 weeks in culture (Tryggvason et al., US Patent U.S. Patent 9,499,794, [82].

Neurobiology

Major laminin isoforms in the peripheral nervous system are: α2, α4, and α5. β1-laminins are ubiquitous, while β2 are restricted to specific locations, like neuromuscular junctions. Deficiency in either of those chains results in severe neural pathologies (reviewed in [15]).

Different cells of peripheral nervous system have specific preferences regarding laminin substrate in vitro. Spinal motoneurons prefer to sprout long axons on LM-211 compared to LM-411, while Schwann cells, in the opposite, form longer sproutings on LM-411 compared to LM-211 [86]. Adult dorsal root ganglion neurons formed longed neuritis on LM-511 and LM-111, but not on LM-411 or LM-211. Notably, though neurons spread axons on LM-511 as well as on LM-111, they engage different integrin receptors [64]. It is possible that different signaling pathways are activated and cells would differ in receptiveness from growth factor cues. We suggest therefore that one has not only considered mere adhesiveness and morphological phenotype as sole evaluation criteria of cell culture system, but also whether the extracellular matrix coating is biologically relevant and whether it can provide relevant niche cues to the cell.

Evaluation Criteria in Developing Cell Culture System

As we have discussed above, synergetic action of specific laminin (or several laminins) with specific growth factor(s) is required to provide the cultured cells imitation of in vivo-like environment. Concentration range for every compound in the system should be estimated experimentally. High-throughput-automated screening with automated quantitative evaluation is often used to find the best possible combination.

When automated screening of numerous samples is performed, the key importance is a set of selection criteria. Biased criteria may result in false positive errors (that would generate cells that would not be able to function correctly after transplantation or even undergo malignant transformation) and false negative errors (good cell culture systems, most closely imitating the healthy natural environment being discarded as “not proliferating with sufficiently high rate”).

Hereby, we discuss several possible pitfalls that should be avoided.

1. Adhesion as sole success criteria for extracellular matrix coating. Extracellular matrix is often perceived as a neutral substance that does not affect cell phenotype, only function of it being able to keep cells attached. However, different extracellular matrix molecules engage different signaling pathways and can drive cell behavior in very distinct ways.
2. High proliferation rate as a success criterion. In the absence of biologically relevant extracellular matrix majority mammalian cells would react by anoikis (apoptosis) or phenotype loss. In the latter case, the cells often attain

fibroblast-like appearance and behavior and, sometimes, high proliferation rate. However, function is lost and after transplantation such cells would be useless. Worst case scenario, however, would be if lack of relevant extracellular matrix would trigger malignant transformation processes in the cultured cells.

3. Expression of specific markers, determined by immunohistochemistry or western blot being a sole criterion of cell functionality. Only the functional assays, specific for each cell type, would be a sufficient proof of the cell identity. Functional assays should be designed for each cell or organotypic culture with regard to their biological role. For example, embryonic stem cells function is to give rise to all the three germ layers. For insulin-producing pancreatic β-cells, it would be to release insulin in glucose-dependent manner.
4. Screening for different growth factors in the absence of biologically relevant extracellular matrix, or screening for different laminins in the absence of biologically relevant growth factors. In vivo, the niche cues are in many cases enabled only by co-signaling from both specific laminin and specific growth factor binding relevant cell receptors. Another pitfall would be to apply stimulatory growth factors and laminins without regard to natural cell polarity [91].

Future Challenges

Use of biologically relevant laminins in regenerative medicine is a new area of research. We have reviewed above some examples of how the "niche-based" concept can be successfully translated into biomedical technologies. We believe that biologically relevant laminins may happen to be a useful tool in:

1. Generating new technologies of
   - In vitro cell culture systems to expand niche-sensitive primary cell types
   - Robust differentiation protocols for embryonic stem cells and adult stem cells
   - 3D artificial tissues and organs with sophisticated architecture
2. Improve efficacy of existing technologies
   - Increase number of doublings that a primary cell culture can undergo without loss of phenotype.
   - Increase rate of proliferation (however, within healthy natural range).
   - Increase functionality of cells cultured in vitro.
3. Improve safety of existing technologies
   - Replace non-defined extracellular scaffolds with defined ones.
   - Replace systems that bear potential source of pathogens, such as feeder cells or xeno compounds, to pathogen-free.

In this chapter, we have focused on laminins' ability to provide general maintenance for specific mammalian cell types, such as to enable survival, healthy proliferation and responsiveness to growth factor stimuli. In vivo, however, laminins have many other advanced biological in vivo roles, which are not yet translated into biomedical technologies. For example, in vivo certain laminins can regulate and maintain organogenesis: e.g., regulate diameter of blood vessels and extent of branching in vasculogenesis and angiogenesis [30, 77], and enable neural crest and brain compartments formation [9, 24]. Laminins can regulate permeability of viable tissue structures, from biomolecules (such as in glomerular basement membrane in kidney [56]) to cells [74, 89]. Laminins may enable mechanosensing by conveying stiffness signals from extracellular matrix to residing cells [25], thus regulating cell behavior [18]. Laminins can guide cell-to-cell contacts with high precision, such as in neuromuscular junction [62]. It is possible that laminins could allow not only to expand isolated cells in vitro, but to design highly organized artificial 3D tissues with natural-like architecture, hosting heterogenic cell communities.

Summary

1. At least 16 laminin isoforms exist in mammals. Each isoform has a unique function. Laminin isoforms are not "similar." In fact, two different isoforms may exert opposite influences on a certain cell type.
2. Golden standard in regenerative medicine technologies would be to expand specific cells in a culture system specifically tailored to mimic their in vivo microenvironment. Such culture would necessarily require niche-specific (1) growth factors, (2) cell contacts (or imitation of those), and (3) tissue-specific extracellular matrix cues.
3. Knowledge of biological roles for laminins in vivo (tissue-specific expression, knockout or mutant models, laminin-associated pathologies, etc.) may provide valuable clues to choose laminin isoforms that would be relevant for culturing specific cell type in vitro. However, knowledge of *laminin trimers* distribution and action in vivo is limited up to date.
4. Specific laminins act in concert with specific growth factors (co-signaling). Use of specific laminin in the absence of specific growth factor, or vice versa, may result in negative result.
5. Criteria for successful in vitro cell culture system should not only include phenotype analysis (such as adhesion, proliferation, and specific markers expression), but also (1) functional assays and (2) side-effects monitoring, such as malignant transformation of the cell lines. Golden standard for establishing a cell culture system should be the in vivo cells within natural environment.
6. Expanding cells approach in regenerative medicine has advantages, compared to traditional organ transplantation approach, but also carries hidden hazards. An isolated cell, when deprived on natural cues, may undergo apoptosis (anoikis), loss of phenotype, or loss of function. One more possibility, which is probably

the worst, is malignant transformation of the cells. Such cells may exhibit abnormally high survival, adhesion, and proliferation properties, which would give them a competitive advantage over non-malignant, healthy cells.

7. Use of tissue-specific laminins for expanding tissue-specific cell cultures is a new trend in regenerative medicine. Natural properties of laminins potentially can enable not only expansion of a specific cell type, but many other advanced technologies, like generation of complex, natural-like 3D tissues.

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References

1. Abrass CK, Hansen KM, Patton BL. Laminin alpha4-null mutant mice develop chronic kidney disease with persistent overexpression of platelet-derived growth factor. Am J Pathol. 2010;176:839–49.
2. Akhmanova M, Osidak E, Domogatsky S, Rodin S, Domogatskaya A. Physical, spatial, and molecular aspects of extracellular matrix of in vivo niches and artificial scaffolds relevant to stem cells research. *Stem Cells Int*. 2015;167025.
3. Amps K, Andrews PW, Anyfantis G, Armstrong L, Avery S, Baharvand H, Baker J, Baker D, Munoz MB, Beil S, Benvenisty N, Ben-Yosef D, Biancotti JC, Bosman A, Brena RM, Brison D, Caisander G, Camarasa MV, Chen J, Chiao E, Choi YM, Choo AB, Collins D, Colman A, Crook JM, Daley GQ, Dalton A, de Sousa PA, Denning C, Downie J, Dvorak P, Montgomery KD, Feki A, Ford A, Fox V, Fraga AM, Frumkin T, Ge L, Gokhale PJ, Golan-Lev T, Gourabi H, Gropp M, Lu G, Hampl A, Harron K, Healy L, Herath W, Holm F, Hovatta O, Hyllner J, Inamdar MS, Irwanto AK, Ishii T, Jaconi M, Jin Y, Kimber S, Kiselev S, Knowles BB, Kopper O, Kukharenko V, Kuliev A, Lagarkova MA, Laird PW, Lako M, Laslett AL, Lavon N, Lee DR, Lee JE, Li C, Lim LS, Ludwig TE, Ma Y, Maltby E, Mateizel I, Mayshar Y, Mileikovsky M, Minger SL, Miyazaki T, Moon SY, Moore H, Mummery C, Nagy A, Nakatsuji N, Narwani K, Oh SK, Olson C, Otonkoski T, Pan F, Park IH, Pells S, Pera MF, Pereira LV, Qi O, Raj GS, Reubinoff B, Robins A, Robson P, Rossant J, Salekdeh GH, Schulz TC, et al. Screening ethnically diverse human embryonic stem cells identifies a chromosome 20 minimal amplicon conferring growth advantage. Nat Biotechnol. 2011;29:1132–44.
4. Aumailley M, Bruckner-Tuderman L, Carter WG, Deutzmann R, Edgar D, Ekblom P, Engel J, Engvall E, Hohenester E, Jones JC, Kleinman HK, Marinkovich MP, Martin GR, Mayer U, Meneguzzi G, Miner JH, Miyazaki K, Patarroyo M, Paulsson M, Quaranta V, Sanes JR, Sasaki T, Sekiguchi K, Sorokin LM, Talts JF, Tryggvason K, Uitto J, Virtanen I, von der Mark K, Wewer UM, Yamada Y, Yurchenco PD. A simplified laminin nomenclature. Matrix Biol. 2005;24:326–32.
5. Barak T, Kwan KY, Louvi A, Demirbilek V, Saygi S, Tuysuz B, Choi M, Boyaci H, Doerschner K, Zhu Y, Kaymakcalan H, Yilmaz S, Bakircioglu M, Caglayan AO, Ozturk AK, Yasuno K, Brunken WJ, Atalar E, Yalcinkaya C, Dincer A, Bronen RA, Mane S, Ozcelik T, Lifton RP, Sestan N, Bilguvar K, Gunel M. Recessive LAMC3 mutations cause malformations of occipital cortical development. Nat Genet. 2011;43:590–4.
6. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nat Protoc. 2007;2:481–5.

7. Chiarugi P, Giannoni E. Anoikis: a necessary death program for anchorage-dependent cells. Biochem Pharmacol. 2008;76:1352–64.
8. Chun SJ, Rasband MN, Sidman RL, Habib AA, Vartanian T. Integrin-linked kinase is required for laminin-2-induced oligodendrocyte cell spreading and CNS myelination. J Cell Biol. 2003;163:397–408.
9. Coles EG, Gammill LS, Miner JH, Bronner-Fraser M. Abnormalities in neural crest cell migration in laminin alpha5 mutant mice. Dev Biol. 2006;289:218–28.
10. Cooper AR, Macqueen HA. Subunits of laminin are differentially synthesized in mouse eggs and early embryos. Dev Biol. 1983;96:467–71.
11. Denes V, Witkovsky P, Koch M, Hunter DD, Pinzon-Duarte G, Brunken WJ. Laminin deficits induce alterations in the development of dopaminergic neurons in the mouse retina. Vis Neurosci. 2007;24:549–62.
12. Dienstag JL, Cosimi AB. Liver transplantation–a vision realized. N Engl J Med. 2012;367:1483–5.
13. Discher DE, Janmey P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. Science. 2005;310:1139–43.
14. Domogatskaya A, Rodin S, Boutaud A, Tryggvason K. Laminin-511 but not -332, -111, or -411 enables mouse embryonic stem cell self-renewal in vitro. Stem Cells. 2008;26:2800–9.
15. Domogatskaya A, Rodin S, Tryggvason K. Functional diversity of laminins. Annu Rev Cell Dev Biol. 2012;28:523–53.
16. Durbeej M. Laminins. Cell Tissue Res. 2010;339:259–68.
17. Dziadek M, Timpl R. Expression of nidogen and laminin in basement membranes during mouse embryogenesis and in teratocarcinoma cells. Dev Biol. 1985;111:372–82.
18. Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. Cell. 2006;126:677–89.
19. Engler AJ, Sweeney HL, Discher DE, Schwarzbauer JE. Extracellular matrix elasticity directs stem cell differentiation. J Musculoskelet Neuronal Interact. 2007;7:335.
20. Georges PC, Hui JJ, Gombos Z, McCormick ME, Wang AY, Uemura M, Mick R, Janmey PA, Furth EE, Wells RG. Increased stiffness of the rat liver precedes matrix deposition: implications for fibrosis. Am J Physiol Gastrointest Liver Physiol. 2007;293: G1147–54.
21. Gu Y, Sorokin L, Durbeej M, Hjalt T, Jonsson JI, Ekblom M. Characterization of bone marrow laminins and identification of alpha5-containing laminins as adhesive proteins for multipotent hematopoietic FDCP-Mix cells. Blood. 1999;93:2533–42.
22. Gu YC, Kortesmaa J, Tryggvason K, Persson J, Ekblom P, Jacobsen SE, Ekblom M. Laminin isoform-specific promotion of adhesion and migration of human bone marrow progenitor cells. Blood. 2003;101:877–85.
23. Hager M, Gawlik K, Nystrom A, Sasaki T, Durbeej M. Laminin {alpha}1 chain corrects male infertility caused by absence of laminin {alpha}2 chain. Am J Pathol. 2005;167:823–33.
24. Halfter W, Dong S, Yip YP, Willem M, Mayer U. A critical function of the pial basement membrane in cortical histogenesis. J Neurosci. 2002;22:6029–40.
25. Hallmann R, Horn N, Selg M, Wendler O, Pausch F, Sorokin LM. Expression and function of laminins in the embryonic and mature vasculature. Physiol Rev. 2005;85:979–1000.
26. Heinonen M, Oila O, Nordstrom K. Current issues in the regulation of human tissue-engineering products in the European Union. Tissue Eng. 2005;11:1905–11.
27. Hewitt ZA, Amps KJ, Moore HD. Derivation of GMP raw materials for use in regenerative medicine: hESC-based therapies, progress toward clinical application. Clin Pharmacol Ther. 2007;82:448–52.
28. Huang CC, Hall DH, Hedgecock EM, Kao G, Karantza V, Vogel BE, Hutter H, Chisholm AD, Yurchenco PD, Wadsworth WG. Laminin alpha subunits and their role in *C. elegans* development. Development. 2003;130:3343–58.
29. Iwao M, Fukada S, Harada T, Tsujikawa K, Yagita H, Hiramine C, Miyagoe Y, Takeda S, Yamamoto H. Interaction of merosin (laminin 2) with very late activation antigen-6 is necessary for the survival of CD4+ CD8+ immature thymocytes. Immunology. 2000;99:481–8.

30. Jakobsson L, Domogatskaya A, Tryggvason K, Edgar D, Claesson-Welsh L. Laminin deposition is dispensable for vasculogenesis but regulates blood vessel diameter independent of flow. FASEB J. 2008;22:1530–9.
31. Jones KJ, Morgan G, Johnston H, Tobias V, Ouvrier RA, Wilkinson I, North KN. The expanding phenotype of laminin alpha2 chain (merosin) abnormalities: case series and review. J Med Genet. 2001;38:649–57.
32. Kao G, Huang CC, Hedgecock EM, Hall DH, Wadsworth WG. The role of the laminin beta subunit in laminin heterotrimer assembly and basement membrane function and development in *C. elegans*. Dev Biol. 2006;290:211–9.
33. Kenne E, Soehnlein O, Genove G, Rotzius P, Eriksson EE, Lindbom L. Immune cell recruitment to inflammatory loci is impaired in mice deficient in basement membrane protein laminin alpha4. J Leukoc Biol. 2010;88:523–8.
34. Klaffky E, Williams R, Yao CC, Ziober B, Kramer R, Sutherland A. Trophoblast-specific expression and function of the integrin alpha 7 subunit in the peri-implantation mouse embryo. Dev Biol. 2001;239:161–75.
35. Klimanskaya I, Chung Y, Becker S, Lu SJ, Lanza R. Human embryonic stem cell lines derived from single blastomeres. Nature. 2006;444:481–5.
36. Knoll R, Postel R, Wang J, Kratzner R, Hennecke G, Vacaru AM, Vakeel P, Schubert C, Murthy K, Rana BK, Kube D, Knoll G, Schafer K, Hayashi T, Holm T, Kimura A, Schork N, Toliat MR, Nurnberg P, Schultheiss HP, Schaper W, Schaper J, Bos E, den Hertog J, van Eeden FJ, Peters PJ, Hasenfuss G, Chien KR, Bakkers J. Laminin-alpha4 and integrin-linked kinase mutations cause human cardiomyopathy via simultaneous defects in cardiomyocytes and endothelial cells. Circulation. 2007;116:515–25.
37. Koch M, Olson PF, Albus A, Jin W, Hunter DD, Brunken WJ, Burgeson RE, Champliaud MF. Characterization and expression of the laminin gamma3 chain: a novel, non-basement membrane-associated, laminin chain. J Cell Biol. 1999;145:605–18.
38. Leivo I, Vaheri A, Timpl R, Wartiovaara J. Appearance and distribution of collagens and laminin in the early mouse embryo. Dev Biol. 1980;76:100–14.
39. Li L, Arman E, Ekblom P, Edgar D, Murray P, Lonai P. Distinct GATA6- and laminin-dependent mechanisms regulate endodermal and ectodermal embryonic stem cell fates. Development. 2004;131:5277–86.
40. Li S, Edgar D, Fassler R, Wadsworth W, Yurchenco PD. The role of laminin in embryonic cell polarization and tissue organization. Dev Cell. 2003;4:613–24.
41. Li S, Harrison D, Carbonetto S, Fassler R, Smyth N, Edgar D, Yurchenco PD. Matrix assembly, regulation, and survival functions of laminin and its receptors in embryonic stem cell differentiation. J Cell Biol. 2002;157:1279–90.
42. Libby RT, Champliaud MF, Claudepierre T, Xu Y, Gibbons EP, Koch M, Burgeson RE, Hunter DD, Brunken WJ. Laminin expression in adult and developing retinae: evidence of two novel CNS laminins. J Neurosci. 2000;20:6517–28.
43. Macdonald PR, Lustig A, Steinmetz MO, Kammerer RA. Laminin chain assembly is regulated by specific coiled-coil interactions. J Struct Biol. 2010;170:398–405.
44. Magner WJ, Chang AC, Owens J, Hong MJ, Brooks A, Coligan JE. Aberrant development of thymocytes in mice lacking laminin-2. Dev Immunol. 2000;7:179–93.
45. Marinkovich MP. Tumour microenvironment: laminin 332 in squamous-cell carcinoma. Nat Rev Cancer. 2007;7:370–80.
46. Martin D, Zusman S, Li X, Williams EL, Khare N, Darocha S, Chiquet-Ehrismann R, Baumgartner S. wing blister, a new Drosophila laminin alpha chain required for cell adhesion and migration during embryonic and imaginal development. J Cell Biol. 1999;145:191–201.
47. Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med. 2005;11:228–32.
48. Miner JH, Cunningham J, Sanes JR. Roles for laminin in embryogenesis: exencephaly, syndactyly, and placentopathy in mice lacking the laminin alpha5 chain. J Cell Biol. 1998;143:1713–23.

49. Miner JH, Li C, Mudd JL, Go G, Sutherland AE. Compositional and structural requirements for laminin and basement membranes during mouse embryo implantation and gastrulation. Development. 2004;131:2247–56.
50. Miner JH, Patton BL, Lentz SI, Gilbert DJ, Snider WD, Jenkins NA, Copeland NG, Sanes JR. The laminin alpha chains: expression, developmental transitions, and chromosomal locations of alpha1-5, identification of heterotrimeric laminins 8-11, and cloning of a novel alpha3 isoform. J Cell Biol. 1997;137:685–701.
51. Miner JH, Yurchenco PD. Laminin functions in tissue morphogenesis. Annu Rev Cell Dev Biol. 2004;20:255–84.
52. Narva E, Autio R, Rahkonen N, Kong L, Harrison N, Kitsberg D, Borghese L, Itskovitz-Eldor J, Rasool O, Dvorak P, Hovatta O, Otonkoski T, Tuuri T, Cui W, Brustle O, Baker D, Maltby E, Moore HD, Benvenisty N, Andrews PW, Yli-Harja O, Lahesmaa R. High-resolution DNA analysis of human embryonic stem cell lines reveals culture-induced copy number changes and loss of heterozygosity. Nat Biotechnol. 2010;28:371–7.
53. Nikolova G, Jabs N, Konstantinova I, Domogatskaya A, Tryggvason K, Sorokin L, Fassler R, Gu G, Gerber HP, Ferrara N, Melton DA, Lammert E. The vascular basement membrane: a niche for insulin gene expression and Beta cell proliferation. Dev Cell. 2006;10:397–405.
54. Nishimune H, Sanes JR, Carlson SS. A synaptic laminin-calcium channel interaction organizes active zones in motor nerve terminals. Nature. 2004;432:580–7.
55. Noakes PG, Gautam M, Mudd J, Sanes JR, Merlie JP. Aberrant differentiation of neuromuscular junctions in mice lacking s-laminin/laminin beta 2. Nature. 1995;374:258–62.
56. Noakes PG, Miner JH, Gautam M, Cunningham JM, Sanes JR, Merlie JP. The renal glomerulus of mice lacking s-laminin/laminin beta 2: nephrosis despite molecular compensation by laminin beta 1. Nat Genet. 1995;10:400–6.
57. Odenthal U, Haehn S, Tunggal P, Merkl B, Schomburg D, Frie C, Paulsson M, Smyth N. Molecular analysis of laminin N-terminal domains mediating self-interactions. J Biol Chem. 2004;279:44504–12.
58. Orlando G, Wood KJ, Stratta RJ, Yoo JJ, Atala A, Soker S. Regenerative medicine and organ transplantation: past, present, and future. Transplantation. 2011;91:1310–7.
59. Patton BL. Laminins of the neuromuscular system. Microsc Res Tech. 2000;51:247–61.
60. Patton BL, Chiu AY, Sanes JR. Synaptic laminin prevents glial entry into the synaptic cleft. Nature. 1998;393:698–701.
61. Patton BL, Connoll AM, Martin PT, Cunningham JM, Mehta S, Pestronk A, Miner JH, Sanes JR. Distribution of ten laminin chains in dystrophic and regenerating muscles. Neuromuscul Disord. 1999;9:423–33.
62. Patton BL, Cunningham JM, Thyboll J, Kortesmaa J, Westerblad H, Edstrom L, Tryggvason K, Sanes JR. Properly formed but improperly localized synaptic specializations in the absence of laminin alpha4. Nat Neurosci. 2001;4:597–604.
63. Pinzon-Duarte G, Daly G, Li YN, Koch M, Brunken WJ. Defective formation of the inner limiting membrane in laminin beta2- and gamma3-null mice produces retinal dysplasia. Invest Ophthalmol Vis Sci. 2010;51:1773–82.
64. Plantman S, Patarroyo M, Fried K, Domogatskaya A, Tryggvason K, Hammarberg H, Cullheim S. Integrin-laminin interactions controlling neurite outgrowth from adult DRG neurons in vitro. Mol Cell Neurosci. 2008;39:50–62.
65. Qian H, Georges-Labouesse E, Nystrom A, Domogatskaya A, Tryggvason K, Jacobsen SE, Ekblom M. Distinct roles of integrins alpha6 and alpha4 in homing of fetal liver hematopoietic stem and progenitor cells. Blood. 2007;110:2399–407.
66. Relan NK, Yang Y, Beqaj S, Miner JH, Schuger L. Cell elongation induces laminin alpha2 chain expression in mouse embryonic mesenchymal cells: role in visceral myogenesis. J Cell Biol. 1999;147:1341–50.
67. Rodin S, Antonsson L, Niaudet C, Simonson OE, Salmela E, Hansson EM, Domogatskaya A, Xiao Z, Damdimopoulou P, Sheikhi M, Inzunza J, Nilsson AS, Baker D, Kuiper R, Sun Y, Blennow E, Nordenskjold M, Grinnemo KH, Kere J, Betsholtz C, Hovatta O, Tryggvason K.

Clonal culturing of human embryonic stem cells on laminin-521/E-cadherin matrix in defined and xeno-free environment. Nat Commun. 2014;5:3195.
68. Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, Hovatta O, Tryggvason K. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol. 2010;28:611–5.
69. Sanes JR, Engvall E, Butkowski R, Hunter DD. Molecular heterogeneity of basal laminae: isoforms of laminin and collagen IV at the neuromuscular junction and elsewhere. J Cell Biol. 1990;111:1685–99.
70. Sarras MP Jr, YAN L, Grens A, Zhang X, Agbas A, Huff JK, St John PL, Abrahamson DR. Cloning and biological function of laminin in Hydra vulgaris. *Dev Biol*. 1994;164:312–24.
71. Scheele S, Falk M, Franzen A, Ellin F, Ferletta M, Lonai P, Andersson B, Timpl R, Forsberg E, Ekblom P. Laminin alpha1 globular domains 4-5 induce fetal development but are not vital for embryonic basement membrane assembly. Proc Natl Acad Sci U S A. 2005;102:1502–6.
72. Shimizu H, Zhang X, Zhang J, Leontovich A, Fei K, Yan L, Sarras MP Jr. Epithelial morphogenesis in hydra requires de novo expression of extracellular matrix components and matrix metalloproteinases. *Development*. 2002;129:1521–32.
73. Siler U, Seiffert M, Puch S, Richards A, Torok-Storb B, Muller CA, Sorokin L, Klein G. Characterization and functional analysis of laminin isoforms in human bone marrow. Blood. 2000;96:4194–203.
74. Sixt M, Engelhardt B, Pausch F, Hallmann R, Wendler O, Sorokin LM. Endothelial cell laminin isoforms, laminins 8 and 10, play decisive roles in T cell recruitment across the blood-brain barrier in experimental autoimmune encephalomyelitis. J Cell Biol. 2001;153: 933–46.
75. Smyth N, Vatansever HS, Murray P, Meyer M, Frie C, Paulsson M, Edgar D. Absence of basement membranes after targeting the LAMC1 gene results in embryonic lethality due to failure of endoderm differentiation. J Cell Biol. 1999;144:151–60.
76. Sorokin LM, Pausch F, Durbeej M, Ekblom P. Differential expression of five laminin alpha (1–5) chains in developing and adult mouse kidney. Dev Dyn. 1997;210:446–62.
77. Stenzel D, Franco CA, Estrach S, Mettouchi A, Sauvaget D, Rosewell I, Schertel A, Armer H, Domogatskaya A, Rodin S, Tryggvason K, Collinson L, Sorokin L, Gerhardt H. Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. EMBO Rep. 2011;12:1135–43.
78. Sunada Y, Bernier SM, Kozak CA, Yamada Y, Campbell KP. Deficiency of merosin in dystrophic dy mice and genetic linkage of laminin M chain gene to dy locus. J Biol Chem. 1994;269:13729–32.
79. Suzuki N, Yokoyama F, Nomizu M. Functional sites in the laminin alpha chains. Connect Tissue Res. 2005;46:142–52.
80. Szot GL, Lee MR, Tavakol MM, Lang J, Dekovic F, Kerlan RK, Stock PG, Posselt AM. Successful clinical islet isolation using a GMP-manufactured collagenase and neutral protease. Transplantation. 2009;88:753–6.
81. Thyboll J, Kortesmaa J, Cao R, Soininen R, Wang L, Iivanainen A, Sorokin L, Risling M, Cao Y, Tryggvason K. Deletion of the laminin alpha4 chain leads to impaired microvessel maturation. Mol Cell Biol. 2002;22:1194–202.
82. TRYGGVASON K., TRYGGVASON K.K. & DOMOGATSKAYA A. U.S. Patent 9,499,794, issued November 22, 2016.
83. Tzu J, Marinkovich MP. Bridging structure with function: structural, regulatory, and developmental role of laminins. Int J Biochem Cell Biol. 2008;40:199–214.
84. Unger C, Skottman H, Blomberg P, Dilber MS, Hovatta O. Good manufacturing practice and clinical-grade human embryonic stem cell lines. Hum Mol Genet. 2008;17:R48–53.
85. Urbano JM, Torgler CN, Molnar C, Tepass U, Lopez-Varea A, Brown NH, de Celis JF, Martin-Bermudo MD. Drosophila laminins act as key regulators of basement membrane assembly and morphogenesis. Development. 2009;136:4165–76.

86. Wallquist W, Plantman S, Thams S, Thyboll J, Kortesmaa J, Lannergren J, Domogatskaya A, Ogren SO, Risling M, Hammarberg H, Tryggvason K, Cullheim S. Impeded interaction between Schwann cells and axons in the absence of laminin alpha4. J Neurosci. 2005;25:3692–700.
87. Wang J, Hoshijima M, Lam J, Zhou Z, Jokiel A, Dalton ND, Hultenby K, Ruiz-Lozano P, Ross J, Jr, Tryggvason K, Chien KR. Cardiomyopathy associated with microcirculation dysfunction in laminin alpha4 chain-deficient mice. *J Biol Chem*. 2006;281:213–20.
88. Wondimu Z, Gorfu G, Kawataki T, Smirnov S, Yurchenco P, Tryggvason K, Patarroyo M. Characterization of commercial laminin preparations from human placenta in comparison to recombinant laminins 2 (alpha2beta1gamma1), 8 (alpha4beta1gamma1), 10 (alpha5beta1gamma1). Matrix Biol. 2006;25:89–93.
89. Wu C, Ivars F, Anderson P, Hallmann R, Vestweber D, Nilsson P, Robenek H, Tryggvason K, Song J, Korpos E, Loser K, Beissert S, Georges-Labouesse E, Sorokin LM. Endothelial basement membrane laminin alpha5 selectively inhibits T lymphocyte extravasation into the brain. Nat Med. 2009;15:519–27.
90. Xu H, Wu XR, Wewer UM, Engvall E. Murine muscular dystrophy caused by a mutation in the laminin alpha 2 (Lama2) gene. Nat Genet. 1994;8:297–302.
91. Xu R, Nelson CM, Muschler JL, Veiseh M, Vonderhaar BK, Bissell MJ. Sustained activation of STAT5 is essential for chromatin remodeling and maintenance of mammary-specific function. J Cell Biol. 2009;184:57–66.
92. Yarnitzky T, Volk T. Laminin is required for heart, somatic muscles, and gut development in the Drosophila embryo. Dev Biol. 1995;169:609–18.
93. Yurchenco PD. Basement membranes: cell scaffoldings and signaling platforms. *Cold Spring Harb Perspect Biol*.2011;3.
94. Yurchenco PD, Cheng YS. Self-assembly and calcium-binding sites in laminin. A three-arm interaction model. J Biol Chem. 1993;268:17286–99.
95. Yurchenco PD, Cheng YS, Colognato H. Laminin forms an independent network in basement membranes. J Cell Biol. 1992;117:1119–33.
96. Yurchenco PD, O'Rear JJ. Basement membrane assembly. Methods Enzymol. 1994;245:489–518.
97. Zhang X, Fei K, Agbas A, Yan L, Zhang J, O'reilly B, Deutzmann R, Sarras MP JR. Structure and function of an early divergent form of laminin in hydra: a structurally conserved ECM component that is essential for epithelial morphogenesis. *Dev Genes Evol*. 2002;212:159–72.

Chapter 4
Extracellular Matrix: Immunity and Inflammation

Amelia Cataldi and Viviana di Giacomo

Abstract The extracellular matrix (ECM) is the non-cellular component of any tissues and organs. It provides not only support, tensile strength, and scaffolding for tissues and cells, but also biochemical signals and specialized proteins. The destabilization or alteration of the ECM structural and chemical composition affects growth, morphogenesis, differentiation, migration, communication, survival of all cells as well as inflammation and immune response. Inflammation is a complex defense mechanism characterized by leukocyte migration from the vasculature to control tissue damage induced by pathogenic (bacterial or viral), traumatic, or toxic injury with subsequent deposition of extracellular matrix resulting in tissue repair. At sites of injury, phagocytic cells, namely macrophages and neutrophils, provide innate cell-mediated immunity, and immune cells are influenced in their migration by the topography and composition of the matrix architecture. The physical and biochemical ECM properties are also able to modulate a number of processes in immune cells, especially lymphocytes that can ultimately lead to inefficient immune response. Among the large number of molecules responsible for ECM homeostasis, matrix metalloproteinases, versican, hyaluronan, and thrombospondins are the most involved in inflammation and immunity.

The Extracellular Matrix

The extracellular matrix (ECM) is the non-cellular component of any tissue and organ [30]. Depending on the origin and on the condition (injury, inflammation, tumor) of the tissue, the three-dimensional (3D) matrix architecture can assume a variety of conformations [131]. In some tissues, the ECM can be dense and stiff, while, in others, it is soft and more porous with gap size of different diameters.

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ECM fibers also display highly variable thickness, straightness, and spatial arrangements. The fibers can be relaxed and non-oriented or, in contrast, linearized and oriented in a specific direction. These physical characteristics determine the architecture of the tissue but, as reported below, they are also essential for regulating immune cell migration and behavior [92].

The extracellular matrix consists of a complex assembly of many proteins and polysaccharides whose specific composition varies from tissue to tissue. The primary components include insoluble fibrous structural proteins (i.e., collagens, laminins, fibronectin, vitronectin, and elastin) and proteoglycans. These large and negatively charged sugars efficiently bind water and fill the space between the fibers. The ECM primarily fulfills a structural role by maintaining an insoluble scaffold, which ultimately defines the shape and stiffness of organs [92].

The extracellular matrix consists in a reinforced composite of collagens and elastic fibers embedded in a viscoelastic gel of proteoglycans, hyaluronan (HA), and water, together with a wide variety and arrangement of assorted glycoproteins [40, 46, 54, 78]. These molecules interact by entanglement and cross-linking to form a bioactive polymer which, in part, regulates the biomechanical properties of tissues and the phenotype of the cells belonging to those tissues. This regulation involves molecular interactions that govern the attachment of cells to their ECM scaffolds through integrin and non-integrin receptors, detachment of cells from those scaffolds, and molecular rearrangements in the matrix that allow cells to change shape during morphogenetic and remodeling events that occur in development and disease. The amount and composition of the ECM is controlled by the coordinated and differential regulation of synthesis and turnover of each of the ECM components. It is becoming increasingly evident that matrix individual components can exert dramatic effects on cell behavior and tissue response to endogenous and exogenous stimuli [129].

The ECM provides not only support, tensile strength, and scaffolding for tissues and cells, but also biochemical signals and specialized proteins (i.e., growth factors, chemokines, cytokines, small matricellular proteins and small integrin-binding glycoproteins) [18].

The destabilization or alteration of the structural and chemical matrix composition affects cell growth, morphogenesis, differentiation, migration, communication and survival [33], along with inflammation and immune response [82].

In addition to providing structural integrity, the extracellular matrix is recognized to play critical roles in regulating progenitor and reparative cell behaviors such as migration, differentiation, proliferation, and survival. The ECM dictates these activities through its binding to adhesion receptors as well as its ability to regulate growth factor bioavailability and signaling. More recently, a key role for mechanical control of cell fate through their interaction with the ECM has emerged [121].

Both mechanical and biochemical molecules influence extracellular matrix dynamics in multiple ways, by releasing small bioactive signaling molecules and growth factors stored within the ECM, by eliciting structural changes to matrix proteins which expose cryptic sites, and by degrading matrix proteins directly [18].

ECM remodeling takes place throughout different phases of disease progression as part of an injury and/or inflammatory response. These phases involve breakdown and disassembly of various matrix components and reassembly of specific components as part of the pathogenesis of these diseases. The sequence of changes is not unlike what is seen during wound repair in which the early ECM changes are characterized by deposits which create a loose, open, and watery matrix (referred to as a "provisional ECM") [17, 130] which allows for cellular invasion and repair. This provisional matrix is then replaced by a more fibrous ECM enriched in collagens and assorted glycoproteins [128].

Among the large number of molecules responsible for ECM homeostasis, matrix metalloproteinases (MMPs), versican, hyaluronan (HA), and thrombospondins are the most involved in inflammation and immunity.

Matrix Metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are a family of proteolytic enzymes involved in physiological processes including tissue homeostasis and repair, and host defense. There is also evidence that MMPs play a role in the pathogenesis of inflammatory diseases with focal tissue destruction, such as rheumatoid arthritis, osteoarthritis, and chronic cutaneous ulcerations, as well as in cancer progression [13, 56, 105]. The expression and activity of metalloproteinases are under strict control in physiological situations, whereas excessive activity of MMPs is often observed in pathological conditions [91]. MMPs were initially characterized as extracellular matrix (ECM) cleaving proteolytic enzymes, but during the past years, a growing number of non-matrix substrates for MMPs have been identified. Metalloproteinases can orchestrate the inflammatory functions at various levels [14, 91], regulating the transmigration of inflammatory cells from vasculature to the site of inflammation in tissue by processing ECM components, growth factors, cytokines, and chemokines.

MMPs belong to the metzincin superfamily, which is characterized by the presence of a highly conserved motif containing three histidine residues, which chelate a zinc ion in the catalytic site [35]. Other families in the metzincin super family are ADAMs (proteinases with a disintegrin and a metalloprotease domain) and ADAM-TSs (ADAM with thrombospondin-like motif), astacins, and serralysins. Metalloproteinases are ubiquitously expressed zinc-dependent endopeptidases with wide substrate specificities. They are produced either as soluble or cell membrane-anchored proteinases that cleave proteins and proteoglycan components of the extracellular matrix. In addition, MMPs cleave a multitude of non-matrix substrates including cytokines, chemokines, growth factors, growth factor receptors, and cell surface adhesion receptors. The members of the MMP family display marked differences in their tissue-specific expression, and substrate specificity makes these proteins a group of proteolytic enzymes with multiple physiological functions [86].

MMPs comprise a family of endopeptidases, which can cleave almost every component of the extracellular matrix proteins. It is documented that many non-ECM proteins can also be cleaved by selected metalloproteinases. Structurally, they all have a zinc ion in the catalytic domain, and their activity is dependent on divalent ions, mainly Zn^{2+} and Ca^{2+} [28, 108]. There are about 27 different MMPs discovered so far, and they are subdivided into groups according to substrate specificity and structural integrity. Induction and expression of these proteins are regulated at the level of transcription and translation, respectively. Further complexity of metalloproteinases is the activation from zymogen to active enzyme and, secondly, the mRNA stability of few MMPs plays a critical role. ProMMPs are converted into active enzymes by intramolecular cleavage of cysteine bridge between thiol group at the prodomain and Zn^{2+} near the catalytic site. The overall activity depends on substrate availability as well as on the presence of inhibitors in pericellular space, though a high concentration of MMPs exists near the plasma membrane [114].

The first metalloproteinase (collagenase) was identified in tadpole tails during metamorphosis by Gross and Lapière [38]. Most MMPs have been studied in vertebrates (25 members), but are also found in lower animals and plants. In humans, these proteins comprise 24 genes, encoding 23 proteins, as one MMP (MMP-23) is coded by two identical genes at chromosome 1 (MMP-23A and MMP-23B). Mammalian metalloproteinases are classified according to:

(I) their localization, soluble (secreted into ECM) or insoluble (anchored to cell membrane);
(II) their similarities in tridimensional structure and substrate affinity, being usually divided into six subgroups: collagenases (MMPs-1, -8, and -13), gelatinases (MMPs-2 and -9), stromelysins (MMPs-3, -10, and -11), matrilysins (MMPs-7 and -26), membrane-type metalloproteinase (MMPs-14, -15, -16, -17, and -24 or MMP-MT1, MT2, MT3, MT4, MT5, and MT6, respectively), and others (MMPs-12, -18, -19, -20, -21, -22, -23, -27, and -28);
(III) numerically listed according to chronological discovery. MMPs-4, -5, and -6 are missing in the list since they were shown to be identical to other members of the family [84].

Metalloproteinase regulation occurs at multiple levels, according to the cell type involved, in a temporal and spatial manner and quantities, by intra- and extracellular mechanisms. Inductive or suppressive signaling from the extracellular matrix (cytokines, growth factors, EMMPRIN, signals from integrins, ECM proteins, cellular stress, morphological changes, etc.) and intracellular signal transduction induce the activation or repression of the MMP genes. In the nucleus, the genes may be transcriptionally controlled by genetic alterations (polymorphisms or mutations, particularly in promoter regions) and by epigenetic control (DNA methylation status and remodeling of chromatin by histone acetylation) as well as post-transcriptionally through mRNA processing. In the cytoplasm, metalloproteinases may be post-transcriptionally regulated by mRNA stability (microRNAs

action and degradation pathway), intracellular activation of furin-susceptible MMPs, insertion of prosthetic groups (N- and O-glycosylation and GPI-anchor) or specific domains in the pro-MMP structure, and, finally, by inducible and constitutive pro-enzyme secretion into the ECM. Several metalloproteinases may be stored in the cytoplasm within granules in specific cell types prior to stimuli, such as inflammatory stimuli, and then secreted while their counterpart on the cellular membrane may be regulated by their localization on specialized membrane microdomains (lipid rafts or caveolae), by endocytosis/recycling (clathrin or caveolin-dependent) and intracellular degradation. In the extracellular matrix, the MMPs may be controlled by proteolytic processing and inactivation, proteolytic activation of pro-MMPs, binding of pro and active forms to inhibitors, and interaction with specific ECM components, leading to specific localization (pericellular perimeter or far from cell secretion point within the matrix), and allosteric control [16, 32, 39, 41, 68, 94, 95, 97, 111, 120, 135] (Table 4.1).

The balance between metalloproteinases and their inhibitors is required for physiological extracellular matrix remodeling, and imbalance in these enzymes leads to pathological states. In the tissues, MMPs are mainly reversibly inhibited in the ECM by their physiologic tissue inhibitors (TIMPs), while the cell surface MMPs are inhibited by the RECK glycoprotein [107].

Versican

Versican, whose production was found increased in many different diseases, is an extracellular matrix (ECM) proteoglycan observed in the pericellular environment of most tissues. Versican, produced by either stromal or myeloid cells, interacts with cells to influence their ability to proliferate, migrate, adhere, assemble, and remodel an ECM and can exert a major role in immunity and inflammation. Versican contributes to the structural integrity of tissues and interacts with cells through direct and indirect means to regulate, in part, cellular events that represent

Table 4.1 MMPs regulation

Step	Regulated by
Genes activation	Cytokines, growth factors, integrins, ECM proteins, cellular stress, morphological changes
Transcription	Polymorphisms, mutations, DNA methylation, histone acetylation
Translation	miRNA degradation, insertion of specific domains
Storage within granules	Localization, endocytosis, recycling, intracellular degradation
Zimogen secretion	Proteolytic activation/inactivation, inhibitors binding, interaction with ECM components, allosteric control

the basis of various diseases. Hence, targeting versican as a way to control cell phenotype offers a novel approach in the treatment of many diseases [129].

Versican is negatively charged due to its glycosaminoglycan (GAG) chains and attracts water, contributing to the viscoelasticity of the pericellular microenvironment [29]. In addition, versican interacts with a number of matrix components near the cell surface including hyaluronan (HA), tenascin-R and -C, thrombospondin 1, fibronectin, and fibrillin [49, 62, 74, 133] to create a mechanically active biopolymer around cells which influences their ability to change shape, adhere, proliferate, migrate, assemble other ECM components, and survive. Versican and extracellular matrix-related molecules may modify the mechanical stiffness around cells contributing to alterations in mechanotransduction influencing cell behavior and phenotype [23, 26, 125]. Versican can also act as a reservoir for cytokines and growth factors to be released at various times, establishing a further subtle control over cell activity and behavior [34, 75, 126].

Versican interacts with several different ECM molecules and, in part, plays a central role in matrix assembly. The domain structure of versican lends itself to multiple types of interactions through either protein–protein or protein–carbohydrate interactions. The best known of these interactions is the binding between the amino-terminal domain of versican (G1) and hyaluronan (HA) [64]. This interaction is stabilized by another protein—link protein—which exhibits selective binding specificity for both HA and versican [20] (Fig. 4.1).

In addition to hyaluronan, versican interacts with other extracellular matrix molecules controlling their organization: it interacts with tenascin-R through its

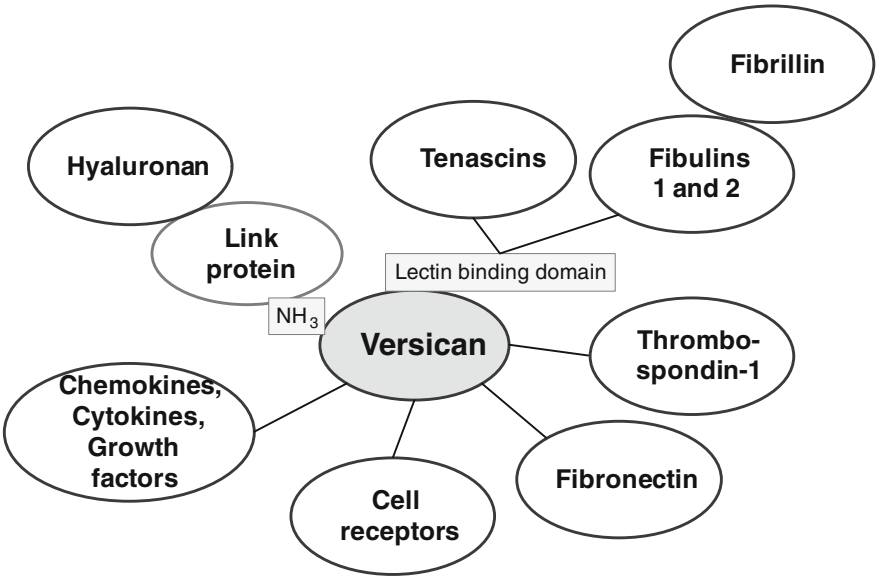

Fig. 4.1 Versican interactions

lectin-binding domain and involves protein–carbohydrate interactions [8]. The lectin-binding domain participates in other ligand interactions as well. For example, versican interacts with fibulin-1 and fibulin-2 [9, 88], a growing family of extracellular matrix proteins highly expressed in the developing heart valve. In adults, however, fibulin-1 and -2 are found associated with microfibrils that are part of elastic fibers. Versican can also interact with proteins associated with elastin, like fibrillin [83, 88], and has been shown to co-localize with elastic fibers in the skin [49]. Furthermore, fibrillins bind fibulin-2, and fibulin is preferentially localized to the elastin/microfibril interface in some tissues, but not in others [96]. Fibulin may represent a bridge between versican and fibrillin, forming high-ordered multimolecular structures important in the assembly of elastic fibers (Fig. 4.1). The relationship between versican and assembly of elastic fibers is interesting and unusual. In fact, elastic fibers are conspicuously absent in newly remodeled extracellular matrix as well as from atherosclerotic and restenotic lesions. The importance of elastic fibers in regulating vascular disease is highlighted by studies of the elastin KO mouse [128].

Hyaluronan

Hyaluronan (HA) is an atypical and relatively simple glycosaminoglycan (GAG); in fact, it is an unsulfated and unbranched polysaccharide not linked to any PG-core proteins. HA is ubiquitously expressed in the extracellular matrix of mammals and is composed of D-glucuronic acid (GlcUA) and N-acetyl- D-glucosamine (GlcNAc) bound together through β 1,3 and β 1,4 glycosidic bonds, respectively [77]. This disaccharide moiety is repeated thousands of times generating a linear polymer with a molecular mass ranging from 5×10^5 to $4–5 \times 10^6$ Da and more. Due to its hydrophilic properties, hyaluronan is very hydrated and makes the extracellular matrix an ideal environment in which cells can move and proliferate. Hyaluronan is an important space-filling molecule as it is evident in humor vitreous, derma and at joint level. Besides its molecular sieving properties related to the chemical and biomechanical characteristics, this polymer interacting with specific proteins called hyaladerins, such as TSG6, and membrane receptors like CD44, RHAMM, HARE, and toll-like receptor (TLR) 4/2, modulates development, morphogenesis, tumorigenesis, migration, apoptosis, cell survival, and inflammation [19, 116–119].

Thrombospondins

Thrombospondins (TSPs) are secreted extracellular matrix proteins from TSP family, which consists of five homologous members. They share a complex domain structure and have numerous binding partners in the extracellular matrix and

multiple cell surface receptors. Information emerged over the past decade identifies TSPs as important mediators of cellular homeostasis, assigning new important roles in cardiovascular pathology to these proteins [102].

Recent studies of the functions of TSP in the cardiovascular system, diabetes, and aging, which placed several thrombospondins in a position of critical regulators, demonstrated the involvement of these proteins in practically each aspect of cardiovascular pathophysiology related to atherosclerosis: inflammation, immunity, leukocyte recruitment and function, function of vascular cells, angiogenesis, and responses to hypoxia, ischemia, and hyperglycemia. TSPs play also a major role in the development and ultimate outcome of the complications associated with atherosclerosis–myocardial infarction, and heart hypertrophy and failure. Their expression and significance increase with age and with the progression of diabetes, two major contributors to the development of atherosclerosis and its complications [102].

Thrombospondins comprise a conserved family of extracellular, oligomeric, multidomain, calcium-binding glycoproteins. In general, basal metazoa and protostomes encode a single TSP in their genomes, and deuterostomes have multiple TSP genes. The mammal thrombospondins have many complex tissue-specific roles, including activities in wound healing and angiogenesis, vessel wall biology, connective tissue organization, and synaptogenesis. These activities derive mechanistically from interactions with cell surfaces, growth factors, cytokines, or components of the extracellular matrix that collectively regulate many aspects of cell phenotype. Emerging evidence on the functions of TSPs in invertebrates suggests that ancient functions include bridging activities in cell–cell and cell–ECM interactions. Knowledge of TSP domain structures provides a rational basis for understanding their roles in vivo and associations with human disease and is assisting ongoing translational applications [2].

Cells

Cells can remodel and reshape the extracellular matrix by degrading and reassembling it, thus playing an active role in sculpting their surrounding environment and directing their own phenotypes. The dynamic reciprocal communication between cells and the ECM plays a fundamental role in tissue development, homeostasis, and wound healing [18].

Cells, in order to change shape during division and migration, must modify their pericellular environment by first degrading the existing matrix and replacing it with new components. Hyaluronan and versican drive the changes leading to expansion of the pericellular matrix and to modifications in the mechanical properties of the ECM that influence cell phenotype [129].

Most matrix components are produced by fibroblasts that also play a role in their assembly into fibers and their spatial disposition. Collagen cross-linking is almost exclusively mediated by an enzyme, the lysyl oxidase (LOX) [134]. The synthesis

and cross-linking of ECM fibers is balanced by the action of matrix metalloproteinases that degrade collagen and other ECM proteins [56].

Fibroblasts are the most common cell type of the connective tissues found throughout the body and the main source of the extensive extracellular matrix characteristic of the tissues. They are also central mediators of pericellular matrix pathological fibrotic accumulation and of cellular proliferation and differentiation which occur in response to prolonged tissue injury and chronic inflammation [55].

Inflammation

Inflammation is triggered when innate immune cells detect infection or tissue injury. Surveillance mechanisms involve pattern recognition receptors (PRRs) on the cell surface and in the cytoplasm. Induction of genes encoding enzymes, chemokines, cytokines, adhesion molecules, and regulators of the extracellular matrix promotes the recruitment and activation of leukocytes, which are critical for eliminating foreign particles and host debris [85].

Inflammation is a complex defense mechanism characterized by leukocyte migration from the vasculature to control tissue damage induced by pathogenic (bacterial or viral), traumatic, or toxic injury [132] with subsequent deposition of extracellular matrix resulting in tissue repair. The inflammatory process is generally categorized into an acute, rapid response, and a persistent but slowly evolving chronic condition, which may progress into inflammatory diseases. An excessive deposition of ECM leads to overgrowth, hardening, and/or scarring of tissues, defined as fibrosis [100].

Fibrosis is the excessive deposition of extracellular matrix proteins into tissues leading to scar formation, disruption of normal tissue architecture, and organ failure. Despite the large clinical impact of fibrosis, treatment options are limited. Adhesion molecules, in particular $\alpha v\beta 6$ and $\alpha 3\beta 1$ integrins and cadherin-11, have been demonstrated to be important mediators of tissue fibrosis. Fibrosis is the final common pathway of a tissue response to injury, including chemical exposures, infections, and autoimmunity [3].

Inflammation is frequently accompanied by capillary permeability and induces endothelial activation, which, when persistent, results in capillary sprouting. The occurrence of such events is in large part mediated by extracellular matrix proteins and proteases. ECM turnover by proteases, in fact, allows the invasion of tissue by specialized endothelial cells and provides specific mechanical forces to expose cryptic adhesion sites and to release factors involved in vascular morphogenesis. Moreover, matrix remodeling and vascular regression contribute to the resolution of inflammation and facilitate tissue repair. In addition, a topic that has recently became of great interest is the degradome, i.e., the battery of proteases and inhibitors express by a cell type under specific conditions, or the protease substrate repertoire of a specific cell type. Its characterization in endothelial, mural, and accessory cells activated in distinct inflammatory contexts promises to identify new

targets for the inhibition or promotion of angiogenesis. Normalization of aberrant vasculature, rather than inhibition of its formation, could offer a better prospect for treating inflammatory diseases characterized by uncontrolled vascularization [6].

Throughout the body, the extracellular matrix provides structure and organization to tissues and also helps regulate cell migration and intercellular communication. In all tissues, inflammation can be induced and propagated by ECM disruption. Extracellular matrix molecules newly liberated by injury or inflammation include hyaluronan fragments, tenascins, and sulfated proteoglycans. These act as "damage-associated molecular patterns" or "alarmins," i.e., endogenous proteins that trigger and subsequently amplify inflammation. Activated inflammatory cells, in turn, further damage the extracellular matrix by releasing degradative enzymes including matrix metalloproteinases [33].

Fibrotic disorders are multistage progressive processes that often arise from different causes and are commonly associated with chronic inflammation. Excessive deposition of extracellular matrix is the hallmark of many fibrotic diseases. This may be due to an excess of fibroblast recruitment and activation, as well as to their differentiation in myofibroblasts. These events may be triggered by cytokines, chemokines, and growth factors released by lymphocytes or macrophages. The excessive production of extracellular matrix is apparently due to alterations of metabolic pathways in activated fibroblasts. It has been suggested that a defective autophagy, an important subcellular process with multiple homeostatic roles, also recognized as a key component of both innate and acquired immunity, could play a role [21].

Normally, the amount of ECM in the tissue is controlled through the balance between its production and degradation. A key role in this balance is played by matrix metalloproteinases.

The ability of MMPs to modify the structural integrity of tissues is essential for certain aspects of normal physiology, such as cell migration, proliferation, growth, and development, as well as for the occurrence of several pathological events (heart remodeling, metastasis, etc.) [73, 113]. Under healthy conditions, their proteolytic activity is precisely regulated by tissue inhibitor of metalloproteinases (TIMP) [16]. The regulation of the ratio MMPs: TIMPs plays an important role in wound healing, and the disruption of this ratio can result in pathological processes [5].

Metalloproteinases and Inflammation

Recent observations provide evidence that matrix metalloproteinases modulate various features of inflammation. They can regulate the integrity of physical barriers and the transmigration of leukocytes from vasculature to tissue as well as the availability and activity of inflammatory mediators, such as cytokines and chemokines. Metalloproteinases also generate chemokine gradients in tissue to recruit inflammatory cells to the site of injury or inflammation and can also regulate survival of inflammatory cells [14, 91]. Studies with MMP knockout mice have

elucidated the specific roles of distinct metalloproteinases in different experimental models involving inflammation. Interestingly, the only inflammatory phenotype without challenge is detected in MT1-MMP-deficient mice, which spontaneously develop arthritis [43]. Among challenges with different experimental models, MMP-9 has been shown to play a role in mouse models of myocardial aging and infarction, cerebral ischemia, asthma, and multiple sclerosis (experimental autoimmune encephalomyelitis (EAE)) [7, 24, 25, 109, 115]. In addition, elevated levels of MMP-2, -7, and -8 have been reported in mice with EAE [36, 58, 87]. The mechanistic role of MMP-12 in a mouse model of emphysema has been elucidated, indicating that defective activation of latent TGF-β regulates the expression of MMP-12 in macrophages [81]. MMP-8 has been shown to regulate inflammation in skin, and MMP-9 may also function as an anti-inflammatory mediator in skin inflammation and in glomerulonephritis [65, 122]. The role of MMP-8 in the regulation of inflammation appears complex. Recruitment of neutrophils is impaired in chemically induced epidermal carcinomas in MMP-8 knockout mice, as compared to wild-type mice [10]. However, in MMP-8-null mice, neutrophil recruitment into alveolar space after lipopolysaccharide stimulation in mouse model of acute lung injury is increased, suggesting an anti-inflammatory potential of MMP-8 [89]. MMP-3 and -9 have also been shown to play an important role in cutaneous inflammation in the mouse model for contact hypersensitivity. Specifically, MMP-3 appears to be required for cutaneous inflammation while MMP-9 may function as an anti-inflammatory mediator in skin. Expression of MMP-9 and -13 is potently upregulated in murine antigen-induced arthritis model, and the expression pattern correlates with the course of synovial inflammation [52].

Furthermore, expression of MMP-13 in mouse synovial tissue induces the onset of inflammation characterized by increased cytokine and chemokine production and inflammatory cell influx [53]. Additional evidence for the pro-inflammatory role of MMP-13 was provided by a recent study, showing that activation of TNF-α by MMP-13 plays an important role in the mouse model of inflammatory bowel disease, suggesting also a possible mechanistic role for MMP-13 in pathogenesis of intestinal ulcerations [112].

The discovery of the disintegrin and metalloproteinase 17 (ADAM17), originally identified as tumor necrosis factor-α converting enzyme (TACE) for its ability as sheddase of TNF-α, inspired scientists to attempt to elucidate the molecular mechanisms underlying ADAM17 implication in diseased conditions. In recent years, it has become evident that this protease can modify many non-matrix substrates, such as cytokines (e.g., TNF-α), cytokine receptors (e.g., IL-6R and TNF-R), ligands of ErbB (e.g., TGF-α and amphiregulin), and adhesion proteins (e.g., L-selectin and ICAM-1). Several recent studies have described experimental model system to better understand the role of specific signaling molecules, the interplay of different signals, and tissue interactions in regulating ADAM17-dependent cleavage of most relevant substrates in inflammatory diseases [70].

Recent literature suggests that ADAM-17 not only is the main protease responsible for the release of TNF-α during the inflammatory response, but it also

appears to be the most important sheddase in terms of the range of its targets. The inflammatory condition is characterized by an increased accumulation of leukocytes in tissue mediated by the transmembrane protein selectins. ADAM-17 also has a role in the shedding of L-selectin, a cellular adhesion molecule, from macrophages, and this process is promoted by leukocyte attachment to endothelial or E-selectins. Thus, ADAM-17 was shown to shed several factors contributing to successful recruitment of leukocytes to the inflammation site. Interestingly, there is some evidence that ADAM17 controls not only pro- but also anti-inflammatory signals. The enzyme was reported to down-regulate the macrophages activation cleaving colony-stimulating factor-1 (CSF-1) from their surface [123]. In addition, a correlation was reported between ADAM-17 expression and IL-15 receptor α activation, which is a soluble form of the receptor implied in the collagen-induced arthritis and cardiac allograph rejection. Also, different stimuli activate ADAM17-mediated IL-6 receptor-α cleavage, contributing to the decline of neutrophil infiltration and to promotion of monocyte recruitment, essential for resolution of the inflammation. Among the tissue inhibitors of matrix metalloproteinases, TIMP-3 is the only one that binds to the extracellular matrix and contains an amino acid sequence (PFG) required to inhibit ADAM-17 [66]. TIMP-3 is induced by molecules involved in inflammation, such as the pro-inflammatory agent PMA and the anti-inflammatory cytokine TGF-β, and has the potential to impact many different branches of haematopoiesis, immunity, and inflammation, primarily as a regulator of ADAM-17, but also as an inhibitor of other proteases which target growth factors, cytokines, and adhesion molecules [70].

Versican and Inflammation

An emerging body of evidence indicates that secreted proteoglycans act as signaling molecules, in addition to their canonical function in maintaining and regulating the architecture of various extracellular matrices. Proteoglycans interact with a number of receptors that regulate growth, motility and immune response. In part, as a consequence of their complex structure, proteoglycans can induce cross talk among various families of receptors and can also interact with natural receptor ligands, often blocking and sequestering their bioactivity. In their soluble form, originating from either partial proteolytic processing or through de novo synthesis by activated cells, some proteoglycans can become potent danger signals, denoting tissue stress and injury. Recently, it has been shown that proteoglycans, especially those belonging to the small leucine-rich and hyaluronan-binding gene families as well as the glycosaminoglycan hyaluronan, act as endogenous ligands of the toll-like receptors, a group of central receptors regulating innate immunity. Furthermore, proteoglycans can activate intracellular inflammasomes and trigger sterile inflammation [31].

A number of studies have identified upregulation of versican gene in monocytes in a number of pro-inflammatory states [34, 127, 129].

Based on its structural complexity, versican is capable of interacting with other ECM components, cell surface proteins (e.g., receptors), proteases, chemokines, and growth factors (Fig. 4.1). Thus, several different mechanisms of controlling inflammation by this proteoglycan have been described [126]. Versican has been suggested to act as a ligand to the toll-like receptors 2/6 heterodimer and to its adaptor CD14, thereby activating tumor-infiltrating myeloid cells to elicit the production of pro-inflammatory cytokines such as TNF-α and IL-6, and promoting tumor metastasis [124]. It is of note that in various inflammatory diseases and in response to lipopolysaccharide, activated macrophages synthesize and secrete versican [126]. Under similar conditions, de novo synthesis of biglycan and decorin by macrophages also occurs. Thus, it is conceivable that macrophages, upon stimulation with pro-inflammatory factors, start to synthesize several proteoglycans that act as endogenous TLR ligands and promote the inflammatory response in an autocrine and paracrine manner. The process is reminiscent of the very late activation antigen in B cells, where immune cells synthesize specific integrins de novo, facilitating their binding to extracellular matrix constituents during inflammation [45].

Furthermore, there is evidence that versican might be involved in M1/M2 macrophage polarization, thereby regulating the phenotype of macrophages. The binding of versican to hyaluronan changes the structure and functional properties of the matrix, which becomes capable of promoting leukocytes adhesion and retention and of stabilizing CD44 signaling [31].

Hyaluronan and Inflammation

Hyaluronan, a major component of the extracellular matrix, together with its receptors and binding partners, is a "keystone molecule" in the inflammatory milieu. HA and its binding proteins regulate the expression of inflammatory genes, the recruitment of inflammatory cells, the release of inflammatory cytokines, and can modulate the course of inflammation. The ability of HA to function as either a pro- or anti-inflammatory molecule is dependent upon its size, microenvironment, localization, and availability of specific binding partners [93].

Accumulation and turnover of extracellular matrix components are the hallmarks of tissue injury. Fragmented hyaluronan stimulates the expression of inflammatory genes by a variety of immune cells at the injury site. Hyaluronan fragments signal through both toll-like receptor (TLR) 4 and TLR-2 as well as CD44 to stimulate inflammatory genes in inflammatory cells. Hyaluronan binds to a number of cell surface proteins on various cell types, and it is also present on the cell surface of epithelial cells to provide protection against tissue damage from the environment by interacting with TLR-2 and TLR-4. Hyaluronan and hyaluronan-binding proteins regulate inflammation, tissue injury, and repair through regulating inflammatory cell recruitment, release of inflammatory cytokines, and cell migration [51].

HA plays a key role in regulating inflammation, being at the center of a complex network of ECM molecules that together exert decisive effects on the nature of inflammation. Hyaluronan has several unique features that qualify it as a keystone molecule in the inflammatory response:

(I) First, HA is known to play important roles in most aspects of the tissue response to injury, including wound healing, angiogenesis, cell trafficking, and proliferation [50].

(II) Second, HA physically interacts with a large and diverse network of ECM molecules present in inflamed tissues. These include HA-binding molecules called hyaladherins such as tumor necrosis factor-stimulated gene (TSG-6), versican, inter-α-inhibitor (I α I), CD38, and others [19]. HA-binding interactions with its receptor CD44 are also impacted by other extracellular matrix molecules, including osteopontin and fibronectin.

(III) Third, hyaluronan is highly abundant in inflamed tissue, and its production can increase as much as 200-fold following injury [27].

(IV) Fourth, fluid shifts caused by HA are responsible for many of the physiologic changes associated with inflammation. Because of the repeating disaccharides of N-acetyl glucosamine and glucuronic acid that make up hyaluronan carry strong negative charges, its synthesis generates oncotic forces that result in edema, vascular permeability changes, and leukocyte egress at sites of injury [63]. Studies utilizing magnetic resonance imaging (MRI) to detect edema associated with islet inflammation [22] may well be tracking the effects of HA.

(V) Fifth, the length of hyaluronan strands in the ECM is a sensitive barometer of the inflammatory milieu. Uninjured tissues are characterized by modest amounts of high molecular weight HA (HMW-HA, > 1000 kDa). Following tissue injury, there is an accumulation of low molecular weight HA (LMW-HA, < 250 kDa) and short HA oligomers (sHA, < 30 kDa) generated through enzymatic degradation of HMW-HA by endogenous hyaluronidases as well as catabolism by a diverse group of microbial hyaluronidases, mechanical forces, and oxidative stress [104]. Normally, LMW-HA is cleared within 14 days of injury, and enhanced hyaluronan production ceases as collagen production increases in conjunction with wound healing. However, chronic wounds are characterized by large amounts of LMW-HA. Both the size and the amount of HA in the tissue environment are therefore tightly linked to the stage of an injury response and to its resolution [51, 103].

Thrombospondins and Inflammation

Inflammation is a defensive process against tissue injury. Once this self-protective strategy is initiated, an effective resolution of the process is crucial to avoid major

and unnecessary tissue damage. If the underlying event inducing inflammation is not addressed and homeostasis is not restored, this process can become chronic and lead to angiogenesis and carcinogenesis. Thrombospondin-1 (TSP-1) is a matricellular protein involved in angiogenesis, cancer, and inflammation [60]. The effects of TSP-1 have been studied in many preclinical tumor models, and mimetic peptides are being tested in cancer clinical trials. However, the molecular mechanisms explaining its role in inflammatory processes are not well understood.

TSP-1 is transiently released early during the acute phase of inflammation, and multiple factors seem to modulate the release of TSP-1 during this process. This protein is strongly expressed in neutrophils, inducing an intense chemotactic response to injured tissues. TSP-1 is secreted in response to inflammation, promoting the resolution of the inflammatory process and facilitating phagocytosis of damaged cells [37]. Thus, enhanced production of TSP-1 could be a compensatory mechanism for controlling the immune response and protecting tissues from excessive damage.

TSP-1 mediates macrophage phagocytosis of apoptotic cells via CD36. This receptor is coexpressed with TSP-1 in macrophages and endothelial cells, and its binding to thrombospondin-1 induces apoptosis in endothelial cells. By activating CD36, TSP-1 also controls blood flow and leukocyte infiltration modulating the action of the nitric oxide (NO) pathway in injured tissues (Fig. 4.2). NO is a gas produced when L-arginine is converted to L-citrulline by the enzyme nitric oxide synthase (NOS). There are four different isoforms of NOS, neuronal (nNOS), endothelial (eNOS), mitochondrial (mtNOS), and the inducible isoform (iNOS). The first two are secreted during normal physiological events, but only iNOS is expressed upon inflammatory stimuli. The effects of nitric oxide in inflammation have been extensively recognized in a variety of studies. NO can modulate leukocyte adhesion in a dose-dependent manner [48]. At low doses, nitric oxide is anti-inflammatory and anti-angiogenic but, after inflammatory stimuli, high levels of NO are secreted promoting angiogenesis and leukocyte adhesion to the endothelium. TSP-1 could inhibit the soluble guanylyl cyclase system in endothelial cells, and consequently the activation of NO by interacting with CD36 and CD47. Through this mechanism, TSP-1 inhibits inflammation by blocking adhesion and activation of leukocytes to the endothelium and by diminishing angiogenesis [47] (Fig. 4.2).

Another factor interacting with TSP-1 during early inflammation is the peroxisome proliferator-activated receptor (PPAR), a member of the nuclear hormone receptor superfamily of transcription factors. When PPAR is absent in leukocytes, the leukocytes secrete high levels of TSP-1. PPAR-γ, one of the isoforms of this receptor, greatly enhances the proapoptotic effects of the TSP-1-derived peptide ABT510. This peptide corresponds to the second type 1 repeat (TSR) of TSP-1 and induces vascular apoptosis in vitro and in vivo through its interaction with CD36. By using a PPAR-γ agonist, the expression of CD36 in endothelial cells is enhanced, improving the anti-angiogenic effects of ABT510 in a CD36-dependent manner [44]. The TSP-1 receptor CD47 is critical for the migration of leukocytes through endothelial and epithelial barriers. CD47 is strongly expressed in

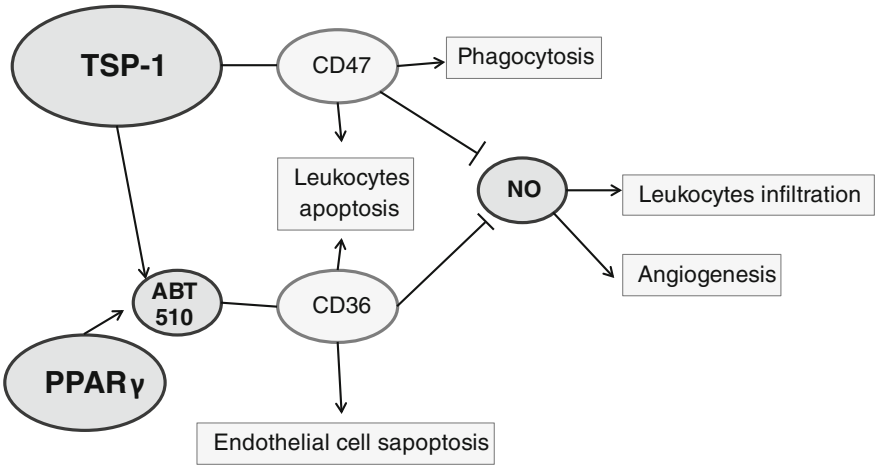

Fig. 4.2 TSP-1 control of inflammation. *NO* nitric oxide

polymorphonuclear cells, and its activation enhances the expression of TSP-1 in leukocytes. TSP-1 also induces leukocytic apoptosis through the CD47 pathway. CD47 can directly cause apoptosis through mitochondrial mechanisms, or by activation of the Fas/CD95 pathway. Expression of CD47 in apoptotic granulocytes can influence the phagocytic functions of the macrophages in inflammatory sites suggesting a critical role of this factor in the resolution of the process [72] (Fig. 4.2).

Immunity

At sites of injury, phagocytic cells, namely macrophages and neutrophils, provide innate cell-mediated immunity and initiate the inflammatory response. Macrophages secrete cytokines that attract neutrophils to leave the blood stream and enter the injured area. The arrival of neutrophils initiates the inflammatory response, by which cells and molecules of innate immunity are recruited into sites of wounding or infection [100].

Immune cells other than T cells are also influenced in their migration by the topography and composition of the matrix architecture. For instance, studies performed in 3D collagen matrices have demonstrated that macrophages can adopt distinct migratory mechanisms according to the extracellular environment. In a recent study, it was argued that the ability of T cells to mount an anti-tumor response is dependent on the matrix structure, more precisely on the balance between pro-migratory reticular fiber networks and unfavorable migration zones composed of dense and aligned ECM structures. Thus, the matrix architecture, that has long been considered to merely provide the structural framework of connective

tissues, can play a key role in facilitating or suppressing the anti-tumor immune surveillance. It is now well accepted that a deregulated ECM favours tumor progression and metastasis. Recent progress in imaging technologies has also highlighted the impact of the matrix architecture found in solid tumor on immune cells and especially T cells. Besides the effect exerted on tumor cells, the physical and biochemical properties of the ECM are also able to modulate a number of processes in immune cells, especially lymphocytes, that can ultimately lead to inefficient tumor killing. Extracellular matrix determinants can indeed promote tumor evasion from the immune system, both by inhibiting the anti-tumor effector activity of T cells, either directly or through the recruitment of immunosuppressive cells, and by limiting cell contact with tumor cells [92].

Along with collagen and fibronectin, some non-structural ECM proteins are often upregulated during tumor development and can modulate cell–cell and cell–ECM interactions, eventually restraining T cell activation [15, 98]. For instance, tenascin C seems to directly inhibit T cell proliferation and IFN-γ production in lung cancer [90] while the glycoprotein SPARC seems to influence the trafficking and the function of immune cells, as SPARC-knockout mice display an increased number of macrophages and neutrophils in tumors and higher cytotoxicity in polymorphonuclear cells [4, 99]. In addition, several ECM fragments are able to recruit and activate macrophages and neutrophils to sites of inflammation, where they could regulate inflammation and adaptive responses [1, 101].

The innate immune system down-regulates effector mechanisms and restores homoeostasis in injured tissue via cytokines from the IL-10 and TGF (transforming growth factor) families mainly released from macrophages, preferentially the M2 subset, which have a capacity to induce regulatory T cells, inhibit the production of pro-inflammatory cytokines, and induce healing of the tissue by regulating extracellular matrix protein deposition and angiogenesis [106]. Proteoglycans are also synthesized by leukocytes and may play a role in the inflammatory response [110].

Metalloproteinases and Immunity

Matrix metalloproteinases are members of the metzincin group of proteases which share the conserved zinc-binding motif in their catalytic active site. It was originally thought that their main function is to degrade the various components of the extracellular matrix, yet recent studies have led us to appreciate their significance as regulators of extracellular tissue signaling networks. Due to the broad spectrum of their substrate specificity, MMPs contribute to the homeostasis of many tissues and participate in several physiological processes, such as bone remodeling, angiogenesis, immunity, and wound healing. Metalloproteinase activity is tightly controlled at the level of transcription, pro-peptide activation, and inhibition by tissue inhibitors of MMPs.

Dysregulation of the activity of these proteins leads to pathological conditions such as arthritis, inflammation, and cancer, thus highlighting MMPs as promising

therapeutic targets. Analysis of MMP mutant mice has proved to be an essential tool for the identification of novel functions and interactions of single matrix metalloproteinase members. Advancing our understanding of the MMP contribution to tissue homeostasis will lead us to identify causal relationships between their dysregulation and the development of disease pathologies, thus guiding us to successful MMP-directed therapies [71].

Over the past 50 years, steady growth in the field of metalloproteinase biology has shown that the degradation of extracellular matrix components represents only a fraction of the functions performed by these enzymes and has highlighted their fundamental roles in immunity. Metalloproteinases regulate aspects of immune cell development, effector function, migration, and ligand–receptor interactions. They carry out ectodomain shedding of cytokines and their cognate receptors. Together with their endogenous inhibitors TIMPs, these enzymes regulate signaling downstream of the tumor necrosis factor receptor and the interleukin-6 receptor, as well as that downstream of the epidermal growth factor receptor and Notch, which are all pertinent for inflammatory responses [57].

Metalloproteinases and their inhibitors affect immunity as early as the first stages of haematopoietic cell development. In adult tissues, specific deficiencies of *Mmp, Adam,* or *Timp* genes in mice culminate in a range of spontaneous or induced inflammatory phenotypes.

Efficient neutrophil migration along chemotactic gradients and extravasation through blood vessels and tissues to sites of infection is important for establishing effective immunity, and metalloproteinases have been shown to contribute to these functions through the modification of chemotactic agents. Chemokine processing by MMPs was simultaneously discovered by two groups; the first reported that MMP-9 processes an amino-terminal fragment of IL-8 (functionally similar to CXCL6 in mice) to increase its potency by a factor of ten; and the second showed that MMP-2 inactivates monocyte chemotactic protein 3 (MCP3; also known as CCL7), which is a mononuclear cell-attracting chemokine 7. *Mmp7* −/− mice are protected from acute lung injury, and histological analysis linked this protection to a decrease in pulmonary neutrophil infiltration. MMP-7-mediated cleavage releases the heparan sulfate proteoglycan syndecan 1, along with its associated CXC-chemokine ligand 3 (CXCL3; also known as KC), which attracts neutrophils to the site of infection. Thus, the absence of MMP-7 compromises the CXCL3 chemokine gradient [69]. Similarly, neutrophils failed to migrate to sites of LPS administration in *Mmp8* −/− mice, as MMP-8 is required for the activation of the neutrophil-recruiting chemokine CXCL6.

Further metalloproteinase-dependent events that contribute to neutrophil chemotaxis and function have been recently described using proteomics, genetic mouse models, or human primary cell cultures; for example, using a double-knockout mouse model, MMP-2 and MMP-9 were shown to function together to cleave CXCL6 and to increase neutrophil migration to the peritoneum during IL-1β-induced peritonitis. As part of a positive feedback loop, meprins can cleave pro-MMP-9 to sensitize it to activation by MMP-3 (also known as stromelysin 1); an identical N-terminal MMP-9 cleavage product has been found in the

conditioned media of neutrophil and monocyte cultures, which suggests that meprins are involved in MMP-9 activation during an immune response [57].

Consistent with the function of TIMPs as metalloproteinase inhibitors, neutrophils migrated more rapidly to the site of infection in *Timp1* −/− mice, and TIMP-1 deficiency amplified the immune response in a mouse model of acute lung injury. Moreover, neutrophil inflammation was sustained in the absence of TIMP-3 in a mouse model of bleomycin-induced lung injury, which indicates that TIMP-3 suppresses neutrophil entry and/or promotes the resolution of inflammation [59].

Once they are proximal to the site of infection, neutrophils extravasate through blood vessels and tissues by making a series of molecular contacts with the vascular endothelium. L-selectin (also known as CD62L) is expressed by neutrophils and participates in their adhesion to vascular endothelial cells. The final step in the neutrophil response is the elimination of the foreign invaders, mainly through phagocytosis, the generation of reactive oxygen species (ROS) and the release of microbicidal substances. As mentioned above, MMP-9 is the major component of gelatinase granules that are secreted from neutrophils following stimulation with IL-8. As MMP-9 cleaves the N-terminal fragment of IL-8 to increase its activity, a positive feedback loop for effective neutrophil degranulation and pathogen clearance seems to be established at the site of infection. In certain pathological settings, this neutrophil-derived metalloproteinase activity has deleterious effects. Chronic obstructive pulmonary disorder (COPD) is characterized by prominent neutrophilic inflammation, and the associated metalloproteinase release is refractory to glucocorticosteroid treatment [57].

Neutrophils normally undergo apoptosis and clearance by phagocytosis, which leads to the resolution of acute inflammation. Recent studies highlight novel functions for metalloproteinases in these processes. Peritoneal neutrophils from *Adamts12* −/− mice showed a reduction in annexin-V staining, which indicates that this protease is associated with neutrophil apoptosis [80]. These examples reiterate the importance of metalloproteinase function in neutrophil-mediated immune responses.

Versican and Immunity

Versican has been suggested to contribute to hyaluronan fragment activation of macrophages, and enhanced cancer metastasis through induction of the hyaluronidases. Several inflammation-associated cytokines, including transforming growth factor-β1, -2, -3, and platelet-derived growth factor (PDGF), have been shown to increase biosynthetic levels of both versican and HA, while IL-1β, and IFN-γ have been shown to reduce levels of versican [11]. Leukocyte trafficking and localization to regions of inflammation mediated by interaction with cell-adhesion receptors functions as a critical initiating step in the inflammatory cascade. Specific chondroitin sulfate (CS) chains on versican preferentially bind to chemokines known to attract mononuclear leukocytes. Versican itself is capable of binding to a

number of cell surface receptors present on leukocytes through interaction also mediated by CS chains, including both L- and P-selectins and CD44 [42]. Direct binding of P-selectin glycoprotein ligand-1 (PSGL-1) by the G3 domain of versican has also been shown to cause aggregation of leukocytes. Together, these HA-binding proteins contribute to the maintenance of tissue integrity and to direct cell–ECM interactions in normal and pathological conditions. Many of the adhesive properties of hyaluronan polymers depend upon the presence of HA-binding proteins, and together with IaI, HCs, TSG-6, and versican contribute to a dynamic extracellular environment capable of directing cell adhesion and the production of inflammatory cytokines [93].

Hyaluronan and Immunity

Within the last year, the role of hyaluronan as an endogenous activator of innate alloimmunity has been investigated [67]. Recent studies have described that the hyaluronan receptor, CD44, modulates these innate immune responses by augmenting regulatory T cell function, and the expression of negative regulators of toll-like receptor signaling. Hyaluronan activates innate alloimmune responses and subsequently influences adaptive alloimmunity.

In regulatory T cells, HMW-HA stimulates STAT5 signaling through CD44 cross-linking, promoting their maintenance and thereby inhibiting their proliferation. Conventional T cell precursors stimulated with HMW-HA produce IL-10, and infusion of these cells attenuates the disease course in a murine model of colitis [12].

CD44 is a type I transmembrane glycoprotein and is widely regarded as the major cell surface HA-binding protein. Widely studied in several contexts, CD44 interactions with HA have important roles in tumor metastasis, lymphocyte adhesion, T cell signaling, angiogenesis, and inflammation. CD44 is expressed on many cell types that contribute to inflammation including leukocytes, neutrophils, macrophages, chondrocytes, fibroblasts, epithelial, and endothelial cells. While the glycosaminoglycans (GAGs) of CD44 can bind to cytokines, growth factors, and extracellular matrix proteins such as fibronectin, the majority of the functions of CD44 depend upon its ability to bind to hyaluronan [93].

Toll-like receptors function as surveillance receptors, interacting with a number of microbial-derived molecules and activating the innate immune system in response to pathogen-associated molecular patterns. Increasingly, the TLRs are also shown to sense damage-associated molecular patterns in response to injury as well. The idea that endogenous matrix degradation products act as regulators of cellular processes is not a new one, but with respect to GAG fragments, the role of HA is the best studied. Hyaluronan is a component of the cellular coat on some pathogens and many of them also express hyaluronidases. The presence of a HA coat likely assists in evasion by the immune system, while the hyaluronidase enzymes may aid in colonization of the host [76].

Thrombospondins and Immunity

During the early stages of injury and inflammation, high levels of thrombospondin-1 increase the tolerance of dendritic cells (DCs) to antigens, ending the inflammatory response. TSP-1 can modulate inflammation by inhibiting or enhancing the secretion of the cytokine interleukin 10; by this way, TSP-1 can also regulate the functions of DCs. Thrombospondin-1 has been recently shown to induce regulatory T cells and impair dendritic cell maturation in a melanoma model [61]. In addition, after adding IL-6, IL-10, or TGF-β1 to cultured DCs, they become immune tolerant and show upregulation of intracellular TSP-1. This protein also inhibits the function of antigen-presenting cells by suppressing their capacity to sensitize T cells in the host. CD47 has also a crucial role in T cell activation. Interaction of TSP-1 with CD47 promotes the activation of thymus-derived $CD4^+$ $CD25^+$ T regulatory cells (Tregs). Through this mechanism, CD47 helps to maintain self-tolerance inducing a suppressive phenotype [37].

It has been recently reported that bacterial pathogenesis may be mediated by CD47. Suppression of CD47 or TSP-1 expression in dendritic cells by using small interfering RNA (siRNA) technique actually protects newborn mice against bacterial (*Escherichia coli*) meningitis. Again, the loss of CD47 activity prevents the maturation of the DCs and the production of inflammatory cytokines [79]. In conclusion, CD47 seems to have pivotal functions in inflammation and immunity and provides a major mechanistic pathway for the functions of TSP-1 in that process.

Finally, the deficiency of CD36 enhances the severity of bacterial and malaria infection. *Cd36* −/− mice exhibit an impaired early pro-inflammatory response to infection, elevation of cytokines, and higher mortality. These findings suggest that CD36 is quite critical for the recognition and clearance of pathogens in acute and chronic infections. By binding to this receptor, TSP-1 could modulate the inflammatory process by activating macrophages and favoring phagocytosis. During chronic inflammation, these adaptive immune mechanisms provide defense against diseases and are regulated by cellular interactions and cytokines. B lymphocytes secrete antibodies that bind to infectious agents and label them for destruction or elimination. Once inside a cell, a pathogen becomes inaccessible to those antibodies and cytotoxic T cells could destroy them by inducing apoptosis of the cell host. Regulatory T cells can modulate the secretion of cytokines enhancing the functions of macrophages and B lymphocytes. Thrombospondin-1 has been reported to decrease immune responses by inhibition of T cell effectors, or by directly inducing T cell apoptosis. In addition, by binding to $\alpha 4\beta 1$ integrin, TSP-1 promotes T cell adhesion and chemotaxis [72].

References

1. Adair-Kirk TL, Senior RM. Fragments of extracellular matrix as mediators of inflammation. Int J Biochem Cell Biol. 2008;40:1101–10.
2. Adams JC, Lawler J. The thrombospondins. Cold Spring Harb Perspect Biol. 2011;3(10): a009712.
3. Agarwal SK. Integrins and cadherins as therapeutic targets in fibrosis. Front Pharmacol. 2014;5:131.
4. Alvarez MJ, Prada F, Salvatierra E, et al. Secreted protein acidic and rich in cysteine produced by human melanoma cells modulates polymorphonuclear leukocyte recruitment and antitumor cytotoxic capacity. Cancer Res. 2005;65:5123–32.
5. Amălinei C, Căruntu ID, Giuşcă SE, et al. Matrix metalloproteinases involvement in pathologic conditions. Rom J Morphol Embryol. 2010;51:215–28.
6. Arroyo AG, Iruela-Arispe ML. Extracellular matrix, inhflammation, and the angiogenic response. Cardiovasc Res. 2010;86:226–35.
7. Asahi M, Wang X, Mori T, et al. Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood–brain barrier and white matter components after cerebral ischemia. J Neurosci. 2001;21:7724–32.
8. Aspberg A, Binkert C, Ruoslahti E. The versican C-type lectin domain recognizes the adhesion protein tenascin-R. Proc Natl Acad Sci U S A. 1995;92:10590–4.
9. Aspberg A, Adam S, Kostka G, et al. Fibulin-1 is a ligand for the C-type lectin domains of aggrecan and versican. J Biol Chem. 1999;274:20444–9.
10. Balbín M, Fueyo A, Tester AM, et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet. 2003;35:252–7.
11. Berdiaki A, Zafiropoulos A, Fthenou E, et al. Regulation of hyaluronan and versican deposition by growth factors in fibrosarcoma cell lines. Biochim Biophys Acta. 2008;1780:194–202.
12. Bollyky PL, Wu RP, Falk BA, et al. ECM components guide IL-10 producing regulatory T-cell (TR1) induction from effector memory T-cell precursors. Proc Natl Acad Sci U S A. 2011;108:7938–43.
13. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci. 2006;11:529–43.
14. Butler GS, Overall CM. Matrix metalloproteinase processing of signaling molecules to regulate inflammation. Periodontol. 2000;63:123–48.
15. Chong HC, Tan CK, Huang RL, et al. Matricellular proteins: a sticky affair with cancers. J Oncol. 2012;2012:351089.
16. Clark IM, Swingler TE, Sampieri CL, et al. The regulation of matrix metalloproteinases and their inhibitors. Int J Biochem Cell Biol. 2008;40:1362–78.
17. Clark RAF, Henson PM. The molecular and cellular biology of wound repair. New York: Plenum Press; 1988.
18. Clause KC, Barker TH. Extracellular matrix signaling in morphogenesis and repair. Curr Opin Biotechnol. 2013;24:830–3.
19. Day AJ, Prestwich GD. Hyaluronan-binding proteins: tying up the giant. J Biol Chem. 2002;277:4585–8.
20. Day AJ, Sheehan JK. Hyaluronan: polysaccharide chaos to protein organization. Curr Opin Struct Biol. 2001;11:617–22.
21. Del Principe D, Lista P, Malorni W, et al. Fibroblast autophagy in fibrotic disorders. J Pathol. 2013;229:208–20.
22. Denis MC, Mahmood U, Benoist C, et al. Imaging inflammation of the pancreatic islets in type 1 diabetes. Proc Natl Acad Sci U S A. 2004;101:12634–9.
23. Discher DE, Mooney DJ, Zandstra PW. Growth factors, matrices, and forces combine and control stem cells. Science. 2009;324:1673–7.

24. Dubois B, Masure S, Hurtenbach U, et al. Resistance of young gelatinase B-deficient mice to experimental autoimmune encephalomyelitis and necrotizing tail lesions. J Clin Invest. 1999;104:1507–15.
25. Ducharme A, Frantz S, Aikawa M, et al. Targeted deletion of matrix metalloproteinase-9 attenuates left ventricular enlargement and collagen accumulation after experimental myocardial infarction. J Clin Invest. 2000;106:55–62.
26. DuFort CC, Paszek MJ, Weaver VM. Balancing forces: architectural control of mechanotransduction. Nat Rev Mol Cell Biol. 2011;12:308–19.
27. Edelstam GA, Laurent UB, Lundkvist OE, et al. Concentration and turnover of intraperitoneal hyaluronan during inflammation. Inflammation. 1992;16:459–69.
28. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer. 2002;2:161–74.
29. Evanko SP, Tammi MI, Tammi RH, et al. Hyaluronan-dependent pericellular matrix. Adv Drug Deliv Rev. 2007;59:1351–65.
30. Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. J Cell Sci. 2010;123:4195–200.
31. Frey H, Schroeder N, Manon-Jensen T, et al. Biological interplay between proteoglycans and their innate immune receptors in inflammation. FEBS J. 2013;280:2165–79.
32. Gabison EE, Huet E, Baudouin C, et al. Direct epithelial-stromal interaction in corneal wound healing: role of EMMPRIN/CD147 in MMPs induction and beyond. Prog Retin Eye Res. 2009;28:19–33.
33. Gaudet AD, Popovich PG. Extracellular matrix regulation of inflammation in the healthy and injured spinal cord. Exp Neurol. 2014;258:24–34.
34. Gill S, Wight TN, Frevert CW, et al. Proteoglycans: key regulators of pulmonary inflammation and the innate immune response to lung infection. Anat Rec (Hoboken). 2010;293:968–81.
35. Gomis-Rüth FX. Structural aspects of the metzincin clan of metalloendopeptidases. Mol Biotechnol. 2003;24:157–202.
36. Graesser D, Mahooti S, Madri JA. Distinct roles for matrix metalloproteinase-2 and α4 integrin in autoimmune T cell extravasation and residency in brain parenchyma during experimental autoimmune encephalomyelitis. J Neuroimmunol. 2000;109:21–131.
37. Grimbert P, Bouguermouh S, Baba N, et al. Thrombospondin/CD47 interaction: a pathway to generate regulatory T cells-from human $CD4^+$ $CD25^-$ T cells in response to inflammation. J Immunol. 2006;177:3534–41.
38. Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: a tissue culture assay. Proc Natl Acad Sci U S A. 1962;48:1014–22.
39. Hadler-Olsen E, Fadnes B, Sylte I, et al. Regulation of matrix metalloproteinase activity in health and disease. FEBS J. 2011;278:28–45.
40. Hay ED. Cell biology of extracellular matrix. New York: Plenum Press; 1991.
41. Hernandez-Barrantes S, Bernardo M, Toth M, et al. Regulation of membrane type-matrix metalloproteinases. Semin Cancer Biol. 2002;12:131–8.
42. Hirose J, Kawashima H, Yoshie O, et al. Versican interacts with chemokines and modulates cellular responses. J BiolChem. 2001;276:5228–34.
43. Holmbeck K, Bianco P, Caterina J, et al. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. Cell. 1999;99:81–92.
44. Huang H, Campbell SC, Bedford DF, et al. Peroxisome proliferator-activated receptor γ ligands improve the antitumor efficacy of thrombospondin peptide ABT510. Mol Cancer Res. 2004;2:541–50.
45. Hynes RO. The extracellular matrix: not just pretty fibrils. Science. 2009;326:1216–9.
46. Hynes RO, Yamada KM. Extracellular matrix biology. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2012.
47. Isenberg JS, Frazier WA, Roberts DD. Thrombospondin-1: a physiological regulator of nitric oxide signaling. Cell Mol Life Sci. 2008;65:728–42.

48. Isenberg JS, Martin-Manso G, Maxhimer JB, et al. Regulation of nitric oxide signalling by thrombospondin 1: implications for anti-angiogenic therapies. Nat Rev Cancer. 2009;9: 182–94.
49. Isogai Z, Aspberg A, Keene DR, et al. Versican interacts with fibrillin-1 and links extracellular microfibrils to other connective tissue networks. J Biol Chem. 2002;277:4565–72.
50. Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol. 2007;23:435–61.
51. Jiang D, Liang J, Noble PW. Hyaluronan as an immune regulator in human diseases. Physiol Rev. 2011;91:221–64.
52. Joronen KR, Ala-aho ML, Majuri H, et al. Adenovirus mediated intra-articular expression of collagenase-3 (MMP-13) induces inflammatory arthritis in mice. Ann Rheum Dis. 2004;63:656–64.
53. Joronen K, Kähäri VM, Vuorio E. Temporospatial expression of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases in mouse antigen induced arthritis. Histochem Cell Biol. 2005;124:535–45.
54. Karamanos N. Extracellular matrix: pathobiology and signaling. Berlin, Boston: Walter de Gruyter; 2012.
55. Kendall RT, Feghali-Bostwick CA. Fibroblasts in fibrosis: novel roles and mediators. Front Pharmacol. 2014;5:123.
56. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010;141:52–67.
57. Khokha R, Murthy A, Weiss A. Metalloproteinases and their natural inhibitors in inflammation and immunity. Nat Rev Immunol. 2013;13:649–65.
58. Kieseier BC, Kiefer R, Clements JM, et al. Matrix metalloproteinase-9 and -7 are regulated in experimental autoimmune encephalomyelitis. Brain. 1998;121:159–66.
59. Kim KH, Burkhart K, Chen P, et al. Tissue inhibitor of metalloproteinase–1 deficiency amplifies acute lung injury in bleomycin-exposed mice. Am J Respir Cell Mol Biol. 2005;33:271–9.
60. Kirk JA, Cingolani OH. Thrombospondins in the transition from myocardial infarction to heart failure. J Mol Cell Cardiol. 2016;90:102–10.
61. Kudo-Saito C, Shirako H, Takeuchi T, et al. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. Cancer Cell. 2009;15: 195–206.
62. Kuznetsova SA, Issa P, Perruccio EM, et al. Versican-thrombospondin-1 binding in vitro and colocalization in microfibrils induced by inflammation on vascular smooth muscle cells. J Cell Sci. 2006;119:4499–509.
63. Laurent TC, Fraser JR. Hyaluronan. FASEB. 1992;J6:2397–404.
64. LeBaron RG, Zimmermann DR, Ruoslahti E. Hyaluronate binding properties of versican. J Biol Chem. 1992;267:10003–10.
65. Lelongt B, Bengatta S, Delauche M, et al. Matrix metalloproteinase 9 protects mice from anti-glomerular basement membrane nephritis through its fibrinolytic activity. J Exp Med. 2001;193:793–802.
66. Lee MM, Yoon BJ, Osiewicz K, et al. Tissue inhibitor of metalloproteinase 1 regulates resistance to infection. Infect Immun. 2005;73:661–5.
67. Lee-Sayer SS, Dong Y, Arif AA, et al. The where, when, how, and why of hyaluronan binding by immune cells. Front Immunol. 2015;6:150.
68. Li L, Li H. Role of microRNA-mediated MMP regulation in the treatment and diagnosis of malignant tumors. Cancer Biol Ther. 2013;14:796–805.
69. Li Q, Park PW, Wilson CL, et al. Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell. 2002;111:635–46.
70. Lisi S, D'Amore M, Sisto M. ADAM17 at the interface between inflammation and autoimmunity. Immunol Lett. 2014;S0165-2478(14):00178-3.

71. Löffek S, Schilling O, Franzke CW. Series "matrix metalloproteinases in lung health and disease": Biological role of matrix metalloproteinases: a critical balance. Eur Respir J. 2011;38:191–208.
72. Lopez-Dee Z, Pidcock K, Gutierrez LS. Thrombospondin-1: multiple paths to inflammation. Mediat Inflamm. 2011;2011:296069.
73. Lu P, Takai K, Weaver VM, et al. Extracellular matrix degradation and remodeling in development and disease. Cold Spring Harb Perspect Biol. 2011;3(ii):a005058.
74. Lundell A, Olin AI, Morgelin M, et al. Structural basis for interactions between tenascins and lectican C-type lectin domains: evidence for a crosslinking role for tenascins. Struct (Camb). 2004;12:1495–506.
75. Macri L, Silverstein D, Clark RA. Growth factor binding to the pericellularmatrix and its importance in tissue engineering. Adv Drug Deliv Rev. 2007;59:1366–81.
76. Matzinger P. Tolerance, danger, and the extended family. Annu Rev Immunol. 1994;12:991–1045.
77. McDonald JA, Camenisch TD. Hyaluronan: genetic insights into the complex biology of a simple polysaccharide. Glycoconj J. 2002;1:331–9.
78. Mecham RP. The extracellular matrix: an overview. Berlin: Springer; 2011.
79. Mittal R, Gonzalez-Gomez I, Prasadarao NV. Escherichia coli K1 promotes the ligation of CD47 with thrombospondin-1 to prevent the maturation of dendritic cells in the pathogenesis of neonatal meningitis. J Immunol. 2010;185:2998–3006.
80. Moncada-Pazos A, Obaya AJ, Llamazares M, et al. ADAMTS-12 metalloprotease is necessary for normal inflammatory response. J Biol Chem. 2012;287:39554–63.
81. Morris DG, Huang X, Kaminski N, et al. Loss of integrin αvβ6-mediated TGF-β activation causes MMP12-dependent emphysema. Nature. 2003;422:169–73.
82. Morwood SR, Nicholson LB. Modulation of the immune response by extracellular matrix proteins. Arch Immunol Ther Exp (Warsz). 2006;54:367–74.
83. Murasawa Y, Watanabe K, Yoneda M, et al. Homotypic versican G1 domain interactions enhance hyaluronan incorporation into fibrillin microfibrils. J Biol Chem. 2013;288:29170–81.
84. Nagase H, Visse R, Murphy G. Structure and function of matrix metalloproteinases and TIMPs. Cardiovasc Res. 2006;69:562–73.
85. Newton K, Dixit VM. Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol. 2012;4:a006049.
86. Nissinen L, Kähäri VM. Matrix metalloproteinases in inflammation. Biochim Biophys Acta. 2014;1840:2571–80.
87. Nygårdas PT, Hinkkanen AE. Up-regulation ofMMP-8 andMMP-9 activity in the BALB/c mouse spinal cord correlates with the severity of experimental autoimmune encephalomyelitis. Clin Exp Immunol. 2002;128:245–54.
88. Olin AI, Morgelin M, Sasaki T, et al. The proteoglycans aggrecan and Versican form networks with fibulin-2 through their lectin domain binding. J Biol Chem. 2001;276:1253–61.
89. Owen CA, Hu Z, Lopez-Otin C, et al. Membrane-bound matrix metalloproteinase-8 on activated polymorphonuclear cells is a potent, tissue inhibitor of metalloproteinase-resistant collagenase and serpinase. J Immunol. 2004;172:7791–803.
90. Parekh K, Ramachandran S, Cooper J, et al. Tenascin-C, over expressed in lung cancer down regulates effector functions of tumor infiltrating lymphocytes. Lung Cancer. 2005;47:17–29.
91. Parks WC, Wilson CW, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 2004;4:617–29.
92. Peranzoni E, Rivas-Caicedo A, Bougherara H, et al. Positive and negative influence of the matrix architecture on antitumor immune surveillance. Cell Mol Life Sci. 2013;70:4431–48.
93. Petrey AC, de la Motte CA. Hyaluronan, a crucial regulator of inflammation. Front Immunol. 2014;5:101.
94. Piperi C, Papavassiliou AG. Molecular mechanisms regulating matrix metalloproteinases. Curr Top Med Chem. 2012;12:1095–112.
95. Ra HJ, Parks WC. Control of matrix metalloproteinase catalytic activity. Matrix Biol. 2007;26:587–96.

96. Reinhardt DP, Sasaki T, Dzamba BJ, et al. Fibrillin-1 and fibulin-2 interact and are colocalized in some tissues. J Biol Chem. 1996;271:19489–96.
97. Rietz A, Spiers J. The relationship between the MMP system, adrenoceptors and phosphoprotein phosphatases. Br J Pharmacol. 2012;166:1225–43.
98. Sangaletti S, Colombo MP. Matricellular proteins at the crossroad of inflammation and cancer. Cancer Lett. 2008;267:245–53.
99. Sangaletti S, Stoppacciaro A, Guiducci C, et al. Leukocyte, rather than tumor-produced SPARC, determines stroma and collagen type IV deposition in mammary carcinoma. J Exp Med. 2003;198:1475–85.
100. Silini A, Parolini O, Huppertz B, et al. Soluble factors of amnion-derived cells in treatment of inflammatory and fibrotic pathologies. Curr Stem Cell Res Ther. 2013;8:6–14.
101. Sorokin L. The impact of the extracellular matrix on inflammation. Nat Rev Immunol. 2010;10:712–23.
102. Stenina-Adognravi O. Thrombospondins: old players, new games. Curr Opin Lipidol. 2013;24:401–9.
103. Stern R, Asari AA, Sugahara KN. Hyaluronan fragments: an information-rich system. Eur J Cell Biol. 2006;85:699–715.
104. Stern R, Jedrzejas MJ. Hyaluronidases: their genomics, structures, and mechanisms of action. Chem Rev. 2006;106:818–39.
105. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol. 2001;17:463–516.
106. Striz I, Brabcova E, Kolesar L, et al. Cytokine networking of innate immunity cells: a potential target of therapy. Clin Sci (Lond). 2014;126:593–612.
107. Takahashi C, Sheng Z, Horan TP, et al. Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc Natl Acad Sci U S A. 1998;95:13221–6.
108. Tezvergil-Mutluay A, Agee KA, Hoshika T, et al. The requirement of zinc and calcium ions for functional MMP activity in demineralized dentin matrices. Dent Mater. 2010;26:1059–67.
109. Toba H, Cannon PL, Yabluchanskiy A, et al. Transgenic overexpression of macrophage matrix metalloproteinase-9 exacerbates age-related cardiac hypertrophy, vessel rarefaction, inflammation, and fibrosis. Am J Physiol Heart Circ Physiol. 2017;312:H375–83.
110. Uhlin-Hansen L, Wik T, Kjellen L, et al. Proteoglycan metabolism in normal and inflammatory human macrophages. Blood. 1993;82:2880–9.
111. Van den Steen PE, Opdenakker G, Wormald MR, et al. Matrix remodelling enzymes, the protease cascade and glycosylation. Biochim Biophys Acta. 2001;1528:61–73.
112. Vandenbroucke RE, Dejonckheere E, Van Hauwermeiren F, et al. Matrix metalloproteinase 13 modulates intestinal epithelial barrier integrity in inflammatory diseases by activating TNF. EMBO Mol Med. 2013;5:932–48.
113. Vargová V, Pytliak M, Mechírová V. Matrix metalloproteinases. EXS. 2012;103:1–33.
114. Verma S, Kesh K, Ganguly N, et al. Matrix metalloproteinases and gastrointestinal cancers: impacts of dietary antioxidants. J Biol Chem. 2014;5:355–76.
115. Vermaelen KY, Cataldo D, Tournoy K, et al. Matrix metalloproteinase-9-mediated dendritic cell recruitment into the airways is a critical step in a mouse model of asthma. J Immunol. 2003;171:1016–22.
116. Vigetti D, Karousou E, Viola M, et al. Hyaluronan: biosynthesis and signaling. Biochim Biophys Acta. 2014;1840:2452–9.
117. Vigetti D, Rizzi M, Moretto P, et al. Glycosaminoglycans and glucose prevent apoptosis in 4-methylumbelliferone-treated human aortic smooth muscle cells. J Biol Chem. 2011;286:34497–503.
118. Vigetti D, Genasetti A, Karousou E, et al. Proinflammatory cytokines induce hyaluronan synthesis and monocyte adhesion in human endothelial cells through hyaluronan synthase 2 (HAS2) and the nuclear factor-kappaB (NF-kappaB) pathway. J Biol Chem. 2011;285:24639–45.

119. Vigetti D, Rizzi M, Viola M, et al. The effects of 4-methylumbelliferone on hyaluronan synthesis, MMP2 activity, proliferation, and motility of human aortic smooth muscle cells. Glycobiology. 2009;19:537–46.
120. Vincenti MP. The matrix metalloproteinase (MMP) and tissue inhibitor of metalloproteinase (TIMP) genes. Transcriptional and posttranscriptional regulation, signal transduction and cell-type-specific expression. Methods Mol Biol. 2001;151:121–48.
121. Volk SW, Iqbal SA, Bayat A. Interactions of the extracellular matrix and progenitor cells in cutaneous wound healing. Adv Wound Care (New Rochelle). 2013;2:261–72.
122. Wang M, Qin X, Mudgett JS, et al. Matrix metalloproteinase deficiencies affect contact hypersensitivity: stromelysin-1 deficiency prevents the response and gelatinase B deficiency prolongs the response. Proc Natl Acad Sci U S A. 1999;96:6885–9.
123. Wang Y, Herrera AH, Li Y, et al. Regulation of mature ADAM17 by redox agents for l-selectin shedding. J Immunol. 2009;182:2449–57.
124. Wang W, Xu GL, Jia WD, et al. Ligation of TLR2 by versican: a link between inflammation and metastasis. Arch Med Res. 2009;40:321–3.
125. Werfel J, Krause S, Bischof AG, et al. How changes in extracellular matrix mechanics and gene expression variability might combine to drive cancer progression. PLoS ONE. 2013;8: e76122.
126. Wight TN. The pathobiology of versican. In: Karamanos N, editor. Extracellular matrix: pathobiology and signaling. KG, Berlin: Walter De Gruyter GMBH & Co.; 2012. p. 154–70.
127. Wight TN, Frevert CW, Debley JS, et al. Interplay of extracellular matrix and leukocytes in lung inflammation. Cell Immunol. 2017;312:1–14.
128. Wight TN, Kang I, Merrilees MJ. Versican and the control of inflammation. Matrix Biol. 2014;35:152–61.
129. Wight TN, Kinsella MG, Evanko SP, et al. Versican and the regulation of cell phenotype in disease. Biochim Biophys Acta. 2014;1840:2441–51.
130. Wight TN, Potter-Perigo S. The extracellular matrix: an active or passive player in fibrosis. Am J Physio Gastrointest Liver Physiol. 2011;301:G950–5.
131. Wolf K, Alexander S, Schacht V, et al. Collagen based cell migration models in vitro and in vivo. Semin Cell Dev Biol. 2009;20:931–41.
132. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol. 2004;4: 583–94.
133. Wu YJ, La Pierre DP, Wu J, et al. The interaction of versican with its binding partners. Cell Res. 2005;15:483–94.
134. Xiao Q, Ge G. Lysyl oxidase, extracellular matrix remodeling and cancer metastasis. Cancer Microenviron. 2012;5:261–73.
135. Yan C, Boyd DD. Regulation of matrix metalloproteinase gene expression. J Cell Physiol. 2007;211:19–26.

Part II
Material Inspired from Nature

Chapter 5
Biologically Inspired Materials in Tissue Engineering

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Abstract The extracellular matrix (ECM) has unique biochemical, mechanical and organisational properties through which it provides a physical scaffolding for cells; a barrier that protects tissues; several signals that affect cell behaviour; and a reservoir for biologically active molecules. Considering the importance of ECM in regulating many fundamental cell processes, a myriad of strategies and materials has been developed to reproduce its properties. The first part of the chapter covers various approaches aiming to generate scaffolds whose fibre size, orientation and stiffness could mimic the ECM nanofibrous structure. In particular, the use of natural fibrous proteins, the application of electrospinning and freeze-drying and examples of tissue engineering applications are presented. The second part discusses strategies aiming to address the ECM ligand-binding function and to reproduce the dynamic, reciprocal, dialogue between cells and their microenvironment; examples of 3D scaffolds for controlled release of growth factors, drugs and genetic material are reported. Researchers have also used native ECM components to recapitulate the biochemical and biophysical properties of ECM. In the third part of the chapter, the use of fibrinogen and fibrin is presented as an example of natural scaffolds recapitulating ECM functions. Fibrinogen and fibrin can be used as provisional matrix in regenerating tissues; moreover, by varying the fabrication method and by blending them with other materials, it is possible to produce biodegradable scaffolds with reasonable control of degradation rate and drug release.

Keywords Fibrous scaffolds · Natural biomaterials · Electrospinning Hydrogels · Freeze-drying · 3D scaffolds · Controlled drug release Growth factors · Gene therapy · Fibrinogen · Fibrin · Crosslinkers Cell carrier

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Functional Fibrous Scaffolds for Tissue Engineering Applications

The term of 'fibrous scaffold' refers to a 3D fibrous network of molecules that closely mimics native tissues. Fibrous structures can present two different architectures: interconnected micropores and nano- or micro-scaled fibres [1, 2] (Fig. 5.1). Both fibrous architectures are characterised by continuous fibres or thin layers, high surface to volume ratio, high porosity, adjustable fibre diameter/fibre distribution/pore size/pore distribution and interconnectivity [3–5]. The attractiveness of fibrous scaffolds for tissue engineering is on the broad range of suitable biomaterials (natural or synthetic) [6]; the possibility of tuning the fibrous structure [7] and biomechanical strength [8] by varying the fabrication method; the ability to enhance nutrient transport [9]; and to support cell ingrowth [10].

Fibrous proteins are attractive natural materials because cells can recognise and attach to specific sites. Moreover, fibrous proteins such as collagen, elastin, keratin or silk consist of highly repetitive amino acid sequences that form secondary structures (e.g. triple helices or beta-sheets) during self-assembling, mimicking native tissue structure. Another advantage is the degradation into non-toxic products [11]. However, some fibrous protein scaffolds display low mechanical properties and exogenous crosslinking does not induce enough stability; therefore, fibrous proteins are mixed with synthetic polymers to increase mechanical performance [12]. Table 5.1 provides examples of natural materials used to produce fibrous scaffolds for tissue engineering applications.

Collagens are the most abundant components in the extracellular matrix (ECM) of connective tissues, constituting 20–30% of total proteins of human body [13]. To date, 29 different collagen types have been identified [14] and all types share a common $[Gly\text{-}X\text{-}Y]_n$ sequence, where frequently X is proline and Y is hydroxyproline [15]. Among all collagen types, collagen type I is the most abundant in the body, and therefore, it is the most widely studied [16, 17], followed by the denatured form of collagen, gelatine [7]. Collagens are attractive biomaterials

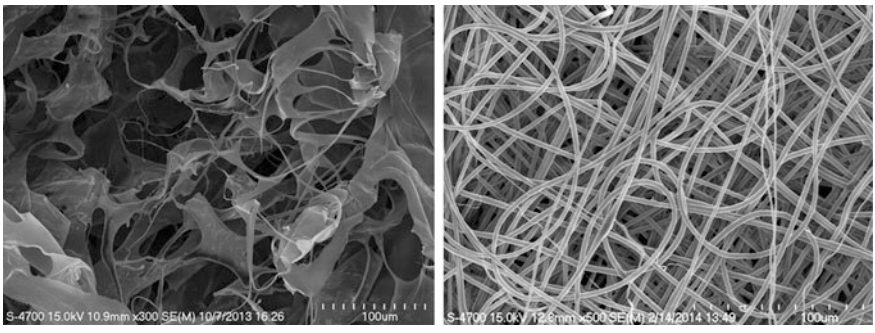

Fig. 5.1 Images obtained by scanning electron microscopy of different fibrous scaffold structure formed by micropores (sponge, left), nano- or micro-fibres (electrospun film, right)

Table 5.1 Example of materials, formulations and applications of fibrous scaffolds

Material	Formulation	Tissue engineering application	Outcome	References
Collagen	Hydrogel	Intervertebral disc repair	It maintained NP cells phenotype over 14 days	[26]
Collagen	Electrospun film	Wound healing	It promoted keratinocytes adhesion and spreading; it accelerated wound healing	[27]
Collagen/ PEUU	Electrospun film	Wound healing	PEUU increased mechanical properties, but reduced cell infiltration and remodelling, delaying wound healing	[12]
Collagen	Film	Wound healing	Crosslinked collagen type I film with increased mechanical and enzymatic resistance that modulates macrophage response	[21]
Elastin	Film	Wound healing	It maintained structural integrity and prevented hernia recurrence during wound healing	[44]
Elastin	Vesicle	Wound healing	Tuneable elastin vesicles for growth factor release with controlled degradation rates	[45]
Elastin/ collagen	Freeze-dried sponge	Wound healing	Non-cross-linked sponge induced wound healing with minimal foreign body reaction	[120]
Keratin	Hydrogel	Wound healing	It promoted in vitro cell proliferation and accelerated re-epithelisation in vivo	[64]
Keratin	Hydrogel	Peripheral nerve repair	It improved functional nerve recovery in a rat model	[61]
Keratin	Hydrogel	Wound healing	Tuneable elastin vesicles for drug release with controlled degradation rates	[62]
Silk	Gel tubes	Vascular grafts	It increased cell attachment and ingrowth of artery smooth muscle and endothelial cells	[71]
Silk	Electrospun film	Wound healing	It promoted keratinocytes adhesion and spreading, especially when combined with collagen coating	[121]
Silk	Electrospun conduit	Vascular grafts	Electrospun silk fibroin grafts enhanced small blood vessels repair	[73]

because they are recognised by the host tissue as normal constituents rather than as a foreign matter [17]. Other advantages of collagen are its abundance and the ease of functionalisation with therapeutic moieties. However, the mechanical and enzymatic stability of collagen produced from animal/human source is minimal because natural crosslinking does not occur in vitro. For that reason, exogenous crosslinking (chemical, biological or physical) is required to control stability and durability in the body [18–21], even though crosslinking has been associated with cytotoxic issues [22], scaffold calcification [23] and foreign body response [24, 25].

Collagen-based materials can be used in different forms—hydrogels, fibres, films and sponges—for tissue engineering. For example, self-assembled collagen hydrogels have been shown to be a promising candidate as cell carrier for adipose stem cells and nucleus pulposus cells [26]. Electrospun collagen has been able to enhance wound healing [27, 28]; however electrospinning processing can denature collagen [29]. Although collagen is mainly extracted from porcine, bovine and human tissues [30], these sources carry the potential risk of disease transmission [31], allergic reactions [32], immunogenicity [33] and variability between batches [30, 34]. In order to avoid these possible issues, fish-extracted collagen and recombinant human collagen have been proposed as possible alternatives [35, 36]. Recombinant collagen from transgenic tobacco plant, mammalian cells, bacteria or silkworm have been employed in research [30, 36, 37], whereas fish-extracted collagen has been rarely used [38] because of its non-mammalian origin.

Elastin is a rubber-like protein that exhibits high elasticity, low stiffness and high resilience [39]. Therefore, elastin is a crucial protein in tissues requiring large stretching and shape recovery, such as blood vessels, ligaments, lungs or skin [39]. In the same way, elastin-based scaffolds are particularly interesting for tissue engineering applications that require large elastic deformation [40, 41]. As for collagen scaffolds, mechanical and biological stability of elastin-based scaffolds can be increased by means of chemical crosslinking [40]. Elastin is composed of approximately 800 amino acids, and it is synthesized from tropoelastin that is formed by two domains: hydrophobic domains—mainly glycine, valine, alanine and proline residues—, and hydrophilic domains—predominantly lysine and alanine residues [42]. The principal sources of elastin are solubilised elastin from mammalian tissues and recombinant elastin-like protein [43]. The main advantage of recombinant elastin is the better control over the physical and chemical characteristics of the resulting scaffold in comparison to solubilised elastin [40]. For example, elastin-like proteins have been used to produce films for hernia repair [44], carrier for growth factor [45] or gene therapy [46].

Elastin has good hemocompatibility, anti-thrombogenic properties and regulatory functions over luminal endothelial cells and vascular smooth muscle cells [47]. Consequently, elastin is an excellent biomaterial for vascular applications [48, 49]. Elastin-coated polymeric or metallic devices have been used for increasing hemocompatibility [50] and for decreasing platelet adhesion, fibrin deposition [51, 52] and implant calcification [53]. Nevertheless, the success of elastin coating may be affected by the chemical modifications required to covalently bond elastin to the device surface [54]. Elastin has been also proposed to construct blood vessels;

however, the use of elastin alone has limited applications due to the low ultimate tensile strength of the scaffold [54]. Therefore, the combination of elastin with polyurethane (PU), poly-lactic-*co*-glycolic acid (PLGA) or polycaprolactone (PCL) has been explored to improve mechanical properties [55–57]. Finally, industrial and clinical use of elastin-based scaffolds has been limited by the issues related to elastin extraction process, which can compromise its biomechanical and biological properties [54].

Keratin is one of the main constituents of skin, hair and nails [58]. Keratin contains cell-binding motifs, such as RGD and LDV, that enhance cell attachment, migration and proliferation [40]. In addition, waste products from the poultry processing can be used as source of keratin. This fact ensures a cheap and continuous source, and therefore, keratin extraction has been widely investigated. Keratin can be extracted by oxidative or reductive methods, but only reductive extraction with DTT or mercaptoethanol ensures long in vivo stability of reconstituted scaffolds [40]. These keratin solutions can be employed to produce hydrogels, sponges, films and fibres [59].

Although keratin-based products are well known in cosmetic industry, its clinical use is limited because of its low mechanical properties and extremely high flexibility [60]. However, keratin hydrogels and films have been demonstrated to promote proliferation of endothelial cells, keratinocytes and fibroblasts, and to regulate Schwann cells activity [61]. Furthermore, keratin-based scaffolds have been successfully used as tuneable drug delivery system [62], to improve nerve repair [63] to accelerate epithelisation and wound remodelling [64] in preclinical models.

Silk is a lightweight, strong and elastic protein produced by several insects and spiders [65]. The two main components of silk are sericin and fibroin; sericin is a glue-like protein that links fibroin fibres, while fibroin is a structural protein commonly used to produce scaffolds [66]. Silk fibroin is composed of glycine-X sequences, where X is often alanine, serine, threonine and valine [67] and is typically extracted from silkworm chrysalis [68]. Silk fibroin can be easily processed in water or other solvents to form hydrogels, fibres, films or sponges [69]. The degradation rate of silk-based scaffolds can be adjusted from few months to up to 1 year by modifying processing parameters, such as the solvent, fibroin concentration and pore size [70].

Silk fibroin has been used for producing blood vessels with successful attachment and ingrowth of both smooth muscle and endothelial cells [71, 72]. Indeed, electrospun silk fibroin grafts have been demonstrated to be an encouraging strategy to repair small blood vessels [73], as they promote endothelial cell and smooth muscle cells infiltration and the development of an elastic lamina on the internal surface of scaffolds in a short term [74]. In addition, silk electrospun films have been studied as potential candidates for wound healing dressings due to their protective capacity against dehydration and microorganisms and their remarkable mechanical properties [75–77]. Clinical data concerning silk, that is mainly used for suturing, suggest that silk is well tolerated at short term with few cases of hypersensitivity associated with sericin residues [69]. However long-term immune response to this non-mammalian derived material is still unknown [69].

To date, a few different fabrication techniques have been established to produce fibrous scaffolds. These techniques include electrospinning, self-assembling and freeze-drying.

Electrospinning is a fabrication method to produce nonwoven films or conduits with high interconnected porosity and controlled fibre diameter in the range of few nanometres to microns [78]; however, distance between fibres cannot be controlled [75]. Electrospinning is a versatile and high cost-efficient technique to mimic natural and fibrillar structures of native tissues [3]. Additionally, electrospun materials exhibit a high surface to mass ratio, which increases the availability of material motifs and the potential effect on cell response. Therefore, it is advocated as a strong technique to produce fibrous scaffolds for several different tissue regeneration applications, for instance neural [79], tendon [80], cornea [81], wound healing [12] and cardiovascular [9] applications.

The basic set-up of electrospinning includes a spinneret (e.g. a hypodermic needle with blunt tip), a syringe pump for ejecting the polymer solution, a power supply and a collector. The polymer needs to be dissolved in a highly volatile solvent. During the extrusion, an electric field gradient is applied between the metallic spinneret and the target collector. This electric gradient induces electrostatic forces that form a jet, accelerate it toward the metallic collector and promote solvent evaporation. As a result, a fibre with electric charge is deposited onto the collector. Fibre orientation can be obtained using rotating drum at different speeds [3, 78, 82]. The modulation of several parameters allows the control over fibre diameter and alignment. Fibre diameter is controlled by concentration [83], voltage [83], spinneret diameter [83], extrusion rate [84] and distance between spinneret and collector [84]. Furthermore, porosity and pore shape have been recently controlled by collectors having a predefined architecture [85]. Although the influence of process parameters has been widely studied, it is still difficult to predict the ease of electrospinning for each material and the resulting fibre characteristics, such as diameter, porosity and stiffness. Moreover, for some materials, it is difficult to obtain fibres displaying an uniform diameter and not presenting globules [69].

The current trend in electrospun fibrous scaffolds is to mimic the fibre size of native tissues [6], like collagen fibrils, which have a fibre diameter of 10–500 nm [18]. Furthermore, fibre orientation has been shown to affect the outcome of the treatment. In tendon repair, parallel-aligned fibres induced in vitro differentiation of tendon progenitor cells into tenocytes and promoted tendon-like tissue in vivo, while random fibres induced mineralisation [86]. Also, parallel-aligned fibre films promoted peripheral nerve regeneration in rats [87]. On the other hand, radially aligned fibres have been employed to generate dura mater substitutes [88], while random fibres have been mainly used for wound healing [12]. Even though the response to electrospun materials has been widely investigated in vitro, the host response in vivo is not yet well defined [69].

Self-assembly is defined as the spontaneous organisation of disordered components into patterns or structures, without external intervention [89]. It usually involves the formation of non-covalent interactions such as van der Waals forces, hydrogen bonds, electrostatic forces and hydrophobic interactions [89]. Some

natural occurring examples are collagen and phospholipids. An aqueous solution of atelocollagen, which is collagen solubilised by proteases, self-assembles into pro-collagen and forms fibrils [90]. By taking advantage of this phenomenon, several collagen forms can be produced, such as hydrogels, single thick fibres, bundles of thick fibres and aligned collagen fibrils. Single collagen fibres can be obtained when collagen solutions are extruded and incubated in polyethylene glycol buffer; this method allows for the production of fibres with diameter ranging between 1 and 300 μm, which imitate the structure of native tendon [91]. Also, bundles of collagen fibres have been used for axonal guidance in nerve repair [92]. Furthermore, in order to control neurite outgrowth, collagen fibrils have been aligned using isoelectric focusing principle [93]. Self-assembled scaffolds can present reproducibility issues due to the sensitivity of self-assembling process to several variables, such as protein purity and concentration, pH, ionic strength and temperature. For example, the self-assembling rate, fibrils structure and isoelectric point of collagen type I are directly modulated by pH [94]. The shape of the scaffold can be controlled by using a mould, and some innovative forms, such as core-shell fibres, have been used as stem cell delivery system and defect-adjustable tissue engineering system [95–98].

Also synthetic peptides have shown self-assembling properties. For instance, the peptide amphiphile molecules are composed by a hydrophobic alkylic tail and a hydrophilic peptidic head and they can self-assemble into hydrogels in response to temperature or pH changes [99, 100]. Peptides containing an alternation of polar and non-polar residues, which form a double-beta sheet structure in water, are another example of self-assembling. These peptides, such as RADA16-I, have been widely used for regenerative medicine purposes [101, 102]. Scaffolds formed by self-assembling sequences can mimick the structure of ECM; however, specific functional sequences can be added to improve cells adhesion or control cell behaviour, for example RGD [103], IKVAV [104] or BMHP1 (Bone Marrow Homing Peptide) [103]. Despite low mechanical properties restrict the use of self-assembling peptides to treat small tissue defects, they have been successfully employed for spinal cord [105, 106], bone [100], cartilage [107] or skin [108] repair in animal models.

Freeze-drying processing, also known as lyophilisation or phase separation, is a simple dehydratation method for producing porous scaffolds. The material is frozen in order to induce the formation of ice crystals, and then ice is removed by sublimation, creating a 3D porous network. With respect to the previous described fabrication methods, some advantages of freeze-drying are reproducibility and the possibility to obtain several engineered shapes, as this technique uses moulds for processing. Similarly to electrospinning and self-assembling, freeze-dried scaffolds display low mechanical properties. These materials can be crosslinked to increase enzymatic resistance, while mechanical properties are slightly improved [18].

Freeze-dried fibrous scaffolds show layers between 50 and 500 nm, pore sizes over 50 μm, high porosity and interconnected pores, which can enhance cell and tissue infiltration [109, 110]. The modulation of temperature gradients during freezing allows the control of pore direction and pore size [111]. Freeze-dried

materials exhibit higher adsorption of protein when compared to bulk/solid materials; this property is related to the high surface to mass ratio. Due to the benefits of this fabrication method, freeze-dried scaffolds have been widely used in tissue engineering, including wound healing [112], spinal cord [113], nerve [114], cartilage [115] and tendon [116] repair. For example, freeze-drying was used to produce sponges with aligned channels that guided linear axonal growth after spinal cord injury [110, 113]. Freeze-dried collagen sponges with oriented channels supported axonal regeneration also in peripheral nerve injury [114]. Moreover, functionalisation of freeze-dried multichannel collagen conduits has been shown to improve nerve repair by increasing aligned axonal regeneration [117]. In the same way, aligned collagen sponges have been used to stimulate tenocytes infiltration and orientation [118, 119]. In order to improve scaffold stability, collagen sponges were reinforced with silk and the resulting sponges supported tendon repair over 18 months [116]. Collagen type II sponges promoted in vivo cartilage formation: chondrocytes infiltrated the sponge and formed new cartilage in 6 weeks [115].

In conclusion, fibrous scaffolds provide structural, biomechanical and biochemical support to cells and new tissue formation; therefore, fibrous scaffolds are a suitable clinical strategy to enhance functional reconstruction of tissues and organs. These materials have the advantage of mimicking ECM characteristics, and their properties can be adapted using different biomaterials and processing techniques. Although results are encouraging, further research is needed to overtake some limitations of each material and technique, for example immunogenicity of biopolymers, reproducibility of some fabrication methods or low mechanical properties of reconstituted scaffolds. Future investigations should evaluate how to scale up extraction of biopolymers and scaffold fabrication, controlling homogeneity between batches. Further research is also required to correlate scaffold characteristics and biological response.

3D Drug Releasing Scaffolds

Advances in proteomics and genomics have led to the identification of several biomacromolecules with therapeutic relevance such as proteins, antibodies, peptides, plasmid DNA and small interfering RNA. This has prompted extensive efforts to develop efficient in vivo drug delivery systems (Table 5.2). However, the influence exerted by the microenvironment in which the cells reside remains underappreciated. The microenvironment includes the ECM and the complex array of cues that actively participate in the crosstalk between cells and the ECM. The ECM, in fact, is not only responsible for maintaining the integrity of the tissues and allowing diffusion of nutrients, but also it provides instructive cues that influence cell behaviour, dictating cell ability to proliferate, differentiate and to produce matrix [122–127]. Also, ECM acts as a natural reservoir of growth factors and other bioactive molecules [128–131].

Table 5.2 Examples of strategies for controlled drug delivery from 3D scaffolds

Bio-molecule	Material	Type of strategy	Outcome	References
VEGF	Type I collagen	Covalent incorporation	The covalent incorporation of VEGF enhanced the angiogenic capabilities of the collagen matrices	[152]
VEGF	Fibrin	Immobilization, use of enzymatically labile sequences	The immobilization of VEGF within the scaffold allowed cell-demanded release with increased formation of new blood vessels with normal morphological appearance	[154]
VEGF	PEG	Covalent incorporation	The subcutaneous implantation of VEGF-containing scaffolds in rats resulted in remodelling of the scaffold and formation of native, vascularised tissue	[156]
bFGF	Type I collagen	Immobilization	Scaffolds in which heparin was immobilized showed increased vascularisation for periods up to 3 weeks in vivo	[164]
SDF-1α	Type I collagen	Immobilization	Scaffolds containing immobilized heparin to trap the stem cell chemo-attractant SDF-1α were implanted subcutaneously in mice. Only few progenitor cells were recruited early after implantation	[166]
BMP-7	PLLA	Particles reservoir system	Scaffolds containing BMP-7-loaded nanospheres allowed for a prolonged maintenance of therapeutic activity of BMP-7 and resulted in improved bone formation in rats	[181]
BMP-7 expressing adenovirus	Fibrin or collagen	Embedding	In vitro tests showed that adenoviruses maintained their therapeutic efficacy for longer when embedded in fibrin gels. In vivo, bone formation was observed in animals implanted with fibrin or collagen virus-loaded gels	[203]

(continued)

Table 5.2 (continued)

Bio-molecule	Material	Type of strategy	Outcome	References
Plasmids	HA/PEG	Controlled degradation	Plasmids were stably incorporated into hydrogels with different HA/PEG content. The cumulative release increased with a decreasing PEG or increasing HA content. The study demonstrated the dependence of plasmid release on the physical properties of the hydrogel	[170]
Radiologic tracer	β-peptides	Enhanced water diffusion	Supramolecular hydrogels were developed by using β-peptides and loaded with a tracer. For 12 h post-injection in rats, the tracer was released from the hydrogel in a uniform and sustained manner	[177]
Lactate	PLDLLA	Controlled degradation	The scaffold was designed with a high degradation rate in order to release lactate. 1 year after implantation into mouse brain, the scaffold induced robust and functional vascularisation and neurogenesis	[178]
IGF-1	Bone marrow aspirate	Adenoviral gene transfer	Bone marrow aspirates were successfully transduced ex vivo. 80% of the cells expressed the transgene over 21 days of culture	[208]

Even though diseases and trauma are often associated with alterations in the structure and properties of ECM, conventional drug delivery approaches focused on restoring cell function without repairing the microenvironment that surrounds the targeted cells [132–134]. This uncontrolled drug delivery often results in poor therapeutic effect or in severe side effects. An altered microenvironment can affect the absorption and retention of the drug into the tissue. Moreover, a hostile microenvironment can accelerate the degradation or denaturation of the bioactive molecule, resulting in a poor outcome of the therapy. In fact, the pharmacokinetics in the host tissue is often characterised by an initial excess followed by a sharp decrease [135–137]. In order to counterbalance this transient effect, biomacromolecules are often delivered at high doses, which can lead to severe side effects, as reported in both animal and clinical studies [138, 139].

Due to these limitations, current research focused on developing 3D drug releasing scaffolds which can mimic ECM and can also restore the altered microenvironment [140, 141]. Scaffolds were found not only to preserve and

prolong the biological activity of biomacromolecules but also to allow controlled drug release according to the needs of the targeted tissue [142, 143]. Furthermore, by using ECM-derived biomacromolecules as building blocks, scaffolds can not only mimic the 3D architecture of the in vivo microenvironment but also influence and control cell behaviour. These biomimetic scaffolds take advantage of the body's inherent ability to heal by guiding the repair process with the delivery of selected bioactive molecules [144–149]. The major challenge when designing ECM-based scaffolds is the identification of the appropriate combination of signals and their spatio-temporal organization to enable formation of functional tissue [143].

An example of such biomimetic approach is given by the delivery of vascular endothelial growth factor (VEGF) in therapies aiming at increasing tissue angiogenesis for the treatment of critical limb ischeamia or simply to increase the formation of new blood vessels within scaffold implants [150–153]. A common problem found in such therapies is the production of malformed and leaky blood vessels as a result of uncontrolled, often excessive, release of VEGF [154]. Therefore, VEGF release needs to be tightly controlled [139, 155]. In the body, VEGF is bound to ECM components until it is released by the cellular enzymatic activity. In an attempt to mimic this phenomenon, Ehrbar et al. used a variant form of VEGF containing the sequence NQEQVSPL, which is a substrate for the transglutaminase FXIII [154]. This strategy mimics the transglutaminase FXIII-mediated incorporation of a α_2-plasmin inhibitor in the blood clot during coagulation. When the modified VEGF was mixed with transglutaminase FXIII, fibrin and thrombin, VEGF was immobilized in the resulting fibrin network. This elegant approach not only allowed the maintenance of VEGF activity for a prolonged period but also it enabled VEGF release according to the spatial and temporal demand of the tissue, leading to formation of morphologically intact blood vessels [151, 154, 156–159]. In this process, which is known as 'dynamic reciprocity', the cells and the surrounding scaffold mutually influenced each other, resulting in scaffold remodelling and progressive release of the immobilized growth factor.

In general, encapsulation of growth factors in enzymatically labile natural hydrogels such as matrigel, fibrin or collagen, enhances their effect on target cells. For example, this was seen in fibrin matrices, where the incorporation of VEGF facilitated the formation of capillary sprouts [151, 156]. The immobilization of heparin to collagen matrices, crosslinked using *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), has been successfully employed for binding and release of growth factors from 3D matrices [160]. These matrices were employed to trap and deliver VEGF [161–163], fibroblast growth factor-2 (FGF-2) [160, 164, 165] and stromal cell-delivered factor-1 (SDF-1α) [166].

Although the sustained delivery of tethered growth factors resulted in an enhanced and prolonged response in vivo [167], 3D scaffolds can be also successfully functionalized by harnessing the affinity between growth factors and ECM components [168]. Since the release of non-immobilized growth factors is not solely dependent on proteolytic activity, these systems offer a tuneable release pattern [169]. For example, it is possible, particularly when using hydrogel systems,

to obtain good control over drug release profile by simply varying scaffold properties such as pore size or swelling ratio, which can be easily modulated by changing scaffold hydrophilicity, crosslinker molecular weight or the extent of crosslinking [170, 171]. For instance, an increase in pore size can favour cell infiltration and proteolytic degradation by immune cells, which in turn accelerates drug release; also, an increase of scaffold hydrophilicity can enhance drug delivery by accelerating bulk degradation via hydrolysis [102]; for example, the degradability of PEG hydrogels can be controlled by varying the length of hydrolytically degradable units within the polymer crosslink [172].

However, a scaffold with controlled biodegradation must degrade into non-toxic products [173], and the method chosen to control the degradation rate should not affect the mechanical properties, in particular the stiffness, of the scaffold. A good example is the approach proposed by Kong and colleagues that aimed at accelerating the degradation rate of alginate hydrogels without affecting their stiffness. Alginate degradation can be accelerated by increasing its oxidation degree; however, it also results in softer gels. To overcome this issue, they adjusted the molecular weight distribution (MWD) of oxidised alginates without varying the number of oxidised uronic acids, thus achieving both regulation of the degradation rate and maintenance of the elastic modulus [174].

Nonetheless, changes in the degradation rate of hydrogels can affect their ability to retain and transport fluids. This issue is particularly important when hydrogels are used as cell carrier, which requires controlled pore size to allow the exchange of nutrients and the release of their paracrine products. Kang et al. [175] developed a 3D hybrid scaffold in which a 3D framework and a hydrogel were combined to enhance the mechanical properties without chemically altering the transport properties of the hydrogel. This system consisted in the injection of a mixture of dopamine-releasing cells and an alginate gel into the internal space of a 3D framework of Ormocomp®. Release studies revealed that the encapsulated cells were able to secrete dopamine for over 8 weeks in vitro and the introduction of RGD peptides into the alginate hydrogel increased the release rate. Moreover, the performance of the system was tested in a mouse model: the subcutaneous implantation of the hybrid scaffold mixed with encapsulated cells showed no signs of immune rejection after 7 days, indicating that the system shielded the exogenous cells from the host immune system while enabling cells to fulfil their paracrine function [175].

Another innovation in the field of 3D drug-releasing scaffolds is the development of supramolecular hydrogels. Conventional hydrogels have a less flexible pore size due to the presence of covalent bonds, thereby limiting the responsiveness to the surrounding environment [176]. Supramolecular hydrogels instead are formed by small molecule hydrogelators that self-assemble into 3D networks of nanofibers. These hydrogels show the ability to imbibe large amounts of water, and also to change their pore size as a result of the reassembly of the hydrogelators during the shrinkage/swelling processes [176]. These properties favour the diffusion of molecules from the delivery system in response to microenvironmental changes. Liang et al. tested the use of supramolecular hydrogels for controlled drug release

in vivo. The hydrogel was developed by using β-peptides composed of unnatural amino acids that act as hydrogelator [177]. As a proof of concept, the hydrogel was loaded with radioactive tracers and implanted in a rat subcutaneous model. The release of the tracer was tracked for 12 h post-injection. Single photon emission computed tomography (SPECT) images revealed that the blood concentration of the tracer was maintained in a narrow range during this period, indicating a uniform and sustained release from the hydrogel [177].

In an in vivo study [178], a poly(L-lactide-*co*-D,L-lactide) (PLDLLA) scaffold was designed with a high degradation rate in order to release lactate, a common cellular cue that induces angiogenesis [179] and supports neuronal progenitor metabolism [180]. These scaffolds were also designed with a controlled topography to mimic the 3D organization of radial glia. Following implantation of these cell-free scaffolds into a mouse model of injured brain, the animals were monitored over 15 months. The radial scaffolds induced robust and functional vascularisation and neurogenesis for more than 1 year due to the delivery of appropriate metabolic cues, the release of lactate and the controlled biophysical environment [178].

Immobilization or absorption of growth factors on 3D scaffolds can undoubtedly increase their biological activity. However, this is generally a short-/medium-term effect as growth factors have a short half-life (in the range of hours/days). If the therapeutic strategy needs a prolonged delivery of the bioactive proteins, it is necessary to provide a protective environment to maintain their biological activity. For instance, poly(L-lactic acid) (PLLA) scaffolds were functionalized by adsorption of bone morphogenetic protein-7 (BMP-7) or by immobilization of BMP-7-loaded nanospheres of poly(lactic-*co*-glycolic acid)(PLGA) [181] and they were implanted in rats. In PLLA scaffolds loaded with adsorbed BMP-7, a failure in bone induction was observed, whereas in scaffolds loaded with immobilized BMP-7-loaded-PLGA nanospheres, new bone formation was found throughout the scaffold, suggesting that nanospheres shielded BMP-7 from denaturation/degradation and released it in a sustained manner, thus allowing osteoblast cells to synthesize functional matrix [181].

Although the encapsulation of growth factors can prolong their effect in vivo, their intrinsic short half-life still limits potential applications, because high doses or repeated administrations are required to obtain the desired effect [182]. These drawbacks can be overcome through the delivery of genetic material to the target tissue, in order to exploit the cell machinery to produce therapeutic proteins in a controlled and sustained manner [124, 183, 184]. Gene therapy is a powerful approach that can stimulate local production of proteins capable of activating autocrine and paracrine loops that influence tissue development and repair [185]. Hence, 3D scaffolds have been tailored to improve the outcome of gene delivery. To increase the cell proliferation rate, which is necessary to allow genetic material to enter the cell nucleus, gene therapy has been associated with delivery of growth factors. For example, the co-delivery of basic fibroblast growth factor (bFGF) and fibroblast cells in a PLGA scaffold was found to enhance fibroblasts proliferation and indirectly, to increase the nucleotide uptake rate with a resulting increase in the levels and duration of transgene expression [124, 186].

The functionalization of the scaffold with cell adhesion molecules was also found to influence the outcome of gene delivery [187]. For this reason, the first scaffold that functioned as gene delivery vehicle was made of collagen, a RGD-rich biomacromolecule [188]. Such collagen scaffolds have been employed for the delivery of both viral [189] and non-viral vectors [190, 191]. With these approaches, it was shown that it is possible to induce transgene expression in vivo which led to tissue repair in a wide range of applications such as bone regeneration [188, 192], wound healing [193–196], muscle repair [197] and optic nerve repair [198]. Other natural polymer-based hydrogels were also used as a platform for gene therapy. For instance, alginate, which is inert to cell adhesion and protein adsorption [199], was functionalized with RGD peptides. The spatial distribution of these cell adhesion peptides influenced the rate of DNA uptake by pre-osteoblasts cells, and RGD-functionalised alginate hydrogels showed the highest transgene expression [187, 200].

3D scaffolds were also found to prolong the half-life of viral vectors and to shield them from the host immune response [201]. Furthermore, delivery of the vector from the scaffold has the advantage of concentrating the transgene expression in the implant site [143]. The method used to functionalise the scaffold with the gene vectors can also determine the gene release profile and the duration of their biological activity [143]. For example, Schek et al. compared in vitro the bioactivity of adenoviruses embedded in fibrin hydrogels or simply resuspended in liquid form. When in solution, the adenoviruses decreased to half-maximal activity after only 15 h, whereas when embedded in fibrin hydrogels the half-life of the adenoviruses was extended to 45 h [201]. The bioactivity of adenoviruses was tested also in mice. In this study, adenoviruses bearing the gene for BMP-7 were implanted intramuscularly: the suspended form of adenoviruses induced bone formation in 50% of the muscles, whereas the efficacy of adenoviruses embedded in fibrin hydrogels was much higher, with bone formation occurring in 80% of the muscles treated [201]. A drawback in the use of viral vectors for gene therapy is the potential damage they can cause when they leak into nearby tissues. Pascher et al. compared the delivery of viral vectors through collagen/glycosaminoglycan matrices or via bone marrow clots (BMC) in a rabbit model. Interestingly, BMC showed superior containment of the vector within the osteochondral defects. Moreover, high levels of transgenic expression and deposition of healthy matrix were observed within the clots [202]. Another interesting aspect of this approach is the use of autologous-derived materials that can overcome the issues related to the manufacturing process and biocompatibility of scaffolds.

To immobilize gene vectors in biomaterials, a number of techniques mimicking the binding of viruses to ECM proteins have been developed. For instance, by using the electrostatic interactions that occur between nucleotides and calcium phosphate (CaP), plasmids (pDNA) can be complexed into a nanocrystalline form and immobilized onto a surface [203]. These complexes have been extensively used as gene vectors for the functionalisation of scaffolds for bone tissue engineering [204, 205].

There are alternatives to viral vectors for gene therapy. Cationic polymers or lipids are often used as complexing agents to condense pDNA to form small particles. These complexes can penetrate the cell membrane and deliver the genetic material intracellularly. Nonetheless, this approach has lower efficacy than viral vectors and higher concentrations of complexes are generally required to obtain substantial biological effects. This can often result in significant toxicity in vitro and in vivo [206]. The use of reservoir systems, such as microspheres and nanoparticles, in collagen hydrogels was found to significantly decrease the toxicity of complexed pDNA [191]. Generally, pDNA delivery is more effective in actively dividing cells as during mitosis the nucleus disassembles temporarily, facilitating the nuclear localisation of the transgene in the newly formed cells. However, tissues that most often are the target of gene therapy are harsh environments in which cell proliferation is often impaired. For this reason, recent research is shifting toward mRNA delivery, as mRNA does not need to enter the nucleus to be expressed, and moreover, its structure can be modified to considerably increase its stability and therapeutic efficacy [207].

Overall, advances in stem cell biology and biomaterial science provide exciting opportunities for tissue engineering. Biomimetic scaffolds can be rationally designed to take advantage of the body's inherent healing capacity and, by incorporating appropriate signals, to use the body as a bioreactor and guide the healing process. However, a more in-depth knowledge of the role of ECM in growth factor binding/release and cell signalling will lead to more effective implants. One of the major challenges is presenting the appropriate combination of signals in the appropriate spatial and temporal patterns. As discussed above, the release pattern can be controlled by modulating the degradation rate and structure of the scaffold. Other options are the use of supramolecular hydrogels, which provide a rapid and uniform release, or the covalent bond of growth factors, which allows a gradual release. As growth factors present a very short half-life, the incorporation into 3D scaffolds of genetically engineered cells or gene vectors could offer a prolonged production in situ. However, improvements in transfection efficiency and biocompatibility of non-viral gene vectors will likely enable a wider adoption of gene therapy in the clinic. Furthermore, a successful tissue engineering strategy will depend on the development of scaffolds able to influence cell behaviour. Hence, the integration of on-demand drug delivery systems with instructive matrices is the future direction of 3D releasing scaffolds in tissue engineering.

Fibrinogen-Based Scaffolds

Fibrinogen and fibrin are attractive polymers for tissue engineering applications because they can provide a natural environment and they have intrinsic healing properties.

Fibrinogen is a 340 kDa plasma glycoprotein involved in formation of blood clots [209]. The molecule is composed of two sets of disulphide-bridged Aα-, Bβ-

and γ-chains. In response to damage to the vascular system, fibrinogen is converted to fibrin by the thrombin-mediated cleavage of fibrinopeptide A from the Aα-chains and fibrinopeptide B from the Bβ-chains, thus initiating fibrin polymerization [210]. Fibrin monomers then self-assemble and form an insoluble fibrin network, which is further stabilized by the factor XIIIa (plasma transglutaminase) that covalently crosslinks the C-terminal of fibrinogen γ-chains. This natural process can be reproduced in vitro by mixing together fibrinogen and thrombin in the presence of calcium ions [209]; then, fibrin gel can be reinforced by introducing a chemical crosslinker, such as genipin [211]. Not only the formation, but also the degradation rate of fibrin gels can be controlled in vitro and in vivo, by addition of fibrinolytic inhibitors. Moreover, fibrin scaffolds can be designed to control the release of growth factors after implantation in response to externally applied stimuli. For example, ultrasound has been used to actively control growth factors release, architecture, and stiffness in a fibrin scaffold doped with sonosensitive emulsion [212]. All these characteristics make fibrin a suitable biomaterial for controlled delivery of genes and biomolecules [213, 214].

In tissue engineering, fibrinogen and fibrin present many other advantages. Since they are naturally designed to provide the temporary scaffold required to support healing and revascularization [215], they can be used as an initial structural support for regenerating tissues. Moreover fibrinogen contains RGD sequences for cell attachment and binding sites for bioactive molecules, such as VEGF, fibroblast growth factor-2 (FGF-2) and interleukin-1 (IL-1) [216].

Furthermore, by varying the fabrication method, fibrinogen and fibrin can be easily shaped into a variety of scaffolds, such as hydrogels, nanofibrous films and microporous matrices, which can be used in combination with cells and biomolecules in a wide range of tissue engineering applications (Table 5.3). At last but not least, fibrinogen and thrombin can be extracted from autologous plasma. The use of autologous fibrin greatly reduces the risk of immune reaction and infection, as fibrin sealants produced from allogenic pooled plasma—although they are available in standardised quality—can still have severe adverse effects, such as hypersensitivity, anaphylactic reaction and transmission of bacteria, viruses and prions [217]. Other issues related to commercial fibrins are the high concentration of fibrinogen, making the fibrin scaffold too dense for cell proliferation and migration and the methods for fibrinogen concentration which can alter the nature of the fibrin. By contrast, an autologous fibrin scaffold has a physiological concentration of fibrinogen, thus providing an appropriate matrix for cell ingrowth [218]. Of note, different fibrin formulations can be used to support different cell functions [219]. For instance, formulations containing a low concentration of fibrinogen supported human mesenchymal stem cells (MSC) growth, while a higher concentration increased their osteogenic differentiation potential [219, 220].

Hereinafter, the fabrication methods of various fibrinogen- and fibrin-based scaffolds and their application in tissue engineering are discussed.

Fibrin hydrogels are produced by mixing fibrinogen with thrombin solution in the presence of calcium ions. Although they have been used in several tissue engineering applications, they present the disadvantage of having low mechanical

Table 5.3 Examples of in vitro and in vivo applications of fibrinogen- and fibrin-based scaffolds

Material	Formulation	Tissue engineering application	Outcome	References
Fibrin/ fibrinogen	Hydrogel	Spinal cord repair	The fibrin/fibrinogen hydrogel integrated with the host spinal cord tissue and supported robust growth of axon	[229]
Fibrin	Hydrogel	Spinal cord repair	Implants of fibrin containing neurotrophic factors enhanced axonal regeneration into transected rat spinal cord	[236]
Fibrin crosslinked with EDC	Hydrogel	Spinal cord repair	Implants of fibrin and MSCs resulted in the formation of longitudinally-aligned neurites	[237]
Platelet-rich fibrin	Glue	Bone repair	Glue mixed with MSCs and BMP-2 induced bone tissue formation in dental implants in dogs	[238]
Fibrin	Hydrogel	Cartilage repair	A significant improvement in knee function was recorded in four patients implanted with fibrin glue and autologous chondrocytes. The graft well integrated with host tissue	[241]
Fibrin	Glue	Wound healing	Fibrin glue combined with fibroblasts and growth factors enhanced wound healing in rabbits	[259]
Fibrin	Glue	Wound healing and vasculogenesis	In vitro, human umbilical vein endothelial cells proliferated and organised themselves into capillary-like structures within the fibrin matrix. The skin substitute showed similar structure to native skin	[247]
Fibrinogen	Electrospun scaffolds	Wound healing	In an in vitro model, fibroblasts migrated into and remodelled the electrospun fibrinogen scaffold with deposition of collagen	[249]

(continued)

Table 5.3 (continued)

Material	Formulation	Tissue engineering application	Outcome	References
Haemoglobin/ gelatin/ fibrinogen crosslinked with phytic acid	Electrospun scaffold	Myocardial repair	The scaffold supported MSC differentiation into functional cardiomyocytes and showed an improved delivery of oxygen	[251]
Fibrin	Electrospun film	Tissue engineering of soft tissues	In vitro, the scaffold supported the attachment, spreading, and proliferation of human umbilical cord blood-derived MSCs. The nanostructure showed the fibre diameter of native ECM	[252]
Fibrin	Bio-printed channels	Microvasculature tissue engineering	The printed endothelial cells proliferated to form a tubular structure inside the fibrin channel	[254]
Fibrin	Fibrin-coated substrate	Orthopaedic tissue engineering	Fibrin can be an ideal bioprinting substrate for numerous growth factors. BMP-2 immobilized to fibrin induced spatially defined muscle-derived stem cell differentiation toward the osteogenic and myogenic lineages	[256]

strength and rapid degradation. Indeed, fibrin and fibrinogen scaffolds lose their structure within a few days through degradation by proteases in vitro and in vivo [209]. To overcome these limitations, fibrin and fibrinogen hydrogels can be (1) blended with synthetic or natural polymers, (2) crosslinked with different chemical and biological agents or (3) functionalized with plasmin inhibitors.

Examples of materials used to achieve adequate mechanical strength are polyurethane (PU), poly(ε-caprolactone) (PCL) and polyethylene glycol (PEG). For instance, a composite construct made from the commercially available fibrin and PU-based scaffold exhibited increased stability in vitro and in vivo as compared to fibrin gels alone: in vitro, the fibrin/PU scaffold was seeded with human adipose-derived stem cells and was shown to be able to maintain its size and weight for 21 days; in vivo, the implantation of fibrin/PU construct resulted in formation of well-vascularized adipose tissue [221]. The same research group used the fibrin/PU scaffold also for cartilage tissue engineering applications [222].

PEG is another material commonly used to increase fibrin stability. Several studies carried out by Seliktar and colleagues showed the advantages of using a hybrid scaffold composed of a fibrinogen backbone and crosslinked with PEG. To include cells in this hydrogel, fibrinogen fragments were PEGylated with PEG-diacrylates, they were mixed with the photoinitiator and cell suspension and finally they were exposed to UV light to form a hydrogel. The mechanical properties were modulated by varying the percent polymeric composition, while the fibrinogen backbone provided the motifs for cell attachment and proteolytic degradation [223]. Further modifications of the PEGylated fibrinogen scaffold, such as blending with a PEGylated albumin hydrogel [224] or incorporation of Pluronic®F127 micelles [225], lead to the development of composite hydrogels for 3D cell culture and controlled drug release. Overall these studies showed that the addition of synthetic materials can improve the control over mechanical stiffness of fibrinogen or fibrin hydrogels. On the other hand, fibrinogen can be used to functionalise synthetic hydrogels. Thrombin and fibrinogen were loaded within PEG hydrogels and the resulting fibrin-loaded hydrogel induced higher vascularisation in vivo in comparison to PEG hydrogels without fibrin [226].

Fibrin and fibrinogen can be also combined with natural materials, such as collagen, alginate and hyaluronic acid, in order to achieve a synergistic effect on cell behaviour. This strategy offers also the possibility to tune the stiffness and degradation rate of the hydrogel. A recent study showed that the degree of vessel-like structure formation was dependent on the collagen/fibrin proportion in a composite hydrogel seeded with endothelial cells, revealing a correlation between matrix stiffness and the degree of vasculogenesis [227]. Furthermore, hyaluronic acid (HA) has been incorporated within fibrin hydrogels to provide structural reinforcement to the fibrin network, as demonstrated by the improved stability in the presence of plasmin and cell-contractile forces of a HA-fibrin hydrogel [228]. An interesting study compared the effect of four injectable hydrogels made of collagen, fibronectin, fibrin and a blend of fibrin/fibronectin in the injured spinal cord of rats: collagen implants resulted in dense inclusions, whereas fibronectin and fibrin well integrated with the host tissue and enhanced neurite elongation; however, the best results in terms of biocompatibility, cell infiltration and axonal growth were displayed by the blend fibrin/fibronectin [229].

As previously mentioned, another strategy to maintain the size and shape of hydrogels for prolonged periods is crosslinking. By varying the concentration of crosslinkers, it is also possible to control the degradation rate. Commonly used crosslinking agents for natural materials are glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and genipin.

In comparison to glutaraldehyde and EDC, genipin presents the advantage to be a naturally occurring compound with a very low level of cytotoxicity. A genipin crosslinked human fibrin hydrogel system was seeded with human articular chondrocytes and implanted subcutaneously into rats, showing promising applications as an autologous scaffold for articular cartilage regeneration, indeed genipin crosslinking improved mechanical strength of the scaffold, enhanced ECM production and did not significantly affect cell viability [211].

The third approach to increase the stability of fibrin- and fibrinogen-based scaffolds is reducing the proteolytic degradation of fibrin by using degradation inhibitors and their stabilizers, such as factor XIII, aprotinin and ε-amino-*n*-caproic acid along with sufficient amounts of thrombin and calcium [209]. However, aprotinin rapidly diffuses out of fibrin matrices and thus do not provide extended matrix protection. A recent study reported that an engineered aprotinin variant can be immobilized within fibrin, then providing extended longevity. The recombinant aprotinin contained the transglutaminase substrate sequence, which allowed to covalently crosslink the aprotinin variant into fibrin matrices during thrombin- and factor XIIIa-mediated polymerisation. Subcutaneously implanted matrices containing this aprotinin variant were detectable in mice for more than twice as long as those containing the wild-type protein [230].

In order to enhance tissue regeneration or replace a damaged organ, cells and biomolecules can be incorporated into fibrin hydrogels. Many research efforts have focused on utilising fibrin hydrogels as cell-supporting scaffolds in tissue engineering of adipose, cardiovascular, ocular, liver, skin, neural, cartilage, tendon and bone tissues.

Recently, a number of preclinical studies have suggested that fibrin patches seeded with cells hold promise for regenerating post-infarcted myocardial tissue [231]. Overall, these studies showed that the fibrin patches could keep cells at the site of injury and improve cardiac function, and they could also reduce the infarct size. In contrast, implanted cells rarely moved out of the patch, emphasising the need for a strategy that could enhance also cell migration toward the site of injury. Of note, the fibrin patches alone, without cells, were able to enhance vascular growth [231]. The beneficial effect of fibrin patches was further improved by incorporation of growth factors, such as IGF-1. For example, a fibrin patch loaded with IGF-encapsulated microspheres and three different types of cells (cardiomyocytes, endothelial cells and smooth muscle cells) derived from human induced pluripotent stem cells were tested in a porcine model of acute myocardial infarction. The tri-lineage cell transplantation combined with IGF-1 significantly improved left ventricular function, myocardial metabolism, and vessel density, while reducing infarct size [232].

In the field of neural tissue engineering, fibrin hydrogels containing growth factors have been demonstrated to enhance axonal growth and cell infiltration in short-gap nerve injuries [233–235]. Implants of fibrin containing neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF) or ciliary neurotrophic factor (CNTF) enhanced axonal regeneration into transected rat spinal cord, whereas only a few regenerated axons were found into the implants that lacked neurotrophic factors [236]. In another study, a novel formulation of fibrin crosslinked with EDC was used to deliver MSCs to the injured spinal cord. The use of the fibrin scaffold resulted in the formation of longitudinally aligned layers of MSCs and in the longitudinally growth of host neurites into this architecture [237].

In bone tissue engineering, fibrin scaffolds have been widely used because of their good adhesive and heamostatic properties. Fibrin glue is often used as beads and microbeads, coating agents on other scaffolds, pre-formed scaffolds, or

injectable hydrogels [219]. In particular, platelet-rich fibrin glue is a promising autologous scaffold for bone repair because platelets release growth factors and bioactive proteins that initiate and accelerate tissue repair. Platelet-rich fibrin glue mixed with bone marrow MSCs and bone morphogenic protein-2 (BMP-2) induced bone tissue formation in dental implants in dogs [238]. However, the role of platelets in the repair of bone is poorly understood and it needs to be further studied. Another issue that deserves further investigation is the addition of other materials, such as osteoconductive ceramics, because it has been shown that fibrin alone is incapable of healing bone defects [232]. In the field of dental pulp tissue engineering, fibrin appeared to be the most suitable material to support viability of dental pulp stem cells and tissue formation among other natural (collagen) and synthetic (PEG-derived scaffolds) materials [239]. Platelet-rich fibrin scaffolds loaded with MSCs have been also employed in cartilage tissue engineering, for instance they have been used in a clinical trial to deliver autologous bone marrow MSCs into patients with full-thickness cartilage defects, and all patients experienced an improvement of symptoms [240]. Fibrin hydrogels have been also used as chondrocyte carrier in articular cartilage repair [241].

A number of studies showed the successful use of fibrin for skin repair. Fibrin glue has been already used in clinical practice as a carrier for keratinocytes in deep, partial and full-thickness wounds. Cell culture studies have shown that the clonogenic ability, growth rate and long-term proliferative potential of keratinocytes is maintained when they are cultured on fibrin which is a naturally occurring substrate in wound healing [242]. In vitro, the production of TGFα which is involved in dermis regeneration and angiogenesis was dramatically increased when keratinocytes were cultured on fibrin-coated substrates [243]. Poly(L-lactide) (PLA) nanofibrous membranes modified with fibrin nanocoating significantly increased cell spreading and expression and synthesis of collagen I in human dermal fibroblasts, in comparison to nonmodified membranes [244]. Growth factors, such as FGF-2, epithelial growth factor (EGF) and keratinocyte growth factor (KGF), can also be added to fibrin scaffolds to improve wound healing, as shown in animal studies [209]. In clinical trials, it has been shown that extensive burned area can be covered with a cultured keratinocyte-fibrin suspension [242]. However, in third-degree burns, the grafts failed to show mechanical stability. To overcome the low mechanical strength of fibrin-based implants while maintaining adhesion and proliferation of keratinocytes, synthetic membranes such as polycaprolactone (PLCL) were coated with fibrin glue [245].

The major drawback of tissue substitutes is the lack of blood supply. One approach to promote angiogenesis is the use of fibrin matrices, because fibrin is involved in healing process, provides specific adhesion sequences for endothelial cells ,and it binds VEGF and FGF-2 with high affinity [246]. In a recent study, human umbilical vein endothelial cells (HUVECs) were seeded with dermal fibroblasts and adipose-derived MSCs in a fibrin matrix to produce a novel artificial skin substitute. This tissue-engineered construct showed a similar structure to native skin, with endothelial cells proliferating and organising themselves into capillary-like structures within the fibrin matrix [247].

Another method to produce fibrin- and fibrinogen-based scaffolds is electrospinning. This process allows for fabrication of fibrous, nonwoven structures, which have been applied in the fields of heart and wound repair. Electrospun fibrinogen scaffolds displayed the geometry of a native provisional wound matrix, and addition of aprotinin maintained the necessary mechanical integrity for use in wound dressing [248]. In an in vitro model, fibroblasts migrated into and remodelled the electrospun fibrinogen scaffold with deposition of collagen. Interestingly, different concentrations of aprotinin modulated scaffold degradation in a predictable fashion, whereas glutaraldehyde vapour fixation was less reliable [249]. As glutaraldehyde vapour crosslinking did not demonstrate any significant improvement in the mechanical properties of electrospun fibrinogen, authors concluded that the process of electrospinning does not expose the lysine residues necessary for glutaraldehyde crosslinking [248, 249]. In a further study, the same group tested three different crosslinkers on an electrospun fibrinogen scaffold. EDC and genipin in ethanol significantly enhanced scaffold mechanical properties and slowed degradation rate in vitro, whereas glutaraldehyde crosslinking failed to produce any significant improvement. However, crosslinking with EDC and genipin was shown to reduce scaffold bioactivity, as fibroblasts migration below the surface and scaffold remodelling were negatively affected [250]. Authors suggested that this poor bioactivity may have been due to the increased scaffold modulus that could have made fibres too stiff to allow for subsurface cell migration, or due to the crosslinking process, that may have masked the integrin binding sites that are normally exposed on fibrinogen.

In order to overcome the limitations of the above-mentioned crosslinking methods, the application of a natural crosslinker has been investigated for the treatment of myocardial infarction. Taking advantage of the high affinity of phytic acid for 2,3-diphosphoglycerate site in haemoglobin, Ravichandra and colleagues used phytic acid to crosslink an electrospun construct composed of haemoglobin/gelatin/fibrinogen, as well as to improve oxygen binding capability: the resulting scaffold supported MSC differentiation into functional cardiomyocytes and showed an improved delivery of oxygen even at higher oxygen tensions, that is a desirable feature in the treatment of ischemic heart [251]. In another study, the need of synthetic crosslinker agents was overcome by combining fibrinogen and thrombin within the syringe of an electrospinning set-up: in this fashion fibrin which is superior to fibrinogen in mechanical properties can be electrospun successfully in a one-step approach. The characterisation of the fibrin-based electrospun nanofibrous scaffold revealed the formation of a structure that mimics the native ECM and enhances cell attachment and proliferation [252].

Fibrin, fibrinogen and thrombin can also be utilised as a bio-ink to produce specific fibrin 3D patterns. Fibrin gels have a low viscosity allowing easy ejection, controllable crosslinking mechanism and fast gelation. Due to the short gelation times and high degradation rates, fibrinogen-/fibrin-based inks are often mixed with other materials [253]. A bio-ink made of thrombin and human endothelial cells was printed into a fibrinogen substrate to form fibrin channels. The printed endothelial cells proliferated to form a tubular structure inside the fibrin channel, suggesting

that thermal inkjet printing technology can be a promising approach for microvasculature tissue engineering [254].

Bioprinting is another strategy to produce cell microenvironments with well-defined spatial patterns of immobilized proteins [255]. In this emerging area of tissue engineering, fibrin can be an ideal printing substrate because of its capacity to bind several growth factors. For example, bone morphogenic protein-2 (BMP2) [256], heparin-binding EGF [257] and FGF-2 [258] were printed and naturally immobilized onto fibrin-coated substrates in order to evaluate the effect of patterned growth factor gradients on cell behaviour.

In the light of the data presented, fibrin and fibrinogen stand out as valuable and versatile natural materials for supplying a provisional matrix and delivering cells, biomolecules and genes to damaged tissue. In comparison to other natural materials, fibrin is particularly appealing because of its excellent biocompatibility, controllable biodegradability, multiple interaction sites for cells and other proteins and ease of fabrication of completely autologous scaffolds. However, some limitations, such as low mechanical strength and high degradation rate, need to be addressed.

References

1. Lim EH, Sardinha JP, Myers S. Nanotechnology biomimetic cartilage regenerative scaffolds. Arch plastic Surg. 2014;41:231–40.
2. Abbah SA, Delgado LM, Azeem A, Fuller K, Shologu N, Keeney M, et al. Harnessing hierarchical nano- and micro-fabrication technologies for musculoskeletal tissue engineering. Adv Healthc Mater. 2015;4:2488–99.
3. Fuller K, Pandit A, Zeugolis DI. The multifaceted potential of electro-spinning in regenerative medicine. Pharm Nanotechnol. 2014;2:23–34.
4. Carbone EJ, Jiang T, Nelson C, Henry N, Lo KWH. Small molecule delivery through nanofibrous scaffolds for musculoskeletal regenerative engineering. Nanomed Nanotechnol Biol Med. 2014;10:1691–9.
5. Woo KM, Chen VJ, Ma PX. Nano-fibrous scaffolding architecture selectively enhances protein adsorption contributing to cell attachment. J Biomed Mater Res A. 2003;67A:531–7.
6. Ma PX. Biomimetic materials for tissue engineering. Adv Drug Deliv Rev. 2008;60:184–98.
7. Barnes CP, Sell SA, Boland ED, Simpson DG, Bowlin GL. Nanofiber technology: designing the next generation of tissue engineering scaffolds. Adv Drug Deliv Rev. 2007;59:1413–33.
8. Shields KJ, Beckman MJ, Bowlin GL, Wayne JS. Mechanical properties and cellular proliferation of electrospun collagen type II. Tissue Eng. 2004;10:1510–7.
9. Heydarkhan-Hagvall S, Schenke-Layland K, Dhanasopon AP, Rofail F, Smith H, Wu BM, et al. Three-dimensional electrospun ECM-based hybrid scaffolds for cardiovascular tissue engineering. Biomaterials. 2008;29:2907–14.
10. Fleischer S, Shapira A, Regev O, Nseir N, Zussman E, Dvir T. Albumin fiber scaffolds for engineering functional cardiac tissues. Biotechnol Bioeng. 2014;111:1246–57.
11. Nicodemus GD, Bryant SJ. Cell encapsulation in biodegradable hydrogels for tissue engineering applications. Tissue Eng Part B Rev. 2008;14:149–65.

12. Hong Y, Takanari K, Amoroso NJ, Hashizume R, Brennan-Pierce EP, Freund JM, et al. An elastomeric patch electrospun from a blended solution of dermal extracellular matrix and biodegradable polyurethane for rat abdominal wall repair. Tissue Eng Part C Methods. 2012;18:122–32.
13. Harkness RD. Biological functions of collagen. Biol Rev Camb Philos Soc. 1961;36:399–463.
14. Soderhall C, Marenholz I, Kerscher T, Ruschendorf F, Esparza-Gordillo J, Worm M, et al. Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. PLoS Biol. 2007;5:e242.
15. Kielty CM, Grant ME. The collagen family: structure, assembly and organization in the extracellular matrix. In: Royce PM, Steinmann B, editors. Connective tissue and its heritable disorders: molecular, genetic and medical aspects. 2nd ed. New York: Wiley; 2002. p. 159–221.
16. Piez KA. Collagen. In: Kroschwitz JI, editor. Encyclopedia of polymer science and engineering. New York: Wiley; 1985. p. 699–727.
17. Zeugolis DI, Raghunath M. Collagen: materials analysis and implant uses. In: Ducheyne P, Healy KE, Hutmacher DW, Grainger DW, Kirkpatrick CJ, editors. Comprehensive biomaterials. Oxford: Elsevier; 2011. p. 261–78.
18. Friess W. Collagen—biomaterial for drug delivery. Eur J Pharm Biopharm. 1998;45:113–36.
19. Zeugolis DI, Paul GR, Attenburrow G. Cross-linking of extruded collagen fibres—a biomimetic three-dimensional scaffold for tissue engineering applications. J Biomed Mater Res A. 2009;89:895–908.
20. Zeugolis DI, Paul RG, Attenburrow G. The influence of a natural cross-linking agent (*Myrica rubra*) on the properties of extruded collagen fibres for tissue engineering applications. Mater Sci Eng C. 2010;30:190–5.
21. Delgado LM, Fuller K, Zeugolis DI. Collagen cross-linking: biophysical, biochemical, and biological response analysis. Tissue Eng Part A. 2017;23:1064–77.
22. Gough JE, Scotchford CA, Downes S. Cytotoxicity of glutaraldehyde crosslinked collagen/poly(vinyl alcohol) films is by the mechanism of apoptosis. J Biomed Mater Res. 2002;61:121–30.
23. Levy RJ, Schoen FJ, Sherman FS, Nichols J, Hawley MA, Lund SA. Calcification of subcutaneously implanted type I collagen sponges. Effects of formaldehyde and glutaraldehyde pretreatments. Am J Pathol. 1986;122:71–82.
24. Brown BN, Londono R, Tottey S, Zhang L, Kukla KA, Wolf MT, et al. Macrophage phenotype as a predictor of constructive remodeling following the implantation of biologically derived surgical mesh materials. Acta Biomater. 2012;8:978–87.
25. Delgado LM, Bayon Y, Pandit A, Zeugolis DI. To cross-link or not to cross-link? Cross-linking associated foreign body response of collagen-based devices. Tissue Eng Part B Rev. 2015;21:298–313.
26. Collin EC, Grad S, Zeugolis DI, Vinatier CS, Clouet JR, Guicheux JJ, et al. An injectable vehicle for nucleus pulposus cell-based therapy. Biomaterials. 2011;32:2862–70.
27. Rho KS, Jeong L, Lee G, Seo BM, Park YJ, Hong SD, et al. Electrospinning of collagen nanofibers: effects on the behavior of normal human keratinocytes and early-stage wound healing. Biomaterials. 2006;27:1452–61.
28. Li M, Mondrinos MJ, Gandhi MR, Ko FK, Weiss AS, Lelkes PI. Electrospun protein fibers as matrices for tissue engineering. Biomaterials. 2005;26:5999–6008.
29. Zeugolis DI, Khew ST, Yew ESY, Ekaputra AK, Tong YW, Yung L-YL, et al. Electro-spinning of pure collagen nano-fibres—just an expensive way to make gelatin? Biomaterials. 2008;29:2293–305.
30. Browne S, Zeugolis DI, Pandit A. Collagen: finding a solution for the source. Tissue Eng Part A. 2013;19:1491–4.
31. IAEA. Trends in radiation sterilization of health care products. Vienna: International Atomic Energy Agency; 2008.

32. Hori H, Hattori S, Inouye S, Kimura A, Irie S, Miyazawa H, et al. Analysis of the major epitope of the α2 chain of bovine type I collagen in children with bovine gelatin allergy. J Allergy Clin Immunol. 2002;110:652–7.
33. Lynn AK, Yannas IV, Bonfield W. Antigenicity and immunogenicity of collagen. J Biomed Mater Res Part B Appl Biomater. 2004;71:343–54.
34. Delgado LM, Shologu N, Fuller K, Zeugolis D. Acetic acid and pepsin result in high yield, high purity and low macrophage response collagen for biomedical applications. Biomed Mater. 2017;12:065009.
35. Tang Y, Yang X, Hang B, Li J, Huang L, Huang F, et al. Efficient production of hydroxylated human-like collagen via the co-expression of three key genes in escherichia coli origami (DE3). Appl Biochem Biotechnol. 2016;178:1458–70.
36. Ruggiero F, Koch M. Making recombinant extracellular matrix proteins. Methods (San Diego, Calif). 2008;45:75–85.
37. Yang C, Hillas PJ, Baez JA, Nokelainen M, Balan J, Tang J, et al. The application of recombinant human collagen in tissue engineering. BioDrugs Clin Immunother Biopharm Gene Ther. 2004;18:103–19.
38. Parenteau-Bareil R, Gauvin R, Berthod F. Collagen-based biomaterials for tissue engineering applications. Materials. 2010;3:1863–87.
39. Gosline J, Lillie M, Carrington E, Guerette P, Ortlepp C, Savage K. Elastic proteins: biological roles and mechanical properties. Philos Trans R Soc Lond B Biol Sci. 2002;357:121–32.
40. Silva R, Fabry B, Boccaccini AR. Fibrous protein-based hydrogels for cell encapsulation. Biomaterials. 2014;35:6727–38.
41. Miranda-Nieves D, Chaikof EL. Collagen and elastin biomaterials for the fabrication of engineered living tissues. ACS Biomater Sci Eng. 2017;3:694–711.
42. Daamen WF, Veerkamp JH, van Hest JC, van Kuppevelt TH. Elastin as a biomaterial for tissue engineering. Biomaterials. 2007;28:4378–98.
43. Werkmeister JA, Ramshaw JA. Recombinant protein scaffolds for tissue engineering. Biomed Mater. 2012;7:012002.
44. Caves JM, Cui W, Wen J, Kumar VA, Haller CA, Chaikof EL. Elastin-like protein matrix reinforced with collagen microfibers for soft tissue repair. Biomaterials. 2011;32:5371–9.
45. Devalliere J, Dooley K, Hu Y, Kelangi SS, Uygun BE, Yarmush ML. Co-delivery of a growth factor and a tissue-protective molecule using elastin biopolymers accelerates wound healing in diabetic mice. Biomaterials. 2017;141:149–60.
46. Monfort DA, Koria P. Recombinant elastin based nanoparticles for targeted gene therapy. Gene Ther. 2017;24:610–20.
47. Waterhouse A, Wise SG, Ng MK, Weiss AS. Elastin as a nonthrombogenic biomaterial. Tissue Eng Part B Rev. 2011;17:93–9.
48. Liu SQ, Tieche C, Alkema PK. Neointima formation on vascular elastic laminae and collagen matrices scaffolds implanted in the rat aortae. Biomaterials. 2004;25:1869–82.
49. Henry JJD, Yu J, Wang A, Lee R, Fang J, Li S. Engineering the mechanical and biological properties of nanofibrous vascular grafts for in situ vascular tissue engineering. Biofabrication. 2017;9:035007.
50. Waterhouse A, Yin Y, Wise SG, Bax DV, McKenzie DR, Bilek MM, et al. The immobilization of recombinant human tropoelastin on metals using a plasma-activated coating to improve the biocompatibility of coronary stents. Biomaterials. 2010;31:8332–40.
51. Woodhouse KA, Klement P, Chen V, Gorbet MB, Keeley FW, Stahl R, et al. Investigation of recombinant human elastin polypeptides as non-thrombogenic coatings. Biomaterials. 2004;25:4543–53.
52. Jordan SW, Haller CA, Sallach RE, Apkarian RP, Hanson SR, Chaikof EL. The effect of a recombinant elastin-mimetic coating of an ePTFE prosthesis on acute thrombogenicity in a baboon arteriovenous shunt. Biomaterials. 2007;28:1191–7.

53. Wachi H, Sugitani H, Murata H, Nakazawa J, Mecham RP, Seyama Y. Tropoelastin inhibits vascular calcification via 67-kDa elastin binding protein in cultured bovine aortic smooth muscle cells. J Atherosclerosis Thromb. 2004;11:159–66.
54. Almine JF, Bax DV, Mithieux SM, Nivison-Smith L, Rnjak J, Waterhouse A, et al. Elastin-based materials. Chem Soc Rev. 2010;39:3371–9.
55. Wong CS, Liu X, Xu Z, Lin T, Wang X. Elastin and collagen enhances electrospun aligned polyurethane as scaffolds for vascular graft. J Mater Sci Mater Med. 2013;24:1865–74.
56. McClure MJ, Sell SA, Simpson DG, Walpoth BH, Bowlin GL. A three-layered electrospun matrix to mimic native arterial architecture using polycaprolactone, elastin, and collagen: a preliminary study. Acta Biomater. 2010;6:2422–33.
57. Foraida ZI, Kamaldinov T, Nelson DA, Larsen M, Castracane J. Elastin-PLGA hybrid electrospun nanofiber scaffolds for salivary epithelial cell self-organization and polarization. Acta Biomater. 2017;62:116–27.
58. Feughelman M. Natural protein fibers. J Appl Polym Sci. 2002;83:489–507.
59. Shavandi A, Silva TH, Bekhit AA, Bekhit AEA. Keratin: dissolution, extraction and biomedical application. Biomater Sci. 2017;5:1699–735.
60. Rouse JG, Van Dyke ME. A review of keratin-based biomaterials for biomedical applications. Materials. 2010;3:999–1014.
61. Sierpinski P, Garrett J, Ma J, Apel P, Klorig D, Smith T, et al. The use of keratin biomaterials derived from human hair for the promotion of rapid regeneration of peripheral nerves. Biomaterials. 2008;29:118–28.
62. Ham TR, Lee RT, Han S, Haque S, Vodovotz Y, Gu J, et al. Tunable keratin hydrogels for controlled erosion and growth factor delivery. Biomacromol. 2016;17:225–36.
63. Pace LA, Plate JF, Mannava S, Barnwell JC, Koman LA, Li Z, et al. A human hair keratin hydrogel scaffold enhances median nerve regeneration in nonhuman primates: an electrophysiological and histological study. Tissue Eng Part A. 2014;20:507–17.
64. Blanchard CR, Timmons SF, Smith RA. Keratin-based hydrogel for biomedical applications and method of production. 1999.
65. Kaplan D, Adams WW, Farmer B, Viney C. Silk: biology, structure, properties, and genetics. Silk Polymers: American Chemical Society; 1993. p. 2–16.
66. Kasoju N, Bora U. Silk fibroin in tissue engineering. Adv Healthc Mater. 2012;1:393–412.
67. Zhou CZ, Confalonieri F, Jacquet M, Perasso R, Li ZG, Janin J. Silk fibroin: structural implications of a remarkable amino acid sequence. Proteins. 2001;44:119–22.
68. Rockwood DN, Preda RC, Yucel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from *Bombyx mori* silk fibroin. Nat Protoc. 2011;6:1612–31.
69. Kundu B, Rajkhowa R, Kundu SC, Wang X. Silk fibroin biomaterials for tissue regenerations. Adv Drug Deliv Rev. 2013;65:457–70.
70. Wang Y, Rudym DD, Walsh A, Abrahamsen L, Kim H-J, Kim HS, et al. In vivo degradation of three-dimensional silk fibroin scaffolds. Biomaterials. 2008;29:3415–28.
71. Lovett ML, Cannizzaro CM, Vunjak-Novakovic G, Kaplan DL. Gel spinning of silk tubes for tissue engineering. Biomaterials. 2008;29:4650–7.
72. Zhang X, Wang X, Keshav V, Wang X, Johanas JT, Leisk GG, et al. Dynamic culture conditions to generate silk-based tissue-engineered vascular grafts. Biomaterials. 2009;30:3213–23.
73. Zamani M, Khafaji M, Naji M, Vossoughi M, Alemzadeh I, Haghighipour N. A biomimetic heparinized composite silk-based vascular scaffold with sustained antithrombogenicity. Sci Rep. 2017;7:4455.
74. Cattaneo I, Figliuzzi M, Azzollini N, Catto V, Fare S, Tanzi MC, et al. In vivo regeneration of elastic lamina on fibroin biodegradable vascular scaffold. Int J Artif Organs. 2013;36:166–74.
75. Zhang X, Reagan MR, Kaplan DL. Electrospun silk biomaterial scaffolds for regenerative medicine. Adv Drug Delivery Rev. 2009;61:988–1006.

76. Song DW, Kim SH, Kim HH, Lee KH, Ki CS, Park YH. Multi-biofunction of antimicrobial peptide-immobilized silk fibroin nanofiber membrane: Implications for wound healing. Acta Biomater. 2016;39:146–55.
77. Gil ES, Panilaitis B, Bellas E, Kaplan DL. Functionalized silk biomaterials for wound healing. Adv Healthc Mater. 2013;2:206–17.
78. Liu W, Thomopoulos S, Xia Y. Electrospun nanofibers for regenerative medicine. Adv Healthc Mater. 2012;1:10–25.
79. Binan L, Tendey C, De Crescenzo G, El Ayoubi R, Ajji A, Jolicoeur M. Differentiation of neuronal stem cells into motor neurons using electrospun poly-L-lactic acid/gelatin scaffold. Biomaterials. 2014;35:664–74.
80. Liu S, Qin M, Hu C, Wu F, Cui W, Jin T, et al. Tendon healing and anti-adhesion properties of electrospun fibrous membranes containing bFGF loaded nanoparticles. Biomaterials. 2013;34:4690–701.
81. Ortega I, Ryan AJ, Deshpande P, MacNeil S, Claeyssens F. Combined microfabrication and electrospinning to produce 3-D architectures for corneal repair. Acta Biomater. 2013;9:5511–20.
82. Hu X, Liu S, Zhou G, Huang Y, Xie Z, Jing X. Electrospinning of polymeric nanofibers for drug delivery applications. J Controlled Release. 2014;185:12–21.
83. Katti DS, Robinson KW, Ko FK, Laurencin CT. Bioresorbable nanofiber-based systems for wound healing and drug delivery: optimization of fabrication parameters. J Biomed Mater Res Part B Appl Biomater. 2004;70:286–96.
84. Cramariuc B, Cramariuc R, Scarlet R, Manea LR, Lupu IG, Cramariuc O. Fiber diameter in electrospinning process. J Electrostat. 2013;71:189–98.
85. Fuller KP, Gaspar D, Delgado LM, Pandit A, Zeugolis DI. Influence of porosity and pore shape on structural, mechanical and biological properties of poly -caprolactone electro-spun fibrous scaffolds. Nanomedicine (London, England). 2016;11:1031–40.
86. Yin Z, Chen X, Chen JL, Shen WL, Hieu Nguyen TM, Gao L, et al. The regulation of tendon stem cell differentiation by the alignment of nanofibers. Biomaterials. 2010;31:2163–75.
87. Lee BK, Ju YM, Cho JG, Jackson JD, Lee SJ, Atala A, et al. End-to-side neurorrhaphy using an electrospun PCL/collagen nerve conduit for complex peripheral motor nerve regeneration. Biomaterials. 2012;33:9027–36.
88. Xie J, MacEwan M, Ray W, Liu W, Siewe D, Xia Y. Radially aligned, electrospun nanofibers as dural substitutes for wound closure and tissue regeneration applications. ACS Nano. 2010;4:5027–36.
89. Whitesides GM, Grzybowski B. Self-assembly at all scales. Science (New York, NY). 2002;295:2418–21.
90. Friess W. Collagen–biomaterial for drug delivery. Eur J Pharm Biopharm. 1998;45:113–36.
91. Zeugolis DI, Paul RG, Attenburrow G. Extruded collagen-polyethylene glycol fibers for tissue engineering applications. J Biomed Mater Res Part B Appl Biomater. 2008;85: 343–52.
92. Daly WT, Yao L, Abu-rub MT, O'Connell C, Zeugolis DI, Windebank AJ, et al. The effect of intraluminal contact mediated guidance signals on axonal mismatch during peripheral nerve repair. Biomaterials. 2012;33:6660–71.
93. Abu-Rub MT, Billiar KL, van Es MH, Knight A, Rodriguez BJ, Zeugolis DI, et al. Nano-textured self-assembled aligned collagen hydrogels promote directional neurite guidance and overcome inhibition by myelin associated glycoprotein. Soft Matter. 2011;7:2770.
94. Li Y, Asadi A, Monroe MR, Douglas EP. pH effects on collagen fibrillogenesis in vitro: electrostatic interactions and phosphate binding. Mater Sci Eng C. 2009;29:1643–9.
95. Olmos Buitrago J, Perez RA, El-Fiqi A, Singh RK, Kim JH, Kim HW. Core-shell fibrous stem cell carriers incorporating osteogenic nanoparticulate cues for bone tissue engineering. Acta Biomater. 2015;28:183–92.

96. Perez RA, Kim HW. Core-shell designed scaffolds for drug delivery and tissue engineering. Acta Biomater. 2015;21:2–19.
97. Perez RA, Kim JH, Buitrago JO, Wall IB, Kim HW. Novel therapeutic core-shell hydrogel scaffolds with sequential delivery of cobalt and bone morphogenetic protein-2 for synergistic bone regeneration. Acta Biomater. 2015;23:295–308.
98. Perez RA, Kim M, Kim TH, Kim JH, Lee JH, Park JH, et al. Utilizing core-shell fibrous collagen-alginate hydrogel cell delivery system for bone tissue engineering. Tissue Eng Part A. 2014;20:103–14.
99. Hartgerink JD, Beniash E, Stupp SI. Peptide-amphiphile nanofibers: a versatile scaffold for the preparation of self-assembling materials. Proc Natl Acad Sci U S A. 2002;99:5133–8.
100. Hartgerink JD, Beniash E, Stupp SI. Self-assembly and mineralization of peptide-amphiphile nanofibers. Science (New York, NY). 2001;294:1684–8.
101. Loo Y, Zhang SG, Hauser CAE. From short peptides to nanofibers to macromolecular assemblies in biomedicine. Biotechnol Adv. 2012;30:593–603.
102. Saracino GA, Cigognini D, Silva D, Caprini A, Gelain F. Nanomaterials design and tests for neural tissue engineering. Chem Soc Rev. 2013;42:225–62.
103. Cunha C, Panseri S, Villa O, Silva D, Gelain F. 3D culture of adult mouse neural stem cells within functionalized self-assembling peptide scaffolds. Int J Nanomed. 2011;6:943–55.
104. Yang H, Xie Z, Wang P, Bi J. Self-assembling nanofibers alter the processing of amyloid precursor protein in a transgenic mouse model of Alzheimer's disease. Neurol Res. 2015;37:84–91.
105. Cigognini D, Satta A, Colleoni B, Silva D, Donega M, Antonini S, et al. Evaluation of early and late effects into the acute spinal cord injury of an injectable functionalized self-assembling scaffold. PLoS ONE. 2011;6:e19782.
106. Cigognini D, Silva D, Paloppi S, Gelain F. Evaluation of mechanical properties and therapeutic effect of injectable self-assembling hydrogels for spinal cord injury. J Biomed Nanotechnol. 2014;10:309–23.
107. Kisiday J, Jin M, Kurz B, Hung H, Semino C, Zhang S, et al. Self-assembling peptide hydrogel fosters chondrocyte extracellular matrix production and cell division: implications for cartilage tissue repair. Proc Natl Acad Sci U S A. 2002;99:9996–10001.
108. Schneider A, Garlick JA, Egles C. Self-assembling peptide nanofiber scaffolds accelerate wound healing. PLoS ONE. 2008;3:e1410.
109. Ma PX, Zhang R. Synthetic nano-scale fibrous extracellular matrix. J Biomed Mater Res. 1999;46:60–72.
110. Stokols S, Sakamoto J, Breckon C, Holt T, Weiss J, Tuszynski MH. Templated agarose scaffolds support linear axonal regeneration. Tissue Eng. 2006;12:2777–87.
111. Stokols S, Tuszynski MH. The fabrication and characterization of linearly oriented nerve guidance scaffolds for spinal cord injury. Biomaterials. 2004;25:5839–46.
112. Garcia Y, Wilkins B, Collighan RJ, Griffin M, Pandit A. Towards development of a dermal rudiment for enhanced wound healing response. Biomaterials. 2008;29:857–68.
113. Stokols S, Tuszynski MH. Freeze-dried agarose scaffolds with uniaxial channels stimulate and guide linear axonal growth following spinal cord injury. Biomaterials. 2006;27:443–51.
114. Bozkurt A, Lassner F, O'Dey D, Deumens R, Bocker A, Schwendt T, et al. The role of microstructured and interconnected pore channels in a collagen-based nerve guide on axonal regeneration in peripheral nerves. Biomaterials. 2012;33:1363–75.
115. Chen H, Yang X, Liao Y, Zeng X, Liang P, Kang N, et al. MRI and histologic analysis of collagen type II sponge on repairing the cartilage defects of rabbit knee joints. J Biomed Mater Res Part B Appl Biomater. 2011;96:267–75.
116. Shen W, Chen X, Hu Y, Yin Z, Zhu T, Hu J, et al. Long-term effects of knitted silk–collagen sponge scaffold on anterior cruciate ligament reconstruction and osteoarthritis prevention. Biomaterials. 2014;35:8154–63.
117. Yao L, Daly W, Newland B, Yao S, Wang W, Chen BK, et al. Improved axonal regeneration of transected spinal cord mediated by multichannel collagen conduits functionalized with neurotrophin-3 gene. Gene Ther. 2013;20:1149–57.

118. Caliari SR. Harley BaC. The effect of anisotropic collagen-GAG scaffolds and growth factor supplementation on tendon cell recruitment, alignment, and metabolic activity. Biomaterials. 2011;32:5330–40.
119. Caliari SR, Ramirez MA, Harley BAC. The development of collagen-GAG scaffold-membrane composites for tendon tissue engineering. Biomaterials. 2011;32:8990–8.
120. Boekema BK, Vlig M, Olde Damink L, Middelkoop E, Eummelen L, Buhren AV, et al. Effect of pore size and cross-linking of a novel collagen-elastin dermal substitute on wound healing. J Mater Sci Mater Med. 2014;25:423–33.
121. Min BM, Jeong L, Nam YS, Kim JM, Kim JY, Park WH. Formation of silk fibroin matrices with different texture and its cellular response to normal human keratinocytes. Int J Biol Macromol. 2004;34:281–8.
122. Daley WP, Peters SB, Larsen M. Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci. 2008;121:255–64.
123. Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane crosstalk between the extracellular matrix–cytoskeleton crosstalk. Nat Rev Mol Cell Biol. 2001;2:793–805.
124. Kong HJ, Mooney DJ. Microenvironmental regulation of biomacromolecular therapies. Nat Rev Drug Discovery. 2007;6:455–63.
125. Aszodi A, Hunziker EB, Brakebusch C, Fassler R. Beta1 integrins regulate chondrocyte rotation, G1 progression, and cytokinesis. Genes Dev. 2003;17:2465–79.
126. Schneiderbauer MM, Dutton CM, Scully SP. Signaling "cross-talk" between TGF-beta1 and ECM signals in chondrocytic cells. Cell Signal. 2004;16:1133–40.
127. Fontana G, See E, Pandit A. Current trends in biologics delivery to restore intervertebral disc anabolism. Adv Drug Deliv Rev. 2015;84:146–58.
128. Teti A. Regulation of Cellular Functions by Extracellular-Matrix. J Am Soc Nephrol. 1992;2:S83–7.
129. Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol. 2011;209:139–51.
130. Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis. Biotechnol Bioeng. 2006;93:1152–63.
131. DeLise AM, Fischer L, Tuan RS. Cellular interactions and signaling in cartilage development. Osteoarthritis Cartilage. 2000;8:309–34.
132. Masuda K, An HS. Growth factors and the intervertebral disc. Spine J. 2004;4:330S–40S.
133. Masuda K, Oegema TR, Jr., An HS. Growth factors and treatment of intervertebral disc degeneration. Spine (Phila Pa 1976). 2004;29:2757–69.
134. Masuda K, Lotz JC. New challenges for intervertebral disc treatment using regenerative medicine. Tissue Eng Part B Rev. 2010;16:147–58.
135. Eppler SM, Combs DL, Henry TD, Lopez JJ, Ellis SG, Yi JH, et al. A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin Pharmacol Ther. 2002;72:20–32.
136. Lee K, Silva EA, Mooney DJ. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. J R Soc Interface. 2011;8:153–70.
137. Saik JE, Gould DJ, Watkins EM, Dickinson ME, West JL. Covalently immobilized platelet-derived growth factor-BB promotes angiogenesis in biomimetic poly(ethylene glycol) hydrogels. Acta Biomater. 2011;7:133–43.
138. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med. 2000;6:389–95.
139. Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM. VEGF gene delivery to myocardium—deleterious effects of unregulated expression. Circulation. 2000;102:898–901.
140. Yang WW, Pierstorff E. Reservoir-based polymer drug delivery systems. Jala-J Lab Autom. 2012;17:50–8.

141. Fontana G, Thomas D, Collin E, Pandit A. Microgel microenvironment primes adipose-derived stem cells towards an NP cells-like phenotype. Adv Healthc Mater. 2014;3:2012–22.
142. Zhang ZP, Hu J, Ma PX. Nanofiber-based delivery of bioactive agents and stem cells to bone sites. Adv Drug Deliver Rev. 2012;64:1129–41.
143. De Laporte L, Shea LD. Matrices and scaffolds for DNA delivery in tissue engineering. Adv Drug Deliver Rev. 2007;59:292–307.
144. Li RH, Wozney JM. Delivering on the promise of bone morphogenetic proteins. Trends Biotechnol. 2001;19:255–65.
145. Chen RR, Mooney DJ. Polymeric growth factor delivery strategies for tissue engineering. Pharm Res. 2003;20:1103–12.
146. Elisseeff J, Puleo C, Yang F, Sharma B. Advances in skeletal tissue engineering with hydrogels. Orthod Craniofac Res. 2005;8:150–61.
147. Lutolf MR, Weber FE, Schmoekel HG, Schense JC, Kohler T, Muller R, et al. Repair of bone defects using synthetic mimetics of collagenous extracellular matrices. Nat Biotechnol. 2003;21:513–8.
148. Place ES, Evans ND, Stevens MM. Complexity in biomaterials for tissue engineering. Nat Mater. 2009;8:457–70.
149. Thomas D, Fontana G, Chen X, Sanz-Nogues C, Zeugolis DI, Dockery P, et al. A shape-controlled tuneable microgel platform to modulate angiogenic paracrine responses in stem cells. Biomaterials. 2014;35:8757–66.
150. Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, et al. Constitutive expression of phVEGF(165) after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. Circulation. 1998;97:1114–23.
151. Zisch AH, Schenk U, Schense JC, Sakiyama-Elbert SE, Hubbell JA. Covalently conjugated VEGF-fibrin matrices for endothelialization. J Control Release. 2001;72:101–13.
152. Koch S, Yao C, Grieb G, Prevel P, Noah EM, Steffens GCM. Enhancing angiogenesis in collagen matrices by covalent incorporation of VEGF. J Mater Sci Mater Med. 2006;17:735–41.
153. Haro H, Kato T, Komori H, Osada M, Shinomiya K. Vascular endothelial growth factor (VEGF)-induced angiogenesis in herniated disc resorption. J Orthop Res. 2002;20:409–15.
154. Ehrbar M, Djonov VG, Schnell C, Tschanz SA, Martiny-Baron G, Schenk U, et al. Cell-demanded liberation of VEGF(121) from fibrin implants induces local and controlled blood vessel growth. Circ Res. 2004;94:1124–32.
155. Post MJ, Laham R, Sellke FW, Simons M. Therapeutic angiogenesis in cardiology using protein formulations. Cardiovasc Res. 2001;49:522–31.
156. Zisch AH, Lutolf MP, Ehrbar M, Raeber GP, Rizzi SC, Davies N, et al. Cell-demanded release of VEGF from synthetic, biointeractive cell-ingrowth matrices for vascularized tissue growth. Faseb J. 2003;17:2260–2.
157. Sakiyama SE, Schense JC, Hubbell JA. Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. Faseb J. 1999;13:2214–24.
158. Sakiyama-Elbert SE, Panitch A, Hubbell JA. Development of growth factor fusion proteins for cell-triggered drug delivery. Faseb J. 2001;15:1300–2.
159. Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. Bioconjugate Chem. 1999;10:75–81.
160. Wissink MJB, Beernink R, Scharenborg NM, Poot AA, Engbers GHM, Beugeling T, et al. Endothelial cell seeding of (heparinized) collagen matrices: effects of bFGF pre-loading on proliferation (after low density seeding) and pro-coagulant factors. J Controlled Release. 2000;67:141–55.
161. Grieb G, Groger A, Piatkowski A, Markowicz M, Steffens GCM, Pallua N. Tissue substitutes with improved angiogenic capabilities: an in vitro investigation with endothelial cells and endothelial progenitor cells. Cells Tissues Organs. 2010;191:96–104.

162. Markowicz M, Heitland A, Steffens GCM, Pallua N. Effects of modified collagen matrices on human umbilical vein endothelial cells. Int J Artif Organs. 2005;28:1251–8.
163. Steffens GCM, Yao C, Prevel P, Markowicz M, Schenck P, Noah EM, et al. Modulation of angiogenic potential of collagen matrices by covalent incorporation of heparin and loading with vascular endothelial growth factor. Tissue Eng. 2004;10:1502–9.
164. van Wachem PB, Plantinga JA, Wissink MJB, Beernink R, Poot AA, Engbers GHM, et al. In vivo biocompatibility of carbodiimide-crosslinked collagen matrices: Effects of crosslink density, heparin immobilization, and bFGF loading. J Biomed Mater Res. 2001;55:368–78.
165. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, et al. Binding and release of basic fibroblast growth factor from heparinized collagen matrices. Biomaterials. 2001;22:2291–9.
166. Bladergroen BA, Siebum B, Siebers-Vermeulen KGC, Van Kuppevelt TH, Poot AA, Feijen J, et al. In vivo recruitment of hematopoietic cells using stromal cell-derived factor 1 alpha-loaded heparinized three-dimensional collagen scaffolds. Tissue Eng Part A. 2009;15:1591–9.
167. Lienemann PS, Lutolf MP, Ehrbar M. Biomimetic hydrogels for controlled biomolecule delivery to augment bone regeneration. Adv Drug Deliver Rev. 2012;64:1078–89.
168. Uebersax L, Merkle HP, Meinel L. Biopolymer-based growth factor delivery for tissue repair: from natural concepts to engineered systems. Tissue Eng Part B Rev. 2009;15: 263–89.
169. Lin CC, Anseth KS. Controlling affinity binding with peptide-functionalized poly(ethylene glycol) hydrogels. Adv Funct Mater. 2009;19:2325–31.
170. Wieland JA, Houchin-Ray TL, Shea LD. Non-viral vector delivery from PEG-hyaluronic acid hydrogels. J Controlled Release. 2007;120:233–41.
171. Kasper FK, Seidlits SK, Tang A, Crowther RS, Carney DH, Barry MA, et al. In vitro release of plasmid DNA from oligo(poly(ethylene glycol) fumarate) hydrogels. J Controlled Release. 2005;104:521–39.
172. Bjugstad KB, Lampe K, Kern DS, Mahoney M. Biocompatibility of poly(ethylene glycol)-based hydrogels in the brain: an analysis of the glial response across space and time. J Biomed Mater Res Part A. 2010;95A:79–91.
173. Jeon O, Alt DS, Ahmed SM, Alsberg E. The effect of oxidation on the degradation of photocrosslinkable alginate hydrogels. Biomaterials. 2012;33:3503–14.
174. Kong HJ, Kaigler D, Kim K, Mooney DJ. Controlling rigidity and degradation of alginate hydrogels via molecular weight distribution. Biomacromol. 2004;5:1720–7.
175. Kang KS, Lee SI, Hong JM, Lee JW, Cho HY, Son JH, et al. Hybrid scaffold composed of hydrogel/3D-framework and its application as a dopamine delivery system. J Controlled Release. 2014;175:10–6.
176. Zhou SL, Matsumoto S, Tian HD, Yamane H, Ojida A, Kiyonaka S, et al. pH-Responsive shrinkage/swelling of a supramolecular hydrogel composed of two small amphiphilic molecules. Chem Eur J. 2005;11:1130–6.
177. Liang GL, Yang ZM, Zhang RJ, Li LH, Fan YJ, Kuang Y, et al. Supramolecular hydrogel of a D-amino acid dipeptide for controlled drug release in vivo. Langmuir. 2009;25:8419–22.
178. Alvarez Z, Castano O, Castells AA, Mateos-Timoneda MA, Planell JA, Engel E, et al. Neurogenesis and vascularization of the damaged brain using a lactate-releasing biomimetic scaffold. Biomaterials. 2014;35:4769–81.
179. Polet F, Feron O. Endothelial cell metabolism and tumour angiogenesis: glucose and glutamine as essential fuels and lactate as the driving force. J Intern Med. 2013;273:156–65.
180. Speder P, Liu J, Brand AH. Nutrient control of neural stem cells. Curr Opin Cell Biol. 2011;23:724–9.
181. Wei GB, Jin QM, Giannobile WV, Ma PX. The enhancement of osteogenesis by nano-fibrous scaffolds incorporating rhBMP-7 nanospheres. Biomaterials. 2007;28:2087–96.
182. Saraf A, Mikos AG. Gene delivery strategies for cartilage tissue engineering. Adv Drug Deliver Rev. 2006;58:592–603.

183. Shimer AL, Chadderdon RC, Gilbertson LG, Kang JD. Gene therapy approaches for intervertebral disc degeneration. Spine. 2004;29:2770–8.
184. Woods BI, Vo N, Sowa G, Kang JD. Gene therapy for intervertebral disk degeneration. Orthop Clin N Am. 2011;42:563–74.
185. Jang JH, Houchin TL, Shea LD. Gene delivery from polymer scaffolds for tissue engineering. Expert Rev Med Devices. 2004;1:127–38.
186. Riddle KW, Kong HJ, Leach JK, Fischbach C, Cheung C, Anseth KS, et al. Modifying the proliferative state of target cells to control DNA expression and identifying cell types transfected in vivo. Mol Ther. 2007;15:361–8.
187. Rowley JA, Mooney DJ. Alginate type and RGD density control myoblast phenotype. J Biomed Mater Res. 2002;60:217–23.
188. Fang JM, Zhu YY, Smiley E, Bonadio J, Rouleau JP, Goldstein SA, et al. Stimulation of new bone formation by direct transfer of osteogenic plasmid genes. P Natl Acad Sci U S A. 1996;93:5753–8.
189. Zhang Y, Song J, Shi B, Wang Y, Chen X, Huang C, et al. Combination of scaffold and adenovirus vectors expressing bone morphogenetic protein-7 for alveolar bone regeneration at dental implant defects. Biomaterials. 2007;28:4635–42.
190. Fontana G, Srivastava A, Thomas D, Lalor P, Dockery P, Pandit A. Three-dimensional microgel platform for the production of cell factories tailored for the nucleus pulposus. Bioconjug Chem. 2014;26:1297–306.
191. Browne S, Fontana G, Rodriguez BJ, Pandit A. A protective extracellular matrix-based gene delivery reservoir fabricated by electrostatic charge manipulation. Mol Pharm. 2012;9:3099–106.
192. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. Nat Med. 1999;5:753–9.
193. Chandler LA, Doukas J, Gonzalez AM, Hoganson DK, Gu DL, Ma CL, et al. FGF2-targeted adenovirus encoding platelet-derived growth factor-B enhances de novo tissue formation. Mol Ther. 2000;2:153–60.
194. Chandler LA, Gu DL, Ma CL, Gonzalez AM, Doukas J, Nguyen T, et al. Matrix-enabled gene transfer for cutaneous wound repair. Wound Repair Regeneration. 2000;8:473–9.
195. Doukas J, Chandler LA, Gonzalez AM, Gu DL, Hoganson DK, Ma CL, et al. Matrix immobilization enhances the tissue repair activity of growth factor gene therapy vectors. Hum Gene Ther. 2001;12:783–98.
196. Tyrone JW, Mogford JE, Chandler LA, Ma CL, Xia YP, Pierce GF, et al. Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. J Surg Res. 2000;93:230–6.
197. Doukas J, Blease K, Craig D, Ma CL, Chandler LA, Sosnowski BA, et al. Delivery of FGF genes to wound repair cells enhances arteriogenesis and myogenesis in skeletal muscle. Mol Ther. 2002;5:517–27.
198. Berry M, Gonzalez AM, Clarke W, Greenlees L, Barrett L, Tsang W, et al. Sustained effects of gene-activated matrices after CNS injury. Mol Cell Neurosci. 2001;17:706–16.
199. Smetana K. Cell biology of hydrogels. Biomaterials. 1993;14:1046–50.
200. Kong HJ, Hsiong S, Mooney DJ. Nanoscale cell adhesion ligand presentation regulates nonviral gene delivery and expression. Nano Lett. 2007;7:161–6.
201. Schek RM, Hollister SJ, Krebsbach PH. Delivery and protection of adenoviruses using biocompatible hydrogels for localized gene therapy. Mol Ther. 2004;9:130–8.
202. Pascher A, Palmer G, Steinert A, Oligino T, Gouze E, Gouze J, et al. Gene delivery to cartilage defects using coagulated bone marrow aspirate. Gene Ther. 2004;11:133–41.
203. Shen H, Tan J, Saltzman WM. Surface-mediated gene transfer from nanocomposites of controlled texture. Nat Mater. 2004;3:569–74.
204. Kumta PN, Sfeir C, Lee DH, Olton D, Choi D. Nanostructured calcium phosphates for biomedical applications: novel synthesis and characterization. Acta Biomater. 2005;1:65–83.

205. Olton D, Li JH, Wilson ME, Rogers T, Close J, Huang L, et al. Nanostructured calcium phosphates (NanoCaPs) for non-viral gene delivery: Influence of the synthesis parameters on transfection efficiency. Biomaterials. 2007;28:1267–79.
206. Newland B, Moloney TC, Fontana G, Browne S, Abu-Rub MT, Dowd E, et al. The neurotoxicity of gene vectors and its amelioration by packaging with collagen hollow spheres. Biomaterials. 2013;34:2130–41.
207. Guan S, Rosenecker J. Nanotechnologies in delivery of mRNA therapeutics using nonviral vector-based delivery systems. Gene Ther. 2017;24:133–43.
208. Frisch J, Rey-Rico A, Venkatesan JK, Schmitt G, Madry H, Cucchiarini M. Chondrogenic differentiation processes in human bone marrow aspirates upon rAAV-mediated gene transfer and overexpression of the insulin-like growth factor I. Tissue Eng Part A. 2015;21:2460–71.
209. Rajangam T, An SS. Fibrinogen and fibrin based micro and nano scaffolds incorporated with drugs, proteins, cells and genes for therapeutic biomedical applications. Int J Nanomed. 2013;8:3641–62.
210. Ahmed TA, Dare EV, Hincke M. Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng Part B Rev. 2008;14:199–215.
211. Dare EV, Griffith M, Poitras P, Kaupp JA, Waldman SD, Carlsson DJ, et al. Genipin cross-linked fibrin hydrogels for in vitro human articular cartilage tissue-engineered regeneration. Cells Tissues Organs. 2009;190:313–25.
212. Moncion A, Arlotta KJ, Kripfgans OD, Fowlkes JB, Carson PL, Putnam AJ, et al. Design and characterization of fibrin-based acoustically responsive scaffolds for tissue engineering applications. Ultrasound Med Biol. 2016;42:257–71.
213. Raut SD, Lei P, Padmashali RM, Andreadis ST. Fibrin-mediated lentivirus gene transfer: implications for lentivirus microarrays. J Control Release. 2010;144:213–20.
214. Lei P, Padmashali RM, Andreadis ST. Cell-controlled and spatially arrayed gene delivery from fibrin hydrogels. Biomaterials. 2009;30:3790–9.
215. Sahni A, Odrljin T, Francis CW. Binding of basic fibroblast growth factor to fibrinogen and fibrin. J Biol Chem. 1998;273:7554–9.
216. Mosesson MW. Fibrinogen and fibrin structure and functions. J Thromb Haemost. 2005;3:1894–904.
217. Tabele C, Montana M, Curti C, Terme T, Rathelot P, Gensollen S, et al. Organic glues or fibrin glues from pooled plasma: efficacy, safety and potential as scaffold delivery systems. J Pharm Pharm Sci. 2012;15:124–40.
218. de la Puente P, Ludena D. Cell culture in autologous fibrin scaffolds for applications in tissue engineering. Exp Cell Res. 2014;322:1–11.
219. Noori A, Ashrafi SJ, Vaez-Ghaemi R, Hatamian-Zaremi A, Webster TJ. A review of fibrin and fibrin composites for bone tissue engineering. Int J Nanomed. 2017;12:4937–61.
220. Gasperini L, Mano JF, Reis RL. Natural polymers for the microencapsulation of cells. J R Soc Interface. 2014;11:20140817.
221. Wittmann K, Storck K, Muhr C, Mayer H, Regn S, Staudenmaier R, et al. Development of volume-stable adipose tissue constructs using polycaprolactone-based polyurethane scaffolds and fibrin hydrogels. J Tissue Eng Regen Med. 2016;10:E409–18.
222. Eyrich D, Wiese H, Mailer G, Skodacek D, Appel B, Sarhan H, et al. In vitro and in vivo cartilage engineering using a combination of chondrocyte-seeded long-term stable fibrin gels and polycaprolactone-based polyurethane scaffolds. Tissue Eng. 2007;13:2207–18.
223. Almany L, Seliktar D. Biosynthetic hydrogel scaffolds made from fibrinogen and polyethylene glycol for 3D cell cultures. Biomaterials. 2005;26:2467–77.
224. Oss-Ronen L, Seliktar D. Polymer-conjugated albumin and fibrinogen composite hydrogels as cell scaffolds designed for affinity-based drug delivery. Acta Biomater. 2011;7:163–70.
225. Frisman I, Seliktar D, Bianco-Peled H. Nanostructuring PEG-fibrinogen hydrogels to control cellular morphogenesis. Biomaterials. 2011;32:7839–46.

226. Jiang B, Waller TM, Larson JC, Appel AA, Brey EM. Fibrin-loaded porous poly(ethylene glycol) hydrogels as scaffold materials for vascularized tissue formation. Tissue Eng Part A. 2013;19:224–34.
227. Rao RR, Peterson AW, Ceccarelli J, Putnam AJ, Stegemann JP. Matrix composition regulates three-dimensional network formation by endothelial cells and mesenchymal stem cells in collagen/fibrin materials. Angiogenesis. 2012;15:253–64.
228. Lee F, Kurisawa M. Formation and stability of interpenetrating polymer network hydrogels consisting of fibrin and hyaluronic acid for tissue engineering. Acta Biomater. 2013;9: 5143–52.
229. King VR, Alovskaya A, Wei DYT, Brown RA, Priestley JV. The use of injectable forms of fibrin and fibronectin to support axonal ingrowth after spinal cord injury. Biomaterials. 2010;31:4447–56.
230. Lorentz KM, Kontos S, Frey P, Hubbell JA. Engineered aprotinin for improved stability of fibrin biomaterials. Biomaterials. 2011;32:430–8.
231. Roura S, Galvez-Monton C, Bayes-Genis A. Fibrin, the preferred scaffold for cell transplantation after myocardial infarction? An old molecule with a new life. J Tissue Eng Regenerative Med. 2017;11:2304–13.
232. Ye L, Chang YH, Xiong Q, Zhang P, Zhang L, Somasundaram P, et al. Cardiac repair in a porcine model of acute myocardial infarction with human induced pluripotent stem cell-derived cardiovascular cells. Cell Stem Cell. 2014;15:750–61.
233. Johnson PJ, Parker SR, Sakiyama-Elbert SE. Controlled release of neurotrophin-3 from fibrin-based tissue engineering scaffolds enhances neural fiber sprouting following subacute spinal cord injury. Biotechnol Bioeng. 2009;104:1207–14.
234. Wilems TS, Pardieck J, Iyer N, Sakiyama-Elbert SE. Combination therapy of stem cell derived neural progenitors and drug delivery of anti-inhibitory molecules for spinal cord injury. Acta Biomater. 2015;28:23–32.
235. Agbay A, Edgar JM, Robinson M, Styan T, Wilson K, Schroll J, et al. Biomaterial strategies for delivering stem cells as a treatment for spinal cord injury. Cells Tissues Organs. 2016;202:42–51.
236. Iwaya K, Mizoi K, Tessler A, Itoh Y. Neurotrophic agents in fibrin glue mediate adult dorsal root regeneration into spinal cord. Neurosurgery. 1999;44:589–95.
237. Hyatt AJT, Wang D, van Oterendorp C, Fawcett JW, Martin KR. Mesenchymal stromal cells integrate and form longitudinally-aligned layers when delivered to injured spinal cord via a novel fibrin scaffold. Neurosci Lett. 2014;569:12–7.
238. Ito K, Yamada Y, Naiki T, Ueda M. Simultaneous implant placement and bone regeneration around dental implants using tissue-engineered bone with fibrin glue, mesenchymal stem cells and platelet-rich plasma. Clin Oral Implan Res. 2006;17:579–86.
239. Galler KM, Brandl FP, Kirchhof S, Widbiller M, Eidt A, Buchalla W, et al. Suitability of different natural and synthetic biomaterials for dental pulp tissue engineering. Tissue Eng Part A. 2018;24:234–44.
240. Haleem AM, Singergy AA, Sabry D, Atta HM, Rashed LA, Chu CR, et al. The clinical use of human culture-expanded autologous bone marrow mesenchymal stem cells transplanted on platelet-rich fibrin glue in the treatment of articular cartilage defects: a pilot study and preliminary results. Cartilage. 2010;1:253–61.
241. Handl M, Trc T, Hanus M, Stastny E, Fricova-Poulova M, Neuwirth J. et al [Therapy of severe chondral defects of the patella by autologous chondrocyte implantation]. Acta Chir Orthop Traumatol Cechoslovaca. 2006;73:373–9.
242. Horch RE, Kopp J, Kneser U, Beier J, Bach AD. Tissue engineering of cultured skin substitutes. J Cell Mol Med. 2005;9:592–608.
243. Yamamoto M, Yanaga H, Nishina H, Watabe S, Mamba K. Fibrin stimulates the proliferation of human keratinocytes through the autocrine mechanism of transforming growth factor-alpha and epidermal growth factor receptor. Tohoku J Exp Med. 2005;207:33–40.

244. Bacakova M, Musilkova J, Riedel T, Stranska D, Brynda E, Zaloudkova M, et al. The potential applications of fibrin-coated electrospun polylactide nanofibers in skin tissue engineering. Int J Nanomed. 2016;11:771–89.
245. Khor HL, Ng KW, Htay AS, Schantz JT, Teoh SH, Hutmacher DW. Preliminary study of a polycaprolactone membrane utilized as epidermal substrate. J Mater Sci Mater Med. 2003;14:113–20.
246. Sahni A, Francis CW. Vascular endothelial growth factor binds to fibrinogen and fibrin and stimulates endothelial cell proliferation. Blood. 2000;96:3772–8.
247. Sanchez Munoz I, Granados R, Holguin Holgado P, Garcia Vela JA, Casares C, Casares M. The use of adipose mesenchymal stem cells and human umbilical vascular endothelial cells on a fibrin matrix for endothelialized skin substitute. Tissue Eng Part A. 2015;21:214–23.
248. McManus MC, Boland ED, Koo HP, Barnes CP, Pawlowski KJ, Wnek GE, et al. Mechanical properties of electrospun fibrinogen structures. Acta Biomater. 2006;2:19–28.
249. McManus MC, Boland ED, Simpson DG, Barnes CP, Bowlin GL. Electrospun fibrinogen: Feasibility as a tissue engineering scaffold in a rat cell culture model. J Biomed Mater Res Part A. 2007;81A:299–309.
250. Sell SA, Francis MP, Garg K, McClure MJ, Simpson DG, Bowlin GL. Cross-linking methods of electrospun fibrinogen scaffolds for tissue engineering applications. Biomed Mater. 2008;3:045001.
251. Ravichandran R, Seitz V, Venugopal JR, Sridhar R, Sundarrajan S, Mukherjee S, et al. Mimicking native extracellular matrix with phytic acid-crosslinked protein nanofibers for cardiac tissue engineering. Macromol Biosci. 2013;13:366–75.
252. Perumcherry SR, Chennazhi KP, Nair SV, Menon D, Afeesh R. A novel method for the fabrication of fibrin-based electrospun nanofibrous scaffold for tissue-engineering applications. Tissue Eng Part C Med. 2011;17:1121–30.
253. Wlodarczyk-Biegun MK, Del Campo A. 3D bioprinting of structural proteins. Biomaterials. 2017;134:180–201.
254. Cui XF, Boland T. Human microvasculature fabrication using thermal inkjet printing technology. Biomaterials. 2009;30:6221–7.
255. Tasoglu S, Demirci U. Bioprinting for stem cell research. Trends Biotechnol. 2013;31:10–9.
256. Phillippi JA, Miller E, Weiss L, Huard J, Waggoner A, Campbell P. Microenvironments engineered by inkjet bioprinting spatially direct adult stem cells toward muscle- and bone-like subpopulations. Stem Cells. 2008;26:127–34.
257. Miller ED, Li K, Kanade T, Weiss LE, Walker LM, Campbell PG. Spatially directed guidance of stem cell population migration by immobilized patterns of growth factors. Biomaterials. 2011;32:2775–85.
258. Campbell PG, Miller ED, Fisher GW, Walker LM, Weiss LE. Engineered spatial patterns of FGF-2 immobilized on fibrin direct cell organization. Biomaterials. 2005;26:6762–70.
259. Mogford JE, Tawil B, Jia SX, Mustoe TA. Fibrin sealant combined with fibroblasts and platelet-derived growth factor enhance wound healing in excisional wounds. Wound Repair Regeneration. 2009;17:405–10.

Part III
Nanotechnologies and Biomimetic

Chapter 6
Advances in Nanotechnologies for the Fabrication of Silk Fibroin-Based Scaffolds for Tissue Regeneration

Nicolò Nicoli Aldini and Milena Fini

Abstract Silks are protein fibers produced by silkworms whose architecture is based on two proteins: fibroin and sericin. Because sericin has been recognized as the main cause of silk's poor performance due to its antigenicity, fibroin alone has now remained popular as a biomaterial, also due to its strength and mechanical properties. Other advantages of this biological product are the water-based processing, biodegradability, and the presence of easily accessible chemical groups for functional modifications. Due to its versatility, fibroin is now widely considered for use in the manufacture of many biological devices and substitutes in different medical fields, with very different biological, physiological, and mechanical properties. In recent years, nanomaterials have gained considerable attention also in tissue engineering, because they exhibit properties that are significantly different to corresponding bulk materials, such as large surface area, increased strength, and enhanced surface reactivity, thus improving material performance. Reviewed studies, mainly in the regeneration of the musculoskeletal system, have been outlined the advantages of fibroin as a scaffold, and the technologies adopted for the nanostructure development of this protein. Further advancements will open up new perspectives in the use of this product in tissue regeneration. Silk-based materials are of particular interest where controlled biodegradation and good mechanical properties are required, such as in tissue engineering of musculoskeletal tissues. Their versatility in processing, biocompatibility properties, ease of sterilization, thermal stability, possibility for surface chemical modifications, and controllable degradation therefore make silk-derived proteins promising biomaterials for many

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clinical functions. Since research into these applications is quite new, we can expect interesting future developments, in which the nanotechnologies might play a decisive role.

Keywords Silk fibroin · Nanotechnologies · Nanomaterials · Biomaterials Biocompatibility · Composite materials · Preclinical studies · Hard tissues In vitro study · In vivo study

Silks are protein fibers produced by silkworms (*Bombyx mori*), but also by spiders and others arthropods. The architecture of silk is based on two proteins: fibroin, which is the core filament, and sericin, which is a protein that glues fibroin fibers together.

Although silk was used extensively in surgery for making suture threads, this application has now been replaced by synthetic materials with greater histocompatibility than natural ones. Benefits and drawbacks of silks for biomaterial applications have been well detailed by Altman, Vepari, and other authors and are summarized in Table 6.1 [1, 2]. More recently, because sericin has been recognized as the main cause of silk's poor performance due to its antigenicity, fibroin alone has remained popular as a biomaterial.

Fibroin is made of highly organized crystals and semi-crystalline regions that account for its elasticity. The primary structure is mainly composed of the amino acids glycine, alanine, serine, valine, and tyrosine with characteristic sequences. These structural elements produce the strength and resiliency of silk fibroin [3]. Indeed, silk has interesting mechanical properties regarding its use as biomaterial. Other advantages of this biological product are its water-based processing, biodegradability, and the presence of easily accessible chemical groups for functional modifications [4].

Nowadays, tissue engineering procedures have become widespread in regenerative medicine for the treatment of diseases when standard medical procedures fail. Regenerative strategies are based on the combination of three main tools: cells (differentiated and not differentiated), scaffolds, and growth factors. Silk fibroin also

Table 6.1 Benefits and drawbacks of silks for biomedical applications

Benefits
Mechanical properties
Prolonged history of use in clinical applications
Manufacturing in water solution and easy insolubilization
Easy chemical modifications
Slow in vivo degradation
Easy functionalization
Drawbacks
Adequate removal of sericin required
Slow degradation of crystalline regions
Possible formation of granuloma
Sensitization and possible allergic response

has received much attention as a scaffold material, due to its above-mentioned biocompatibility, processability, biodegradability, and mechanical and thermal properties [5]. Scaffolds should mimic tissue extracellular matrix (ECM) in biological and chemical composition and physical structure [6]. Mimicking the nanofibrous structures of ECM to achieve better biocompatibility remains a challenge [7].

In recent years, nanomaterials have gained considerable attention also in tissue engineering, because they exhibit properties that are significantly different to corresponding bulk materials, such as large surface area, increased strength, and enhanced surface reactivity, thus improving material performance. The definition adopted by the International Organization for Standardization (ISO/TS 27687:2008) is: "Material with any external dimension in the nanoscale or having internal structure in the nanoscale" defining "Nanoscale" as a size range from approximately 1 to 100 nm. Polymeric nanofiber matrix is similar to fibrous ECM proteins and is thus a candidate as ECM-mimetic biomaterial [6].

Due to its above-mentioned properties, fibroins now being widely considered for use in the manufacture of many biological devices and substitutes. Table 6.2 shows several experimental and clinical studies on the possible use of this product in different medical fields.

Such a wide range of applications, with very different biological, physiological, and mechanical properties, requires of course an equally wide range of ways to manage the product to make it suitable for soft and hard tissue substitution. Following the physicochemical characterization, appropriate preclinical investigations, with both in vitro and in vivo tests, must be planned to validate novel production, based on fibroin alone or as a composite. Some studies mainly in the

Table 6.2 Applications of silk fibroinbiomaterials for tissue regeneration

Organ/apparatus	Applications[a]	Authors
Vascular tissue	Flow diverting devices, stents (C)	[8, 9]
Neural tissue	Peripheral nerve conduits (E)	[10]
Skin	Composite scaffolding membranes	[11]
Bone	Composite scaffolds with/without addition of biological factors (E)	[12, 13, 14]
Cartilage	Scaffolds to support chondrocytes (E)	[15, 16]
Ligament and Tendon	Scaffolds and scaffold-free ligaments, composites (E)	[17, 18]
Cardiac tissue	Cardiac patch composite + mesenchymal cells (E)	[19, 20]
Ocular tissue	Cornea replacement (E)	[21, 22]
Hepatic tissue	Silk fibroin–collagen blended films (E)	[23]
Intervertebral tissue	Porous scaffolds (E)	[24]
Bladder	Wall repair and reconstruction (E)	[25]
Eardrum tissue	Silk-based membranes (E)	[26]

[a]C *clinical*; E *experimental*

regeneration of the musculoskeletal system have been reviewed to focus on the use of fibroin as a scaffold and the technologies adopted for the nanostructure development.

Following a preclinical protocol, for example, in an in vitro and in vivo study Fini et al. [27] evaluated the behavior of an injectable silk fibroin hydrogel through osteoblast cultures and after implantation in critical size defects of rabbit distal femurs, using synthetic poly (D, L-lactide–glycolide) copolymer as control material. In vitro biocompatibility was evaluated by measuring cytotoxicity and cytocompatibility on human osteoblast-like cell line (MG 63), whereas in vivo the bone defect healing rate and quality of the newly formed bone inside the defects were determined by measuring histomorphometric parameters, such as trabecular bone volume, trabecular thickness, trabecular number, and trabecular separation. In vitro tests indicated that both materials significantly increased cell proliferation in comparison with the negative control. Both materials promoted bone healing when used to fill critical size defects in rabbit femurs, but the histomorphometry showed better results in new-formed bone of the silk fibroin hydrogel-treated defects in comparison with the control gel. The regrown bone of the Silk fibroin hydrogel-treated defects appeared to be more similar to normal bone than that of the control synthetic polymeric material-treated defects, in comparison with controls treated with a synthetic polymeric material, thus suggesting that silk fibroin hydrogel can accelerate remodeling processes. Like this study, which is aimed at hard tissue repair, many others describe a range of scaffold preparation procedures and the tissues to be replaced.

Electrospinning technology, which uses an electrical charge to draw very fine fibers on the micro- or nanoscale, enables porous nanofibrous scaffolds to be obtained, which are able to mimic the ECM. Considering the physical–chemical properties and the structure of the scaffolds, micro- and nanoparticles can be obtained from silk solutions by various procedures, such as freeze-drying and grinding procedures, spray drying, jet breaking, self-assembly, and freeze-thawing. Milling of silk fibers is also an option to obtain silk particles using any chemicals. According to Kundu et al. [4], these particles can play the dual role of improving mechanical properties of scaffolds and at the same time act as a carrier of growth factors for rapid tissue regeneration. Indeed to improve mechanical and biological performances, inorganic or organic fillers have been incorporated in silk 3D scaffolds during or after fabrication to obtain composites. The main advantage is in this case the compatibility between the components. Consequences of a poor compatibility may result in inhomogeneous mixtures, phase separation, and adverse tissue reactions. To obtain better compatibility, silk–silk composite scaffolds are made by incorporating milled silk particles in porous silk sponge, resulting in a significant improvement in mechanical properties.

With respect to the material porosity, different methods of processing are presented in the literature. The importance of the processing method is highlighted by Kuboyama et al. These authors evaluated the porosity of scaffolds prepared using regenerated *Bombyx mori* silk fibroin dissolved either in water (AF) orin hexafluoroisopropanol (HFIP). The two preparations were comparatively analyzed

in an animal model in which the formation and growth of new bone in the implantation site (rabbit femoral epicondyle) was examined by means of micro-CT and histology. The AF scaffold exhibited significantly greater osteoconductivity than that obtained by the protein dissolved in HFIP. Micro-CT analysis showed that the morphology of the newly formed bone differed significantly in the two types of silk fibroin scaffold. After 4 weeks of implantation, new trabecular bone was seen inside the pores of the AF scaffold implant, whereas the HFIP scaffold only contained necrotic cells. No trabecular bone was seen within the pores of the latter scaffolds, although the pores of these did contain giant cells and granulation tissue [28].

Lin et al. [29] evaluated silk fibroin with different nanostructures, self-assembled in aqueous solution to improve porous structure formation. In comparison with scaffolds derived from fresh solution, the nanofibrous silk scaffolds showed better cell compatibility in vitro. These observations suggest that the control of silk nanoscale can regulate matrix features including porous structure and nanostructure, which are important in regulating cell and tissue outcomes.

In the specific field of bone tissue engineering, silk fibroin gained much interest as a scaffold material and various strategies were developed to create a three-dimensional (3D) structure with high porosity and osteoconductivity. Salt-leaching or freeze-drying methods were used to create porous scaffolds. Moreover, in attempts to mimic bone properties, the incorporation of ceramic components into silk fibroin scaffolds has also been shown. Fibrous silk fibroin scaffolds were obtained using the electrospinning technique and the bone regeneration in the scaffolds was confirmed; they are considered to be effective in replacing collagen for many tissue engineering applications [30].

The use of non-mulberry tropical silk fibroin as a bioactive polymer in blended nanofibrous matrices resulted in osteoconductive scaffolds. The blending of 2 wt% silk fibroin exhibited higher cell attachment, growth, and ECM formation, when compared to unmodified polyvinyl alcohol as control and the other blend ratio. The mechanical robustness of constructs resembled the original bone tissue, thus indicating a promising future for this blend in bone regeneration and reconstruction. This in vitro study, therefore, lays the foundation for designing clinically relevant orthopedic grafts in vivo [31]. Considering in particular, the behavior of scaffolds interacting with cells, Teuschl et al. [32] found that surface modifications of fibroin with carbohydrate-binding protein lectin enhanced the adhesion of cells, in particular adipose-derived stromal cells; the proliferation and differentiation in osteogenic lineages were however not influenced. Considering that silk fibroin is used to obtain scaffolds for bone tissue engineering, this possibility may be of practical interest. Uchida et al. [33] also showed that plasma-irradiated silk fibroin was a regulator of bone matrix properties in an animal model based on the placement of scaffolds in critical size defects in rabbit femurs, which increased bone matrix, mineral concentration, cortical thickness, and trabecular bone volume.

Fibroin fibers can also be used as composites with other polymers. Yang et al. [34] studied different types of scaffolds based on silk fibroin and poly (L-lactide-*co*-caprolactone) blends intended for tendon tissue engineering. Biocompatibility

analysis revealed that bone marrow-derived mesenchymal stem cells exhibited a higher proliferation rate when cultured on nanofibrous scaffolds compared with the other scaffolds. The mechanical testing results indicated that the tensile properties of the nanofibrous scaffolds were reinforced in the direction parallel to the nano-yarns and fulfilled the mechanical requirements for tendon repair.

Teimouri et al. [35] proposed a nanocomposite with silk fibroin–chitosan/Nano ZrO_2 for tissue engineering. The scaffold was found to possess a porous nature with pore dimensions suitable for cell infiltration and colonization.

Kishimoto et al. [36] observed that the incorporation of montmorillonite, a very soft crystalline silicate mineral in silk fibers would improve their physical properties. This nanocomposite might be considered for new biocompatible applications, such as scaffolds for tissue engineering like bone regeneration, because of the osteoinductive montmorillonite properties and biodegradable and biocompatible silk presence.

Hydrogel products constitute a group of polymeric materials, the hydrophilic structure of which renders them capable of holding large amounts of water in their 3D networks. Hydrogels can be formulated in different physical forms, such as micro- and nanoparticles, coatings, and films. Kim et al. [37] studied silk fibroin/hydroxyapatite composite hydrogels obtained with different hydroxyapatite contents in fibroin matrix. Previous studies have shown that fibroin can be easily manufactured into hydrogel without additional crosslinking reagents or toxic chemicals. Therefore, the fibroin hydrogel manufacturing process is not only physiologically safe but also biocompatible. For bone regeneration, this offers interesting properties for silk-based scaffolds to be used in critical size bone defects and cartilage.

Chen et al. [5] studied the potential of a Silk Fibroin 3D scaffold produced by additive manufacturing technology, which can be used to engineer tissues that are structurally complex, because it is capable of producing precise architectural features using a layer-by-layer approach based on computer-aided design, to obtain a scaffold for cartilage engineering. These authors verified the presence of micropores and interconnected channels within the scaffold by scanning electron microscopic observations. In vitro cell culture within the fibroin scaffold using porcine articular chondrocytes showed a steady increase in cell numbers, thus giving positive indications for the use of the scaffold for cartilage repair.

Again in the field of osteoarticular apparatus, considering its application as a tendon and ligament substitute, Farè et al. [38] performed studies on a novel structure made of silk fibroin able to match the mechanical performance requirements of anterior cruciate ligament. In particular, these authors evaluated in vitro cell ingrowth in sericin-free, silk fibroin knitted sheath with braided core structure. Micro-CT analysis confirmed that the core was highly porous and had a higher degree of interconnectivity than that observed for the sheath. The in vivo cell colonization of the scaffolds is thus expected to penetrate even the internal parts of the structure. Tensile mechanical tests confirmed the scaffold's suitability for anterior cruciate ligament reconstruction. In vitro tests with fibroblasts revealed the absence of cytotoxic substances in the extracts. ESM obtained with human

periodontal ligament Fibroblasts cultured in direct contact with fibroin, compared to control samples, displayed an increased secretion of aggrecan and fibronectin (FBN) at 3 and 7 days of culture, and no change in IL-6 and TNF-α secretion, thus suggesting the usefulness of this novel scaffold for tendon tissue regeneration.

Nanotechnology and tissue engineering are widely involved in the achievement of constructs for skin tissue regeneration. Gandhimathi et al. [39] performed a study to evaluate the applications of composite copolymers of polylactic acid and poly-(ε-caprolactone) with silk fibroin, vitamin E, and curcumin, as nanofibrous scaffolds, to assess their potential as substrates for the culture of human dermal fibroblasts for skin tissue engineering. Scaffolds were made by electrospinning and characterized by fiber morphology, membrane porosity, wettability, mechanical strength, and chemical properties. Human dermal fibroblasts were cultured on these scaffolds, and the cell–scaffold interactions were evaluated. The results showed that human dermal fibroblasts cultured on nanofibrous scaffolds proved to be a potential support for skin regeneration.

Suganya et al. [40] also evaluated a hybrid biomaterial for dermal substitutes, based on nanofibrous silk fibroin scaffold. The scaffold was made by the electrospinning technique and the physical–chemical characterization showed improved hydrophilic properties and favorable tensile strain, as well as a favorable cell proliferation.

Finally, magnetic fibroin scaffolds were also evaluated, by integrating magnetic materials and fibroin scaffolds, for potential use in magnetic-field-assisted tissue engineering. Magnetic nanoparticles were introduced into scaffolds at different strengths of magnetization. The scaffolds were evaluated in vitro and were found not to be toxic to cells and improved cell adhesion and proliferation. These findings suggest that magnetized silk-based biomaterials can be engineered with interesting features for biomaterials and tissue engineering applications [41].

Besides these applications as a support for tissue regeneration, fibroin shows interesting properties for implantable systems for the controlled release of drugs.

Indeed, nanostructured materials are now frequently used in drug delivery systems.

Achieving efficient drug delivery systems is a way to improve new routes of administration of therapeutic agents. Silk fibroin is a suitable material for incorporation into a variety of drug delivery vehicles capable of delivering a range of therapeutic agents. Indeed, silk fibroin matrices have been shown to successfully deliver anticancer drugs, small molecules, and biomolecules [42]. In a study by Subia [43], silk fibroin–albumin blended nanoparticles were made using the desolvation method and evaluated as carriers for the delivery of methotrexate. They found promising properties as a nanocarrier for the delivery of drugs and other bioactive molecules.

Conclusions

The manifold investigations that span through very different fields of applications concur that fibroin is a promising biomaterial. Further advancements will open up new perspectives in the use of this product in tissue regeneration. Silk-based materials are of particular interest where slow biodegradation and good mechanical properties are required, such as in tissue engineering of bone, ligaments, and musculoskeletal tissues.

Their versatility in processing, biocompatibility properties, ease of sterilization, thermal stability, possibility for surface chemical modifications, and controllable degradation therefore make silk-derived proteins promising biomaterials for many clinical functions. Since research into these applications is quite new, we can expect interesting future developments, in which the nanotechnologies might play a decisive role.

References

1. Altman GH, Diaz F, Jakuba C, Calabro T, Horan RL, Chen J, Lu H, Richmond J, Kaplan DL. Silk-based biomaterials. Biomaterials. 2003;24:401–16.
2. Vepari C, Kaplan DL. Silk as a biomaterial. Prog Polym Sci. 2007;32:991–1007.
3. Meinel L, Hofmann S, Karageorgiou V, Kirker-Heade C, McCoole J, Gronowicz G, Zichner L, Langer R, Vunjak-Novakovica G, Kaplan DL. The inflammatory responses to silk films in vitro and in vivo. Biomaterials. 2005;26:147–55.
4. Kundu B, Rajkhowa R, Kundu SC, Wang X. Silk fibroin biomaterials for tissue regenerations. Advanc Drug Deliv Rev. 2013;65:457–70.
5. Chen CH, Liu JM, Chua CK, Chou SM, Shyu VBH, Chen JP. Cartilage tissue engineering with silk fibroin scaffolds fabricated by indirect additive manufacturing technology. Materials. 2014;7:2104–19.
6. Ma Z, Kotaki M, Inai R, Ramakrishna S. Potential of nanofiber matrix as tissue-engineering scaffolds. Tissue Eng. 2005;11(1–2):101–9.
7. Lu G, Liu S, Lin S, Kaplan DL, Lu Q. Silk porous scaffolds with nanofibrous microstructures and tunable properties. Coll Surf B Biointerfaces. 2014;120:28–37.
8. Causin F, Pascarella R, Pavesi G, Marasco R, Zambon G, Battaglia R, Munari M. Acute endovascular treatment (48 hours) of uncoilable ruptured aneurysms at non-branching sites using silk flow-diverting devices. Interv Neuroradiol. 2011;17(3):357–64.
9. Leonardi M, Cirillo L, Toni F, Dall'Olio M, Princiotta C, Stafa A, Simonetti L, Agati R. Treatment of intracranial aneurysms using flow-diverting silk stents (BALT): a single centre experience. Interv Neuroradiol. 2011;17(3):306–15.
10. Wang CY, Zhang K-H, Fan CY, Mo XM, Ruan HJ, Li FF. Aligned natural–synthetic polyblend nanofibers for peripheral nerve regeneration. Acta Biomater. 2011;7:634–43.
11. Yoo CR, Yeo IS, Park KE, Park JH, Lee SJ, Park WH, Min BM. Effect of chitin/silk fibroin nanofibrous bicomponent structures on interaction with human epidermal keratinocytes. Int J Biol Macromol. 2008;42:324–34.
12. Meinel L, Betz O, Fajardo R, Hofmann S, Nazarian A, Cory E, Hilbe M, McCool J, Langer R, Vunjak-Novakovic G, Merkle HP, Rechenberg B, Kaplan DL, Kirker-Head C. Silk based biomaterials to heal critical sized femur defects. Bone. 2006;39:922–31.

13. Li C, Vepari C, Jin HJ, Kim HJ, Kaplan DL. Electrospun silk-BMP-2 scaffolds forbone tissue engineering. Biomaterials. 2006;27:3115–24.
14. Kweon H, Lee K-G, Chae C-H, Balázsi C, Min S-K, Kim JY, Choi JY, Kim SG. Development of nano-hydroxyapatite graft with silk fibroin scaffold as a new bone substitute. J Oral Maxillofacc Surg. 2011;69:1578–86.
15. Wang Y, Blasioli DJ, Kim HJ, Kim HS, Kaplan DL. Cartilage tissue engineering with silk scaffolds and human articular chondrocytes. Biomaterials. 2006;27:4434–42.
16. Gellynck K, Verdonk PCM, Nimmen EV, Almqvist KF, Gheysens T, Schokens G, Langenhove LV, Kiekens P, Mertens J, Verbruggen G. Silkwormand spider silk scaffolds for chondrocyte support. J Mater Sci Mater Med. 2008;19:3399–409.
17. Chen X, Qi YY, Wang LL, Yin Z, Yin GL, Zou XH, Ouyang HW. Ligament regeneration using a knitted silk scaffold combined with collagen matrix. Biomaterials. 2008;29:3683–92.
18. Sahoo S, LokToh S, Hong JC. Goh PLGA nanofiber-coated silk microfibrous scaffold for connective tissue engineering. J Biomed Mater Res B Appl Biomater. 2010;95B:19–28.
19. Yang MC, Wang SS, Chou NK, Chi NH, Huang YY, Chang YL, Shieh MJ, Chung TW. The cardiomyogenic differentiation of rat mesenchymal stem cells on silk fibroin– polysaccharide cardiac patches in vitro. Biomaterials. 2009;30:3757–65.
20. Patra C, Talukdar S, Novoyatleva T, Velagala SR, Mühlfeld C, Kundu B, Kundu SC, Engel FB. Silk protein fibroin from Antheraea mylitta for cardiac tissue engineering. Biomaterials. 2012;33:2673–80.
21. Yang T, Zhang M. Potential of nanofiber matrix as tissue-engineering scaffolds. Int J Ophthalmol. 2008;8:1557–9.
22. Lawrence BD, Marchant JK, Pindrus MA, Omenetto FG, Kaplan DL. Silk film biomaterials for cornea tissue engineering. Biomaterials. 2009;30:1299–308.
23. Cirillo B, Morra M, Catapano G. Adhesion and function of rat liver cells adherentto silk fibroin/collagen blend films. Int J Artif Organs. 2004;27:60–8.
24. Chang G, Kim HJ, Vunjak-Novakovic G, Kaplan DL, Kandel R. Enhancing annulusfibrosus tissue formation in porous silk scaffolds. J Biomed Mater Res A. 2010;92A:43–51.
25. Cannon GM Jr, Mauney JR, Gong EM, Yu RN, Adam RM, Estrada CR. Silk Asa novel biomaterial in bladder tissue engineering. J Pediatr Urol. 2010;6(S1):S82.
26. Ghassemifar R, Redmond S, Chirila TV, Zainuddin. Advancing towards a tissue-engineered tympanic membrane: silk fibroin as a substratum for growing human eardrum keratinocytes. J Biomater Appl. 2010;24:591–606.
27. Fini M, Motta A, Torricelli P, Giavaresi G, Nicoli Aldini N, Tschon M, Giardino R, Migliaresi C. The healing of confined critical size cancellous defects in the presence of silk fibroin hydroge. Biomaterials. 2005;26:3527–36.
28. Kuboyama N, Kiba H, Arai K, Uchida R, Tanimoto Y, Bhawal UK, Abiko Y, Miyamoto S, Knight D, Asakura T, Nishiyama N. Silk fibroin-based scaffolds for bone regeneration. J Biomed Mater Res Part B Appl Biomater. 2013;2013(101B):295–302.
29. Lin S, Lu G, Liu S, Bai S, Liu X, Lu Q, Zuo B, Kaplan DL, Zhu H. Nanoscale control of silks for nanofibrous scaffold formation with improved porous structure. J Mater Chem B Mater Biol Med. 2014;2(17):2622–33.
30. Kim H, Che L, Ha Y, Ryu W. Mechanically-reinforced electrospun composite silk fibroin nanofibers containing hydroxyapatite nanoparticles. Mater Sci Eng. 2014;40:324–35.
31. Bhattacharjee P, Kundu B, Naskar D, Maiti TK, Bhattacharya D, Kundu SC. Nanofibrous Nonmulberry Silk/PVA Scaffold for Osteoinduction and Osseointegration. Biopolymers. 2015;103:271–84.
32. Teuschl AH, Neutsch L, Monforte X, Rünzler D, van Griensven M, Gabor F, Redl H. Enhanced cell adhesion on silk fibroin via lectin surface modification. Acta Biomaterialia. 2014;10:2506–17.
33. Uchida R, Bhawal UK, Kiba H, Arai K, Tanimoto Y, Kuboyama N, Asakura T, Nishiyama N. Effect of plasma-irradiated silk fibroin in bone regeneration. J Biosci Bioeng. 2014;118(3): 333e–40e.

34. Yang C, Deng G, Chen W, Ye X, Mo X. A novel electrospun-aligned nanoyarn-reinforced nanofibrous scaffold for tendon tissue engineering. Coll Surf B Biointerfaces. 2014;122:270–6.
35. Teimouri A, Ebrahimi R, Emadi R, Beni BH, Chermahini AN. Nano-composite of silk fibroin–chitosan/Nano ZrO_2 for tissue engineering applications: fabrication and morphology. Int J Biol Macromol. 2015;76:292–302.
36. Kishimoto Y, Ito F, Usamia H, Togawa E, Tsukada M, Morikawa H, Yamanaka S. Nanocomposite of silk fibroin nanofiber and montmorillonite: fabrication and morphology. Int J Biological Macromol. 2013;57:124–8.
37. Kim H, Park JB, Kang MJ, Park YH. Surface-modified silk hydrogel containing hydroxyapatite nanoparticle with hyaluronic acid–dopamine conjugate. Int J Biological Macromol. 2014;70:516–22.
38. Farè S, Torricelli P, Giavaresi G, Bertoldi S, Alessandrino A, Villa T, Fini M, Tanzi MC, Freddi G. In vitro study on silk fibroin textile structure for Anterior Cruciate Ligament regeneration. Mater Sci Eng C. 2013;33:3601–8.
39. Gandhimathi C, Venugopal JR, Bhaarathy V, Ramakrishna S, Kumar SD. Biocomposite nanofibrous strategies for the controlled release of biomolecules for skin tissue regeneration. Int J Nanomed. 2014;9:4709–22.
40. Suganya S, Venugopalc J, Ramakrishnac S, Lakshmib BS, Giri VR. Naturally derived bio functional nanofibrous scaffold for skin tissue regeneration. Int J Biological Macromol. 2014;68:135–43.
41. Samal SK, Dash M, Shelyakova T, Declercq HA, Uhlarz M, Bañobre-López M, Dubruel P, Cornelissen M, Herrmannsdörfer T, Rivas J, Padeletti G, De Smedt S, Braeckmans K, Kaplan DL, Dediu VA. Biomimetic magnetic silk scaffolds. ACS Appl Mater Interfaces. 2015;7(11):6282–92.
42. Mottaghitalab F, Farokhi M, Shokrgozar MA, Atyabi F. Silk fibroin nanoparticle as a novel drug delivery system. J Controll Release. 2015;206:161–76.
43. Subia B, Kundu SC. Drug loading and release on tumor cells using silk fibroin-albumin nanoparticles as carriers. Nanotechnology. 2013;24(3):035103 (Epub 21 Dec 2012).

Chapter 7
Nanoscale Architecture for Controlling Cellular Mechanoresponse in Musculoskeletal Tissues

Francesco Oliva, Clelia Rugiero, Umberto Tarantino and Nicola Maffulli

Abstract Cellular mechanoresponse is not very known yet, especially if we consider the function of nanoscale architecture. First, we need understand the tissue behavior on macroscale and how this feature is transduced in microscale. How the musculoskeletal system (bone, cartilage, tendons, muscles, and ligaments) responses to prestress and to external forces still is unknown for several aspects. Furthermore, focusing the attention to macroscale and microscale changes in the musculoskeletal system after injuries seems very interesting. Try to clarify this knowledge; it is very important to create nanoscale scaffolds able to better improve musculoskeletal tissue healing.

Keywords Musculoskeletal tissues healing · Mechanotransduction Scaffolds · Nanoscale engineering · Bone · Tendon · Muscle · Ligament

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Nanometric Architecture

Mechanoresponsiveness is a complex feature of all living tissues [1, 2]. Experiments with cultured cells confirm that mechanical stresses can directly alter many cellular processes, including signal transduction, gene expression, growth, differentiation, and survival [3–9]. But *which is the mechanism by which mechanical stresses applied on the macroscale are transmitted to individual cells on the microscale and transduced into a biological response*? To understand the mechanoregulation process, we must consider that living organisms, such as humans, are composed of several organs (bone, muscle, blood vessels, nerves) that, in turn, are constructed from tissues (e.g., muscle fibers, vascular endothelium, connective tissue) which are composed of groups of living cells and their associated extracellular matrix (ECM). ECMs are macromolecular complexes composed of different collagens, glycoproteins, proteoglycans, and many others proteins that represent the in vivo scaffolds for cell anchorage [10]. Each cell contains intracellular organelles, a nucleus, lipid membranes, and a viscous cytosol permeating a filamentous cytoskeleton [11, 12]. Each of these subcellular components is composed of aggregates of different molecules. In other words, living systems are neither homogeneous nor isotropic and therefore require the development of appropriate inhomogeneous and anisotropic engineering models to describe their behaviors. The question of how the body senses and responds to mechanical stresses is not simply an issue of the material properties of its components, also it is a problem related to the architectural arrangement of its microstructure [13].

Recent advances in molecular biology have focused the attention on the molecular factors in tissue development. There are many regulatory signals, such as mechanical stresses, that are equally critical for control of tissue form and function. This is perhaps most evident in orthopedics where it is well-known that muscle and bone actively remodel in response to changes in exercise or altered gravity as experienced in spaceflight [14–19].

Nanoscale Engineering

Tissue engineering is aimed at implant or “seeded” living cells as engineering materials into artificial biopolymer structures capable of supporting three-dimensional growth. These structures, called *scaffolds*, are often capable to influence their own microenvironments. Successful regeneration necessitates the development of three-dimensional (3D) tissue-inducing scaffolds that mimic the hierarchical architecture of native tissue extracellular matrix (ECM). Cells in nature recognize and interact with the surface topography they are exposed to via ECM proteins. The interaction of cells with nanotopographical features such as pores, ridges, groves, fibers, nodes, and their combinations has proven to be an important signaling modality in controlling cellular processes. Integrating nanotopographical

cues is especially important in engineering complex tissues that have multiple cell types and require precisely defined cell–cell and cell–matrix interactions on the nanoscale. Thus, in a regenerative engineering approach, nanoscale materials/scaffolds play a paramount role in controlling cell fate and the consequent regenerative capacity. Advances in nanotechnology have generated a new toolbox for the fabrication of tissue-specific nanostructured scaffolds. For example, biodegradable polymers such as polyesters, polyphosphazenes, polymer blends, and composites can be electrospun into ECM-mimicking matrices composed of nanofibers, which provide high surface area for cell attachment, growth, and differentiation [20]. It is also possible to add to the scaffolds different factors that improve the ECM generation.

Tensegrity

Tensegrity is the physiological tension that is generated in the musculoskeletal system, and it is due to the internal stress. The recent convergence between physics and biology has led many physicists to enter the fields of cell and developmental biology. One of the most exciting areas of interest has been the emerging field of mechanobiology that investigates how cells control their mechanical properties and how physical forces regulate cellular biochemical responses, a process that is known as mechanotransduction. Tensegrity (tensional integrity) architecture, which depends on tensile prestress for its mechanical stability, has a central role in biology. ***Prestress*** is the internal stress prior to application of external force, it is a critical governor of cell mechanics and function, and tensegrity can be used by cells contributing to mechanotransduction. Tensegrity also predicts both quantitative and qualitative behaviors of living cells. In addition, tensegrity is used to both stabilize three-dimensional form and to channel forces from the macroscale to the nanoscale, thereby facilitating mechanochemical conversion at the molecular level [21].

Architecture and Prestress

The musculoskeletal system supports the weight of our bodies, allows us to rapidly adjust to resist external forces, and permits us to move freely in our environment. Selective pressures demand that the construction of such a machine minimizes mass with flexibility and without compromising its structural integrity to handle unexpected forces. The musculoskeletal system has evolved to address these demands through optimization of both material properties (how the individual support elements are designed) and architecture (how the different elements are oriented and connected together).

The human skeleton is actually strongly influenced by the architecture of our bodies. Their mechanical behavior depends on how the surrounding tensile

muscles, tendons, and ligaments are joined and oriented in. The internal tension and/or prestress stabilizes the joints: Even when the bones are pulled away from each other, the joint does not dislocate. Actually, there are multiple muscles, tendons, and ligaments that contribute to the structure of each joint and the number and position of these tensile elements play a critical role in defining the joint's potential strength, power, speed, and range of motion.

The local stress patterns are clearly visualized in sections or radiographs of the human femur which demonstrate that the network of trabecular bone is organized to approximate the principal stress directions. This is known by 1892, when Julius Wolff and others realized that mechanical loads can affect bone architecture in living beings, but the mechanisms responsible for this effect were unknown. By 2003, we know how this effect occurs and some of its applications. Our load-bearing bones include tibias, femurs, humeri, vertebrae, radii, mandibles, maxillae, wrists, hips, etc. The strength of such bones and their trabeculae would represent their most important physiologic feature but in the special sense of relative to the size of the typical peak voluntary loads on them. The biologic "machinery" that determines whole-bone strength forms a tissue-level negative feedback system called the mechanostat. Two thresholds make a bone's strains determine its strength by switching on and off the biologic mechanisms that increase or decrease its strength. The largest voluntary loads on load-bearing bones determine most of their strength after birth, and these loads come from muscle. This process affects, in part, the healing of fractures, bone grafts, osteotomies, and arthrodeses; the bone's ability to endure load-bearing joint and dental endoprostheses; why healthy bones are stronger than the minimum needed to keep voluntary loads from breaking them suddenly or from fatigue; some general functions and disorders of bone modeling and basic multicellular unit-based bone remodeling; some limitations of in vitro data and of pharmaceutical actions; and the fact that many bone-active humoral and local agents have permissive roles in a bone's adaptations and healing, instead of forcing them to occur [22].

This observation suggests that the living cells that continually remodel bone are able to sense changes in mechanical stresses in their local environment and that they respond by depositing new ECM where it is needed and removing it from where it is not. This process results in deposition of bone ECM in specific patterns that correspond precisely to engineering lines of tension and compression characteristic for elastic structure of this size and shape with similar loading conditions. It is an example of the importance of cellular mechanotransduction for regulation of tissue morphogenesis. The specialized microarchitecture of cancellous bone further optimizes its structural efficiency (strength/mass ratio). Architectural organization on a smaller size scale (the molecular level) also contributes significantly to the mechanical strength of biologic tissue [23]. In the bone, the matrix of each trabeculum consists of a composite material containing hydroxyapatite crystals embedded within a network of collagen fibrils [24]. The collagen augments the tensile strength of the bone, while the minerals contribute largely to its compressive stiffness and strength. In the living organism, the stress in the bone ECM is influenced by the shape of the entire bone, the pull of the surrounding muscles and

tendons, and its loading conditions. Contractile fibroblasts also prestress the collagen network during the process of tissue development and remodeling, before the surrounding ECM calcifies. Prestress also plays an important role in determining the mechanics of cartilage, tendons, and ligaments [23]. In cartilage, the loose collagen network is stretched open and prestressed by the osmotic force of hydration of embedded proteoglycan molecules [9, 25, 26]; however, the cellular components (chondrocytes) and their internal support elements (cytoskeleton, nucleus) may also bear some mechanical loads [27, 28]. In soft tissues, that are composed mostly of parallel collagen fibers and elastin [29, 30], such as ligaments and tendons, the prestress results from the active contraction of living cells (myofibroblasts) that are embedded within its ECM. The cell contractions pull the collagen into an undulating, buckled structure and keep the ligament under tension at all times [31]. Thus, these soft tissues remodel and adjust their fiber orientations to optimize their load-bearing capacity much like bone and cartilage [23].

Tensegrity and Mechanochemical Transduction

Tensegrity structures are characterized by use of continuous tension and local compression; architecture, prestress, and triangulation play the most critical roles in terms of determining their mechanical stability. In living organisms, mechanical stresses applied at the macroscale result in structural rearrangements at the cell and molecular level. The demonstration of discrete mechanical linkages between cells and their ECM via integrins suggests how mechanical signals resulting from ECM deformation may be transferred across cell surface integrin receptors to distinct structures in the cell and nucleus, including ion channels, nuclear pores, nucleoli, chromosomes, and perhaps even individual genes, independently of ongoing chemical signaling mechanisms [8, 9, 27, 28]. In fact, different studies have demonstrated that signal transduction pathways can be activated within milliseconds after cell surface integrins and associated cytoskeletal connections are mechanically stressed [32]. This type of physical coupling between intracellular structures, cell surface receptors, and the ECM could serve to coordinate, complement, and constrain slower diffusion-based chemical signaling pathways and thus explain how mechanical distortion of ECM caused by gravity or other mechanical stresses can change cell shape, alter nuclear functions, and switch cells between different genetic programs [8, 33]. The question remains: ***how could these tensegrity-based structural rearrangements be transduced into a biochemical response***? Several potential mechanisms can be envisioned. For example, a recent work suggests that much of the cell's metabolic and signal transduction machinery effectively functions in a "solid state" [34]. The enzymes and substrates that mediate these pathways are physically immobilized on the insoluble molecular scaffolds that comprise the cytoskeleton and nuclear matrix (nucleoskeleton). In fact, many signal-transducing molecules that are activated by cell binding to growth factors and ECM actually appear to be concentrated at the site of integrin binding,

on the cytoskeletal backbone of the focal adhesion [35, 36]. Thus, mechanical signals may be integrated with other environmental signals and transduced into a biochemical response through stress-dependent changes in cytoskeletal scaffold geometry or mechanical deformation [9]. For example, one potential mechanism for mechanochemical transduction is through stress-dependent cytoskeletal rearrangements that result in changes in proximity between different immobilized enzymes and substrates. If a protein kinase and its physiological substrate were both immobilized on the cytoskeleton and physically separated, then no phosphorylation would result. However, if there is a mechanical deformation of the tissue, ECM, cytoskeletal composite results in structural rearrangements that bring these two molecules into direct apposition and then phosphorylation might proceed causing a downstream signaling cascade to initiate. In addition, the transfer of focused mechanical energy to soluble molecules in the cytosol will alter their shape and hence their electrochemical potential through mechanical distortion. Stress-induced changes in molecular mechanics (stiffness and conformation) can then produce direct mechanochemical transduction by altering thermodynamic (association and dissociation constants) or kinetic (molecular motion) parameters. Regulation of tubulin polymerization (microtubule formation) by mechanical stresses balanced between microtubules, contractile microfilaments, and ECM provides one example of a thermodynamic transduction mechanism [37]. Stress-dependent changes in the frequency of opening and closing "stretch-sensitive" ion channels are an excellent example of the kinetic form of transduction [33]. Living cells sense multiple simultaneous inputs and are able to organize a single, concerted response. The tensegrity structure may be used to focus mechanical energy on critical transducing molecules and to "tune" the entire cellular response to stress by mechanically coupling biologic structures at different size scales and in different locations within living cells, tissues, and organs. This tuning function may be accomplished by altering the prestress in the system (e.g., by varying cytoskeletal tension), remodeling architecture, or modifying the mechanics of individual structural components. Specificity results from local changes in material properties of the structural elements (e.g., stress will rapidly dissipate in highly viscous regions), and from how the different elements are mechanically coupled (e.g., linkage of the cytoskeleton to the ECM by integrins and to the cytoskeleton of neighboring cells by cell–cell adhesion molecules, such as cadherins) [13].

Also in tendon studies, it is evident the important role of ECM. Tendon ruptures are increasingly common, repair can be difficult, and healing is poorly understood. Tissue engineering approaches often require expansion of cell numbers to populate a construct, and maintenance of cell phenotype is essential for tissue regeneration. Total collagen, the ratio of collagen types I and III, and decorin can be used as indicators of matrix formation in human tendon, and expression of the integrin beta 1 subunit as a marker of cell–matrix interactions, for example in patients receiving surgery for rupture. During the healing, cells become more rounded and are more widely spaced at confluence, and confluent cell density declines. No change to total cell layer collagen is observed, but the ratio of type III to type I collagen increases at the end. Decorin expression significantly decreases, while integrin expression does

not change. Thus, the phenotype of tenocytes in culture rapidly drifts during the healing [38].

This architectural model of biologic organisms may help to explain one of the most fundamental properties of living creatures: how the parts and the whole function as a single integrated system. Due to use of tensegrity architecture, mechanical stress is concentrated and focused on signal-transducing molecules that physically associate with cell surface molecules that anchor cells to extracellular matrix, such as integrins, and with load-bearing elements within the internal cytoskeleton and nucleus. Mechanochemical transduction may then proceed through local stress-dependent changes in molecular mechanics, thermodynamics, and kinetics within the cell. In this manner, the entire cellular response to stress may be orchestrated and tuned by altering the prestress in the cell, just as changing muscular tone can alter mechanical stability and structural coordination throughout the whole musculoskeletal system [13].

The finding that cells are not bits of viscous cytoplasm surrounded by an elastic membrane but instead structured as tensegrities with internal load-bearing struts and tensed cables also has led to novel insights into developmental control and pathobiology also in other fields, and not only for the musculoskeletal system. For example, the finding that microtubules bear compression in living cells is extremely relevant for heart physiology because an increased density of the microtubule component of the extramyofilament portion of the cardiocyte cytoskeleton caused by pressure overload can physically interfere with inward-directed shortening of the myofibrillar bundle and hence lead to contractile dysfunction associated with cardiac hypertrophy. The integrated nature of biological architecture also helps to explain why cardiac diseases and developmental abnormalities can be caused by mutations in various ostensibly unrelated molecules, including integrins, cytoskeletal filaments, ion channels, or nuclear components. Forces channeled over ECMs and to integrins are converted into biochemical changes by producing changes in deformation of other load-bearing mechanotransducer molecules, such as stress-sensitive ion channels, protein kinases, G proteins, and other signaling molecules, inside the cell. Recent advances in mathematics, engineering, and statistical mechanical models of tensegrity structures, and in the use of nanotechnology to create artificial cell–material control interfaces, may provide new ways to investigate, model, manipulate, probe, and control these fundamental mechanotransduction mechanisms in living cells, tissues, and organs, including heart, in the future [39].

Hormesis

Hormesis is a dose–response phenomenon, characterized by a low-dose stimulation and a high-dose inhibition. The term hormesis was first introduced into the scientific literature in 1943 by Chester Southam and John Ehrlich, mycology researchers at the University of Idaho. Reactive oxygen species (ROS) are emerging as

intracellular redox signaling molecules involved in the regulation of bone metabolism, including receptor activator of nuclear factor-κB ligand-dependent osteoclast differentiation, but they also have cytotoxic effects that include lipoperoxidation and oxidative damage to proteins and DNA. ROS generation, which is implicated in the regulation of cellular stress response mechanisms, is an integrated, highly regulated, process under control of redox-sensitive gene coding for redox proteins called vitagenes. Vitagenes, encoding for proteins such as heat shock proteins (Hsps) Hsp32, Hsp70, the thioredoxin, and the sirtuin protein, represent a system controlling a complex network of intracellular signaling pathways relevant to life span and involved in the preservation of cellular homeostasis under stress conditions. The biological relevance of dose–response affects those strategies pointing to the optimal dosing to patients in the treatment of numerous diseases. Thus, the heat shock response has become an important hormetic target for novel cytoprotective strategies focusing on the pharmacological development of compounds capable of modulating stress response mechanisms [40]. Hormesis is a type of stress often related to reactive oxygen species (ROS) originating from the mitochondrial respiratory chain (mitochondrial hormesis or mitohormesis). The accumulation of transient low doses of ROS either through chronic physical activity or caloric restriction influences signaling from the mitochondrial compartment to the cell, reduces glucose metabolism, induces mitochondrial metabolism, increases stress resistance, and increases life span. Mitochondrial formation of presumably harmful levels (chronic and/or excessive) of ROS within skeletal muscle has been observed in insulin resistance of obese subjects, type 2 diabetes mellitus and in impaired muscle function associated with normal aging. Advances in mitochondrial bioimaging combined with mitochondrial biochemistry and proteome research have broadened the knowledge of specific cellular signaling and other related functions of the mitochondrial behavior. Mitochondria are dynamically involved in several muscle cellular activities including signaling, proliferation, differentiation, autophagy, and death. The dynamics, size, number, and location of mitochondria in muscle cells vary significantly according to the cellular conditions and energetic needs. Mitochondria are generally considered as the main source of ROS in skeletal muscle cells. Several physiological and pathological conditions may result in ROS production by mitochondria. For example, high intense contractile activity, disuse muscle atrophy or inflammation increase mitochondrial ROS generation. Although the excessive ROS production is associated with the etiopathology of several human disease, low levels of ROS are important mediators for a variety of cellular process, including cell adhesion, immune response, apoptosis, cell growth, and differentiation. ROS also act as second messengers in intracellular signaling. However, chronic and excessive ROS production may lead to muscle cell damage and death. The final effect of ROS depends on intrinsic and extrinsic factors such as the level and the duration of ROS targeting muscle cells, the source, the site of ROS generation, the antioxidant status of target cells, the DNA repair capacity, the differentiation stage of muscle cells, and the proliferative and myogenic capacity of satellite cells. Due to their hormetic nature, in muscle tissue mitochondrial ROS may trigger different signaling pathways leading to different and diverging

responses, from adaptation to cell death. For example, physical exercises and chronic contractile activity induce a number of physiological adaptations that ameliorate muscle function and exercise performance. Trained muscle undergoes a remodeling toward a more oxidative phenotype altering the ultrastructural and subcellular organization. One of the most important effects of endurance training is mitochondrial biogenesis with an increase in mitochondrial content. It is well-known that physical exercise increases ROS generation in skeletal muscle cells. During exercise, the increased ROS production is mainly due to the high oxygen consumption that takes place during increased mitochondrial activity. There is probably generation of ROS at multiple subcellular locations in response to a multiplicity of mechanical and metabolic stimuli in muscle cells [41]. Thus, contraction-induced production of ROS has been shown to cause oxidative stress to skeletal muscle. As an adaptive response, muscle antioxidant defense systems are upregulated after heavy exercise. Nuclear factor (NF) kappaB and mitogen-activated protein kinases (MAPKs) are the major oxidative stress-sensitive signal transduction pathways. Activation of NF-kappaB signaling cascade has been shown to enhance the gene expression of important enzymes, such as mitochondrial superoxide dismutase (MnSOD) and inducible nitric oxide synthase (iNOS). MAPK activations are involved in a variety of cellular functions including growth, proliferation, and adaptation. The exercise has effects on NF-kappaB and MAPK signaling, as well as on the time course of activation, in rat skeletal muscle. In addition, ROS have a role in the exercise-induced upregulation of MnSOD and iNOS, and in the potential interactions of NF-kappaB and MAPK in the signaling of these enzymes. It is clear that ROS may serve as messenger molecules to activate adaptive responses through these redox-sensitive signaling pathways to maintain cellular oxidant–antioxidant homeostasis during exercise [42]. Furthermore, physical exercise shares common metabolic paths with caloric restriction and glucose restriction increasing mitohormesis and inducing a positive adaptive response that ends with stress resistance, antioxidant defense, and prolonged life span [41]. In addition, the mild stress-induced hormesis stimulates maintenance and repair systems and strengthens the homeodynamic space of cells and organisms. Hormesis through mild heat shock, natural and synthetic hormetins, and other stressors brings about several antiaging effects in human fibroblasts, keratinocytes, and telomerase-immortalized bone-marrow stem cells. Depending on the cell type, these antiaging hormetic effects include extension of replicative life span, enhanced proteasomal activities, increased chaperone levels, and improved wound healing, angiogenesis, and differentiation. The main molecular pathways for achieving such hormetic effects are through targeting the processes for the repair and removal of molecular damage, which can slow aging [43].

Hormesis has a role not only in the muscle but also in the bone and in its diseases such as Alzheimer pathology and osteoporosis [40]. Epidemiological studies also indicate that patients suffering from atherosclerosis are predisposed to develop osteoporosis. Atherogenic determinants such as oxidized low-density lipoprotein (oxLDL) particles have been shown both to stimulate the proliferation and promote apoptosis of bone-forming osteoblasts. OxLDL particles cause hormesis-like

response with the stimulation of both proliferation and cellular NAD (P) H-dependent reduction potential by low concentrations, whereas high concentrations are associated with the cell death. The induction of hormesis-like response by oxLDL in osteoblastic cells is associated with the stimulation of cell proliferation and ROS production by low concentrations of oxLDL. It is generally accepted that the stimulation of osteoblastic proliferation may compromise their differentiation into competent bone-forming cells. In accordance, low concentrations of oxLDL reduce the alkaline phosphatase activity, a marker of osteoblastic maturity. In addition, oxLDL compromises the migration of osteoblastic cells. Both functions have been shown to play a critical role in bone formation, remodeling, and fracture repair. Therefore, low concentrations of oxLDL may alter the bone metabolism by reducing osteoblastic differentiation in favor of uncontrolled cell proliferation and by affecting cell migration. On the other hand, high concentrations of oxLDL cause osteoblastic cell death that will result in reduced bone formation. In summary, oxLDL particles alter osteoblastic cell proliferation, migration, and apoptosis rate and thereby may contribute to alteration of bone metabolism equilibrium and may be responsible for the reduction of bone mass associated with atherogenic conditions [44].

Bone and Osteoinduction

Mesenchymal stem cells (MSCs) have the potential to replace or restore the function of damaged tissues and offer much promise in the successful application of tissue engineering and regenerative medicine strategies [45]. It has been demonstrated that adult mesenchymal stem cells (MSCs) are capable of giving rise to several different cell types, including myoblasts, adipocytes, fibroblasts, chondrocytes, and osteoblasts [46–49]. MSCs exist in a highly specialized microenvironment in which their maintenance, proliferation, migration, and differentiation involve a complex interplay of many local and systematic signals between stem cells and adjacent cells [50–55]. Osteoblasts are derived from MSCs and exist in close proximity to the bone marrow, which serves as a major source of MSCs and hematopoietic stem cells. They are located on the surface of bone, express multiple hormone receptors and various cell surface molecules, and secrete hormones and enzymes that maintain the balance between bone formation and resorption [56, 57]. However, it remains unclear whether and how osteoblasts affect the self-renewal and differentiation of another major stem cell population in bone-marrow MSCs. Co-cultures with MSCs and with a higher density of osteoblasts express greater mRNA expressions of Runx2, type I collagen, and osteocalcin [58]. Human bone-marrow-derived MSCs, by virtue of their capacity for self-renewal and multipotent differentiation, are considered promising candidate cells for regenerative medicine and tissue engineering applications. The multipotent differentiation capabilities of MSCs into various types of cell lineage, such as osteoblasts, chondrocytes, and adipocytes, have been characterized in numerous studies [46, 47]. Osteoblasts,

which are derived from bone-marrow MSCs, are the main cell type responsible for bone formation and are one of the neighboring cell types close to the bone marrow. It is believed that interaction between bone-marrow MSCs and their neighboring cells may lead to MSC osteogenesis via cell–cell contact or osteoblast-derived soluble factors [59]. It has been demonstrated that Runx2 is as an essential transcription factor for the induction of early osteogenic differentiation. Studies of the expression of Runx2 demonstrate that the early osteogenesis of co-cultures is highly dependent on osteoblast numbers co-cultured in an osteoinductive environment. Co-cultures with MSCs and osteoblasts demonstrated that direct cell–cell contact is sufficient to induce osteogenic differentiation by enhancing the gene expressions of Runx2, type I collagen, and osteocalcin. More significant and distinct osteogenic differentiation is promoted by additional osteogenic supplements. The quality of osteogenesis, as evidenced by protein expression, is proportional to the quantity of osteoblasts in the co-cultures. A recent study has also suggested that primary bone-derived cells promote the osteogenesis of human embryonic stem cells in a co-culture model by releasing bone morphogenetic proteins 2 and 4 [60]. These findings demonstrate that the osteogenesis of stem cells may be induced or promoted in a co-culture system through either direct cell–cell contact or secreted soluble factors derived from osteoblasts. These findings show that direct cell–cell contact between MSCs and osteoblasts contributed little to MSC osteogenic differentiation. In addition, the co-cultures required at least one osteoinductive factor to induce or enhance the osteogenic differentiation in MSCs: either an appropriate number of osteoblasts or treatment with osteogenic supplements. Culture conditions that included both a large quantity of osteoblasts and osteogenic supplements resulted in synergistic inhibition during osteogenesis by shrinking the process of osteogenic differentiation. The direct cell–cell contact and soluble osteogenic factors might act as synergistic modulators to promote or inhibit osteogenic differentiation in MSCs. Understanding the possible synergistic interactions between MSCs and osteoblasts might be an alternative approach to manipulating the fates of stem cells and for differentiating lineages in cell-therapeutic strategies and regenerative medicine [58]. Mesenchymal tissues are subjected to mechanical stimuli in vivo, and terminally differentiated cells from the mesenchymal lineage respond to mechanical stimulation in vivo and in vitro. MSCs have also been shown to be highly mechanosensitive, and this may present an ideal method for controlling MSC differentiation. External mechanical forces are able to induce or enhance MSC differentiation into a wide variety of tissue-specific cells; however, precisely controlling the timing and outcome of MSC differentiation is still a large challenge. Characterization of the exact force MSC experience in loading systems is important to know what mechanisms are actually inducing the observed responses and to help simplify the design of future loading systems. While most bioreactor systems employ a common force type (e.g., tension or compression), there are also likely to be other mechanisms at work causing significant secondary effects. Mathematical and computer modeling is important for characterizing the forces that are being experienced by the cells [61].

In conclusion, mechanical stress plays a vital role in maintaining bone architecture and this was studied for the first time by Julius Wolff: Mechanical loads can affect bone architecture in living beings [23]. However, the process by which osteogenic cells convert the mechanical signal into a biochemical response governing bone modeling is not clear. For example, in a recent study, was investigated how Atlantic salmon (Salmo salar) vertebra responds to exercise-induced mechanical loading. Bone formation in the vertebrae was favored through increased expression of genes involved in osteoid production. Fourier transform infrared spectroscopy (FT-IR) showed that bone matrix secreted both before and during sustained swimming had different properties after increased load compared to control, suggesting that both new and old bones are affected. Concomitantly, both osteoblasts and osteocytes in exercised salmon showed increased expression of the receptor nk-1 and its ligand, substance P (SP), both known to be involved in osteogenesis. The functional role of SP was investigated in vitro using osteoblasts depleted for SP. The cells showed severely reduced transcription of genes involved in mineralization, demonstrating a regulatory role for SP in salmon osteoblasts. Investigation of α-tubulin stained osteocytes revealed cilia-like structures. Together with SP, cilia may link mechanical responses to osteogenic processes in the absence of a canaliculi network. These results imply that salmon vertebral bone responds to mechanical load through a highly interconnected and complex signal and detection system, with SP as a key factor for initializing mechanically induced bone formation in bone lacking the canaliculi system [62].

Thus, it is probably that the bone development is mediated by neurotransmitters. In fact, SP and calcitonin gene-related peptide (CGRP) have been also found in the perichondrium and within the cartilage canals. It is still unknown whether they exert a direct effect on chondrocytes during joint development. The presence of CGRP and SP indicates the presence of nerves fibers and precedes the development of cartilage canals. Nerve fibers may play a role in the development of synovial joints before and during the presence of cartilage canals. The presence of CGRP and SP in the cartilage at birth may be involved in the early postnatal maturation of synovial joints [63]. Substance P is part of the superfamily of tachinins. SP exerts a trophic influence on neuronal tissue. The release of SP can lead to vasodilatation, plasma extravasion, and leukocyte recruitment, with a clear proinflammatory action which continues through the release of collagenase, prostaglandin E2, interleukin 1, TNFα, and oxygen metabolites [64]. Peripheral nervous fibers containing SP have been identified in the synovial membrane, in ligaments, in the menisci, the subchondral bone of human or rat and in some feline and horse joints [65]. Recent studies showed neurofilaments in arthritic cartilage and in osteophytes. This suggests a role of this neuropeptide in pain transmission and during degenerative and reparative processes occurring in arthritic joints [66]. CGRP is a peptide produced by tissue-specific alternative processing of the primary RNA transcripts of the calcitonin gene. Nervous fibers containing CGRP have been identified in spinal motoneurons [67], in the motor plates of voluntary muscles, in the bone marrow, close to the epiphyseal plates in contact with osteoblasts, in the periosteum, around the vessels in Volkman's channels and the Havers's channels, in the synovial

membrane, in ligaments, and in subchondral bone of human joints [68]. CGRP also seems to be involved in tendon and fracture healing [68, 69]. SP and CGRP have been identified in the perichondrium and in periarticular neurons [70]. Neuronal involvement may play an important role in the development of degenerative joint disease, consequent to progressive age-related loss of joint innervation. Autonomic nerve fibers may also actively participate in bone repair [71]. It is possible that the periarticular autonomic nerve fibers, through their neuropeptides, exert a trophic effect on joint development.

ECM and Tendons

On the other hand, it is necessary to focus the attention on the soft tissues, such as tendons. Only through the clarification of the microanatomy, pathophysiology, genetics of ECM, we may be able to improve current therapeutic knowledge on tendon diseases. Unfortunately, today too often the media and some areas of medicine offer to the patients new medical therapy based on tissue regeneration of tendons without scientific certainty of the effectiveness. The main effort of the research at this time should be focused not only on the in vivo application of new potential therapies but also and especially on the mechanisms that regulate the homeostasis of the ECM during exercise and pathologic conditions [72].

Tendon structure. The structure of a tendon is an important example of complexity of ECM three-dimensional organization. The extracellular matrix (ECM) is a macromolecular network with both structural and regulatory functions. Tendons are made by a fibrous, compact connective tissue that connects muscle to bone designed to transmit forces and withstand tension during muscle contraction. The extracellular matrix (ECM) is a macromolecular network with both structural and regulatory functions. Indeed, ECM furnishes mechanical and biochemical signals that cooperate in the integrated control of cell proliferation, survival, migration, and differentiation. ECM components belong to four major types of macromolecules: the collagens, elastin, proteoglycans, and noncollagenous glycoproteins (e.g., laminins, entactin/nidogen, fibronectin, and tenascins). The most copious proteins in the ECM are members of the collagen family. The collagens are involved in the formation of ECM fibrillar and microfibrillar networks and play a key role in determining tissue-specific mechanical properties.

Tendon's ultrastructure and ECM. The apparently simple tendon's structure and composition provide both rigidity and flexibility. This ability is based on tendon nonlinear, viscous-elastic, anisotropic, and heterogeneous mechanical properties. Similar to other tissues, there is a clear relationship between structure and function of the tendon [73]. Indeed, different tissue microenvironments provide specific characteristics to the different three-dimensional organization of the ECM since embryogenesis [74]. The organization of the ECM in the tendon is peculiar as well as within different tendons. The dialogues between different cells (fibroblasts, muscle, and cartilage cells) or better the dialects between different cells mediated by

various signaling cascades dependent on FGFs and TGF beta are used at decisive stages of development to make specific the phases of induction, organization, aggregation, or differentiation of cells [75]. The structure of the tendon is so complex that it is very difficult to maintain its function in the healing since this process is reparative rather than regenerative in the adult [76, 77]. A tendon consists of 70% of water and 30% of dry mass, which is composed by 60–80% of type I collagen and 2% of elastin. Among collagens, the most abundant component is collagen type I (95%), while type III and type V collagens represent the remaining 5% of the total collagens [77]. There are many types of collagen, and each of these has a specific function; every year one new type or one new function is discovered. Tendons in addition to collagen contain proteoglycans, glycosaminoglycans, and glycoproteins including fibronectin, thrombospondin, and tenascin-C immersed in different compositions of ECM lying cellular elements represented (90/95%) by tenoblasts and tenocytes. The remaining 5–10% of the cells consists of chondrocytes, synovial and vascular cells [73]. This information can be useful to create appropriated nanoengineering techniques [78].

Proprioception: Muscle, Tendon, and Ligament

Mechanoreceptors in healthy muscles, ligaments, and tendons procure the sensation of the joint movement, joint position, and stability. Loss of mechanoreceptors not only causes mechanical instability, but also leads to a disturbance in the neuromuscular control due to the loss of mechanoreceptors. Proprioception is the sensation of the joint movement and joint position [79].

Ligaments and proprioception. Research has shown that four types of nerve endings can be listed in ligaments: Ruffini corpuscles, Pacinian corpuscles, Golgi tendon organ-like endings, and free nerve endings [80]. These four types of receptors were also found in the fibrous joint capsule. Ruffini endings are intertwined with collagen fibers, and they seem to be activated by the displacement of the fibers. Ruffini corpuscles are both static and dynamic mechanoreceptors which are able to signal static joint position, changes in intra-articular pressure as well as movement direction, amplitude, and velocity [80, 81]. Pacinian corpuscles are dynamic, rapidly adapting mechanoreceptors with a low threshold. They are inactive on immobile joints, though becoming active at the acceleration and deceleration. Golgi tendon organ endings have an high threshold and are slowly adapting. They are inactive in immobile joint, and it has been suggested that they measure the ligament tension. They have been discovered in the cruciate ligaments, medial collateral ligament, the medial patellar ligament. Finally, free nerve endings are the articular nociceptor endings [82].

The sensory role of the cruciate ligaments. Knee joint ligaments have been traditionally considered passive structures only involved in mechanical functions [83]. A variety of studies concerning the presence of mechanoreceptors in cruciate ligaments have been reported [84]. Three different types of nerve fibers and of nerve

endings have been described in normal ACL: (1) fibers of large diameter which are fast-conducting mechanoreceptive sensory afferents; (2) fibers of small diameter slow-conducting nociceptive sensory afferents, and (3) sympathetic efferent vasomotor fibers. How we said before, four nerve endings have been identified in human cruciate ligaments: Ruffini corpuscles, Pacinian corpuscles, Golgi tendon organ-like endings, and free nerve endings [82, 85]. Ligament mechanoreceptors influence movements via the γ-muscle spindle system. They contribute to the stiffness preprogramming around the joint and are crucial for joint stability maintenance. Therefore, the knee ligament injury would damage the mechanoreceptors and alter neuromuscular functions secondary to diminished somatosensory information. ACL rupture causes considerable changes in stretch reflex excitability, so the "giving way" of the knee is not simply related to the decrease in mechanical joint stability, but is closely associated with altered stretch reflex excitability that most probably takes place on the spinal level. Reduction of mechanoreceptors is not only due to a lesion of the soft tissue. Mechanoreceptors are not immutable structures, but their properties change with aging. Aging affects numbers and morphology of mechanoreceptors. This phenomenon could explain why normal aging process is associated with deficits in proprioception.

It was left to Abbott et al. [86] to attribute more importance to knee structures, as they first described the knee ligaments as having rich sensory innervation, which allowed them to act as the first link in the kinetic chain. Ever since, our understanding of this complex functioning has evolved, and it is now widely accepted that movement or charge in knee position stimulates receptors in knee ligaments that allow the conscious appreciation of limb position in space. Proprioception is receptor and neural arc mediated. The stimulation of mechanoreceptors in the knee ligaments initiates different types of reflex muscle contractions through the neural arc involving the dorsal root ganglion sensory neurons. Many studies over the last 30 years have demonstrated significant presence of mechanoreceptors in the fibers of an intact anterior cruciate ligament (ACL). These were first described by Schultz et al. [87] in 1984, and it was subsequently established that the receptors included not just Pacinian, Ruffini, or Golgi tendon-type bodies, but also numerous nerve endings distributed all over the ACL. These receptors play an important role in the complicated neural network of proprioception. They are capable of detecting changes not only in tension, speed, acceleration, and direction of movement, but also allow a subconscious determination of the position of knee joint in space. It becomes a corollary that damaged mechanoreceptors would alter neuromuscular functions secondary to diminished somatosensory information (proprioception and kinesthesia). In modern orthopedics, this has become a key factor in understanding functional instability after ACL injuries and methods to treat it. Subsequent to an ACL injury, it has been observed that the relationship between passive stability and the functional stability of the knee joint is often ambiguous. It is possible that the functional instability that occurs after an injury to the ACL is due to the combined effects of excessive tibial translation and a lack of "coordinated muscle activity" to stabilize the knee joint. This lack of coordinated muscle stabilization of the knee

joint is thought to be due to diminished or absent sensory feedback from the ACL to the neuromuscular system.

Mechanoreceptors in intact ACL. The first histological demonstration of mechanoreceptors in the human ACL was done by Schultz et al. [87]. The cruciate ligaments were obtained at the time of total knee replacement and from autopsy and amputation specimens. The ultrastructure of nerve endings in a human knee joint capsule was subsequently described by Halala et al. [88]. These authors found the three types of nerve endings: free nerve endings, Ruffini corpuscles, and Pacinian corpuscles. In the joint capsule, free nerve endings were located below the synovial layer and within the fibrous layer near blood vessels. These nerve terminals are derived from myelinated A-delta fibers or from unmyelinated C-fibers. Ruffini corpuscles were present within the fibrous layer and the ligaments of the capsule in three variations: small Ruffini corpuscles without a capsule, small corpuscles with a connective tissue capsule, and large Ruffini corpuscles with an incomplete perineural capsule. Their afferent axons were myelinated, and, inside the corpuscle, nerve terminals were anchored in the connective tissue belonging to the fibrous layer or to the ligaments, respectively. The presence of an incomplete perineural capsule depended on the structure of the surrounding connective tissue. In ligaments with collagenous fibrils oriented in a parallel fashion, the perineural capsule was well-developed and the Ruffini corpuscle resembled a Golgi tendon organ; in areas where the fibrils showed no predominant orientation, Ruffini lacked a capsule. Small Pacinian corpuscles were situated within the fibrous layer near the capsular insertion at the meniscus articularis or at the periosteum. Larger Pacinian corpuscles with one or several inner cores and a perineural capsule were found on the outer surface of the fibrous layer. By the turn of the century, it was becoming clear that the mechanoreceptors located in the ACL constitute an afferent source of information toward the central nervous system. The ACL deficiency can cause a disturbance in neuromuscular control and affects central programs and consequently the motor response resulting in serious dysfunction of the injured limb. It was examined brain activation by using functional magnetic resonance imaging technique (1.5-T scanner): ACL deficiency can cause reorganization of the central nervous system, suggesting that such an injury might be regarded as a neurophysiologic dysfunction, not a simple peripheral musculoskeletal injury [89]. This evidence could explain the variation of clinical symptoms that accompany this type of injury, and the degrees of dysfunctions in different individuals with an ACL-deficient knee.

Mechanoreceptors in the stump of an injured ACL. In untreated ACL lesions in humans, morphologically normal mechanoreceptors persisted in the ACL remnant for about 3 months after injury. Beyond that time, the number of receptors gradually decreased. By the ninth month after injury, only a few nerve endings were found, and they were totally absent after 1 year. These results indicate that the proprioceptive potential of the stump may diminish with the passage of time, and this may have a potential bearing on surgical outcomes in cases where reconstruction is delayed. Thus, preserving the ACL remnants might improve functional

outcome after ACL reconstruction as some reinnervation and recovery of proprioception are likely in such cases.

Previous studies of ACL anatomy and histology have demonstrated that the maximum concentration of the nerve endings is at the attachment sites of the ligament to the bone. This serves as the main tract for proprioceptive feedback. These are the stumps that are seen at arthroscopy and are routinely removed, thereby aggravating the sensory damage to the knee joint. In addition, preserving remnant provided synovium for the reconstructed ACL, and it could accelerate revascularization of the graft. The remnants not only improved proprioception but also provided mechanical stability in certain cases. However, there is always a risk of developing a cyclops lesion if the remnant is preserved and this could lead to impingement. In conclusion, remnant preservation in ACL reconstruction, although technically demanding, can provide better clinical results as compared to remnant-sacrificing techniques.

With presence of degenerative changes, the proprioceptive potential of the injured stump decreases more, further substantiating the fact that delays in treatment negatively affect the mechanoreceptor and proprioceptive fibers in the stump. In ACL-deficient knees of long duration, repeated episodes of giving way and reinjury due to instability may render the stump prone to degeneration and decrease its proprioceptive potential.

In conclusion, mechanoreceptors in intact ACL contribute toward functional stability of the knee joint. Injury to ACL not only causes mechanical instability, but also leads to a disturbance in the neuromuscular control of the injured knee due to loss/damage to mechanoreceptors. ACL reconstruction restores proprioceptive potential of the knee to some extent, but the results vary. Although the remnant ACL contains residual mechanoreceptors, the number and functionally of these receptors is dependent, to some extent, on the physical characteristics of the remnant and duration of injury. Nevertheless, these remnants are worth preserving during ACL reconstruction and can play an important role in restoration of proprioception of knee following ACL reconstruction [90].

Tendinous mechanoreceptors. Golgi tendon organ-like endings have been found in tendons. Stimulus response suggests they are high-threshold, slowly adapting mechanoreceptors that are completely inactive in immobile joints. They become active when joints are at the extreme of their ranges of movement and considerable stresses are generated in the joint ligaments [80, 82].

Healing of tendon mechanoreceptors. The dynamic response of the Golgi tendon organs is made physiologically by two components: an initial peak and a second main component showing correlation between tension rate and discharge frequency. During first part of the healing, motor units had to come to a full recovery. Speed contraction and absolute tension were not fully restored yet. Almost all receptors were reinnervated. Probably after a complete nerve section, occurs a reinnervation by inappropriate axons. However, abnormal responses seem more due to an immaturity of the transduction mechanism. After the nerve transection, the structure of the motor units is modified; this can lead to an increase in

the mechanical input to the Golgi tendon organs. Missing of the responses coincides with an alteration of the dynamic sensitivities [91].

Muscle mechanoreceptors. Golgi tendon organs co-work with another mechanoreceptor: the muscle spindle, which is the most innervated receptor, with afferent and efferent fibers. These receptors are aligned parallel to the muscle fibers, and they primarily detect changes in the length and the speed of muscles [92]. Mechanoreceptors function as transducer devices converting a signal in one form of energy to another one (i.e., the physical stimulus of tension into a specific nerve signal). The summation of receptor discharges forms a frequency-modulated code that the central nervous system uses to analyze joint position, motion, and acceleration [93]. The transduction of mechanical stimuli into electrical responses is essential for audition, kinesthesis, proprioception, and automatic sensation of pressure and volume. Seems that Pacinian corpuscles and Golgi tendon organs are more represented than Ruffini endings in ligaments, both Pacinian corpuscles and Golgi tendon organs are physiologically reflexogenic. Pacinian corpuscles discharge at the onset or cessation of movement and are involved in quick movements; this afferent feedback to the central nervous system would have a stabilizing effect on the joints. Golgi tendon organs are found in ligaments, myotendinous and myoaponeurotic junctions of mammalian skeletal muscle. Their discharges are responsible for tension and joint position; thus, they are also responsible for joint stabilization [80, 94].

Healing of muscle mechanoreceptors. Few reports focused the healing of mechanoreceptors. Some of these showed that muscle spindle afferents reinnervate their original ending sites with the spindle after a nerve injury but the afferents that had been regenerated are smaller than the control group. In contrast Haftel and coworkers showed that not all the regenerated afferences were able to restore a pathway to central nervous system. Moreover, there were some differences in muscle spindle responses to stretch, showing that reinnervated afferences had an increased length in threshold, probably due to an incomplete recovery of gamma motoneurons drive to reinnervated muscle spindles [95].

In conclusion, mechanoreceptors in normal tissues contribute to stability of the joints. Injuries of ligaments, tendons, and muscles contribute to the mechanical instability and to a disturbance in neuromuscular control due to the loss of mechanoreceptors. This field remains to be investigated more deeply in the way to clarify the complex relationship between soft tissues and nervous system in normal and pathological conditions. Further studies need to be conducted on mechanoreceptor functions in normal and pathological conditions and in the close future maybe our surgical procedures; rehabilitation protocols for conservative or post-surgical treatments will become modified if proprioception sense will be better addressed [96].

Nanoscale Biotechnology

Although further studies and new techniques are necessary, there are already different types of nanoscale biotechnology studied and experimented.

Electrospinning. Electrospinning has emerged to be a simple, elegant, and scalable technique to fabricate polymeric nanofibers. Pure polymers as well as blends and composites of both natural and synthetics have been successfully electrospun into nanofiber matrices. Physiochemical properties of nanofiber matrices can be controlled by manipulating electrospinning parameters to meet the requirements of a specific application. Such efforts include the fabrication of fiber matrices containing nanofibers, microfibers, combination of nano- and microfibers, and also different fiber orientations/alignments. Polymeric nanofiber matrices have been extensively investigated for diversified uses such as filtration, barrier fabrics, wipes, personal care, biomedical and pharmaceutical applications. Recently electrospun nanofiber matrices have gained a lot of attention and are being explored as scaffolds in tissue engineering due to their properties that can modulate cellular behavior. Electrospun nanofiber matrices show morphological similarities to the natural extracellular matrix (ECM), characterized by ultrafine continuous fibers, high surface-to-volume ratio, high porosity, and variable pore-size distribution. Efforts have been made to modify nanofiber surfaces with several bioactive molecules to provide cells with the necessary chemical cues and a more in vivo-like environment. The current paper provides an overlook on such efforts in designing nanofiber matrices as scaffolds in the regeneration of various soft tissues including skin, blood vessel, tendon/ligament, cardiac patch, nerve, and skeletal muscle [97].

Nanofiber scaffolds. Nanofiber scaffolds, produced by the electrospinning technique, have gained widespread attention in tissue engineering due to their morphological similarities to the native extracellular matrix. For cartilage repair, studies have examined their feasibility; however, these studies have been limited, excluding the influence of other scaffold design features. It evaluated the effect of scaffold design, specifically examining a range of nano- to micron-sized fibers and resulting pore size and mechanical properties, on human mesenchymal stem cells (MSCs) derived from the adult bone marrow during chondrogenesis. MSC differentiation was examined on these scaffolds with an emphasis on temporal gene expression of chondrogenic markers and the pluripotent gene, Sox2, which has yet to be explored for MSCs during chondrogenesis and in combination with tissue engineering scaffolds. Chondrogenic markers of aggrecan, chondroadherin, sox9, and collagen type II were highest for cells on micron-sized fibers (5 and 9 μm) with pore sizes of 27 and 29 μm, respectively, in comparison with cells on nano-sized fibers (300 nm and 600 to 1400 nm) having pore sizes of 2 and 3 μm, respectively. Undifferentiated MSCs expressed high levels of the Sox2 gene but displayed negligible levels on all scaffolds with or without the presence of inductive factors, suggesting that the physical features of the scaffold play an important role in differentiation. Micron-sized fibers with large pore structures and mechanical

properties comparable to the cartilage ECM enhanced chondrogenesis, demonstrating architectural features as well as mechanical properties of electrospun fibrous scaffolds enhance differentiation [98].

For example, ***polycystin-1*** (PC1) is a large membrane-associated protein that interacts with polycystin-2 in the primary cilia of renal epithelial cells to form a mechanosensitive ion channel. Bending of the cilia induces calcium flow into the cells, mediated by the polycystin complex. Antibodies to polycystin-1 and polycystin-2 abolish this activation. Based on this, it has been suggested that the extracellular region of polycystin-1, which has a number of putative binding domains, may act as a mechanosensor. A large proportion of the extracellular region of polycystin-1 consists of beta sandwich PKD domains in tandem array. Atomic force microscopy was used to investigate the mechanical properties of the PKD domains of polycystin-1. These domains, despite having a low thermodynamic stability, exhibit a remarkable mechanical strength, similar to that of immunoglobulin domains in the giant muscle protein titin. Molecular dynamic simulations performed at low constant force show that the first PKD domain of polycystin (PKDd1) has a similar unfolding time as titin I27, under the same conditions. The simulations suggest that the basis for this mechanical stability is the formation of a force-stabilized intermediate. Thus, these domains will remain folded under external force supporting the hypothesis that polycystin-1 could act as a mechanosensor, detecting changes in fluid flow in the kidney tubule [99].

In other studies, PKD1 gene was stably silenced in osteoblastic cell line MC3T3-E1 by using lentivirus-mediated shRNA technology to investigate the involvement of PC1 in mechanical strain-induced signaling cascades controlling osteogenesis. It was showed that mechanical tensile strain sufficiently enhanced osteogenic gene expressions and osteoblastic proliferation. However, PC1 deficiency resulted in the loss of the ability to sense external mechanical stimuli, thereby promoting osteoblastic osteogenesis and proliferation. The signal pathways implicated in this process were intracellular calcium and Akt/b-catenin pathway. The basal levels of intracellular calcium, phospho-Akt, phospho-GSK-3b, and nuclear accumulation of active b-catenin were significantly attenuated in PC1-deficient osteoblasts. In addition, PC1 deficiency impaired mechanical strain-induced potentiation of intracellular calcium, and activation of Akt-dependent and Wnt/b-catenin pathways, which was able to be partially reversed by calcium ionophore A23187 treatment. Furthermore, applications of LiCl or A23187 in PC1-deficient osteoblasts could promote osteoblastic differentiation and proliferation under mechanical strain conditions. Therefore, these results demonstrated that osteoblasts require mechanosensory molecule PC1 to adapt to external mechanical tensile strain, thereby inducing osteoblastic mechanoresponse, partially through the potentiation of intracellular calcium and downstream Akt/b-catenin signaling pathway [100].

Scaffolds for chondrogenic differentiation. How we said before, chondrogenic differentiation of mesenchymal stem cells is strongly influenced by the surrounding chemical and structural milieu. Since the majority of the native cartilage extracellular matrix is composed of nanofibrous collagen fibrils, much of recent cartilage

tissue engineering research has focused on developing and utilizing scaffolds with similar nanoscale architecture. However, the current literature lacks consensus regarding the ideal fiber diameter, with differences in culture conditions making it difficult to compare between studies. It was studied how to develop a more thorough understanding of how cell–cell and cell–biomaterial interactions drive in vitro chondrogenic differentiation of bone-marrow-derived mesenchymal stem cells (MSCs). Electrospun poly(ε-caprolactone) microfibers (4.3 ± 0.8 μm diameter, 90 μm^2 pore size) and nanofibers (440 ± 20 nm diameter, 1.2 μm^2 pore size) were seeded with MSCs at initial densities ranging from 1×10^5 to 4×10^6 cells cm^{-3} -scaffold and cultured under transforming growth factor-β (TGF-β) induced chondrogenic conditions for 3 or 6 weeks. Chondrogenic gene expression, cellular proliferation, as well as sulfated glycosaminoglycan and collagen production were enhanced on microfiber in comparison with nanofiber scaffolds, with high initial seeding densities being required for significant chondrogenic differentiation and extracellular matrix deposition. Both cell–cell and cell–material interactions appear to play important roles in chondrogenic differentiation of MSCs in vitro, and consideration of several variables simultaneously is essential for understanding cell behavior in order to develop an optimal tissue engineering strategy [101].

The objective of the scientific studies is to develop a scaffold for mesenchymal stromal cell (MSC) recruitment, proliferation, and chondrogenic differentiation. The concept behind the design is to mimic the cartilage matrix and contain stimulatory agents that make continuous supply of inductive factors redundant.

The use of electrospun extracellular matrix (ECM)-mimicking nanofibrous scaffolds for tissue engineering is limited by poor cellular infiltration. Cell penetration could be enhanced in scaffolds by using a hierarchical structure where nanofibers are combined with micron-scale fibers while preserving the overall scaffold architecture. To assess this, were fabricated electrospun porous poly(lactic acid) (PLA) scaffolds having nanoscale, microscale, and combined micro/nanoarchitecture and evaluated the structural characteristics and biological response in detail. Although the bioactivity was intermediate to that for nanofiber and microfiber scaffold, a unique result of this study was that the micro/nano combined fibrous scaffold showed improved cell infiltration and distribution than the nanofibrous scaffold. Although the cells were found to be lining the scaffold periphery in the case of nanofibrous scaffold, micro/nanoscaffolds had cells dispersed throughout the scaffold. Further, as expected, the addition of nanoparticles of hydroxyapatite (nHAp) improved the bioactivity, although it did not play a significant role in cell penetration. Thus, this strategy of creating a three-dimensional (3D) micro/nanoarchitecture that would increase the porosity of the fibrous scaffold and thereby improving the cell penetration can be utilized for the generation of functional tissue-engineered constructs in vitro [102].

Thus, it is possible to combine nanofibrous (*N*: ∼400 nm) and microfibrous (*M*: ∼10 μm) poly-ε-caprolactone (PCL) scaffolds; for example, they were combined with 1% high-molecular-weight sodium hyaluronate (NHA/MHA), 1% hyaluronan (HA) and 200 ng transforming growth factor beta 1 (TGF-β1; NTGF/MTGF), or 0.1% bovine serum albumin (N/M). Scaffolds were seeded with MSCs from bone

marrow and cultured without growth factors in vitro. Cultures with chondrogenic medium supplemented with TGF-β1 served as controls. Proliferation, migration, and release of TGF-β1 were investigated. Cell differentiation was evaluated by polymerase chain reaction (PCR) and real-time PCR. NTGF and MTGF exhibited primarily an initial release of TGF-β1. None of the factors released by the scaffolds recruited MSCs. The expression of aggrecan was dependent on the scaffold ultrastructure with nanofibers promoting increasing and microfibers decreasing expression levels. Composites containing HA demonstrated elevated seeding efficiency and lower type I collagen expression. Expression of type II collagen was dependent on continuous or late supply of TGF-β1, which was not provided by the scaffold design. The initial release of TGF-β1 induced an expression of type I collagen and osteogenic marker genes. Thus, nanofibrous PCL scaffolds with or without augmentation are suitable for chondrogenic initiation of MSCs. Initial release of HA is sufficient in terms of directing the implanted MSCs toward a chondrogenic end, whereas a late release of TGF-β1 is preferred to foster type II and avoid type I collagen expression [103].

Hyaluronic acid (HA) is a molecule of particular interest for producing scaffold for tissue engineering. It has been reported that bone marrow (BM) is the MSC source an appropriate in the setting of cartilage regeneration to treat cartilage focal lesions. Thus, can be used HA membranes reached of BM-MSCs, aspirated by the posterior iliac crest, as scaffolds. The interaction of MSCs with the scaffolds such as HA membranes may contribute to positively influence the differentiation process toward chondrogenesis. Many other factors may help MSC differentiation into chondrocytes as the spatial conformation of the culture system, additional reagents as platelet rich plasma (PRP), PL or TGF-b1, but the influence of the structure and composition of the scaffold is particularly relevant since it could provide favorable microenvironment for MSC chondrogenesis [104].

PRP and MSCs for tendon regeneration. PRP and mesenchymal stem cells can be used also for tendon injuries. Tendon tissue shows limited regeneration potential with formation of scar tissue and inferior mechanical properties. Several growth factors have the capacity to improve the healing response and decrease scar formation. PRP and MSC work as efficient growth factor carriers. Platelets contained in PRP produce a number of relevant cytokines participating in physiological tendon healing (e.g., platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF)). The angiogenic effect is vital for tendon and ligament healing, and PRP may contribute to increase it. In vitro experiments and animal studies showed promising results for the use of PRP; however, clinical controlled studies have shown a tendency to reduce the pain-related symptoms but no significant differences in overall clinical scores. Unfortunately is yet unknown which dose and which type of PRP should be used to have more significant results. On the other hand, MSCs can contribute to tendon healing in different ways. First, they can provide tenocytes by direct differentiation into these cells. Secondly, they can provide a number of anabolic cytokines by their extraordinary paracrine activity. Thirdly, they show significant anti-inflammatory activity that may contribute to the healing process. MSCs are not totally arrived in

clinical use so that there is still a lack of randomized controlled trials. In basic research experiments, they show an extraordinary paracrine activity, anti-inflammatory effect, and the possibility to differentiate in tenocytes when different activating factors are added [105].

PAM and SCF. For some specific tissue regeneration, to overcome problems such as cell survival, lack of cell differentiation, and integration in the host tissue, a new tool described as pharmacologically active microcarriers (PAM) has been described. PAMs are biocompatible and biodegradable microdevices coated with cell adhesion molecules, conveying cells on their surface and presenting a controlled delivery of growth factor. For other specific tissue regeneration, micro- and nanoporous scaffolds with a specific 3D-shape serve as temporary support for cells to grow into a new tissue, before it is transplanted back to the host tissue. PAM production can be performed by using an innovative continuous process that involves the use of supercritical fluid (SCF) (mainly supercritical carbon dioxide), for the treatment of simple or multiple emulsions at relative low temperatures. Scaffolds with a specific 3D shape require several characteristics, such as high regular and reproducible 3D structure, porosity exceeding 90% and an open pore geometry, high internal surface areas, and specific mechanical properties. To produce interconnected microcells and a nanometric substructure, a new supercritical-fluid-assisted technique for the formation of 3D scaffolds has been proposed. It consists of three steps: formation of a polymeric gel loaded with a solid porogen, drying of the gel using SCF, washing with water to eliminate the porogen. When gel drying is performed by SCF, the supercritical mixture formed during the process has no surface tension and can be easily eliminated in a single step. One of the major problems in gel drying is the possibility of gel collapse; using SCF, the absence of surface tension avoids this problem preserving the nanoporous structure. "Injectable scaffold" or temporary scaffold was also tested, respectively, for the culture of mesenchymal stem cell in order to study their growth and differentiation. The result showed higher biocompatibility with respect to similar product obtained by conventional technology [106–110].

Conclusions

The complexity and well-orchestrated structure of musculoskeletal tissues reacts physiologically and pathologically to mechanical stresses. Thousands of molecular events occur during these events; understanding both the nanophysiology of cellular mechanoresponse and how to use this knowledge is the key point of tissue engineering. New information is required to introduce new types of nanotechnology or to modify those already used, which will be able to improve the reparation of injuries in various systems such as the musculoskeletal system.

References

1. Ingber DE. Integrins as mechanochemical tranducers. Curr Opin Cell Biol. 1991;3:841–8.
2. Ingber DE. Tensegrity: the architectural basis of cellular mechanotranduction. Ann Rev Physiol. 1997;59:575–99.
3. Rodan GA, Bourret LA, Harvey A, Mensi T. Cyclic AMP and cyclic GMP: mediators of the mechanical effects on bone remodeling. Science. 1975;189:467–9.
4. Davies PF, Remuzzi A, Gordon EJ, Dewey CF, Gimbrone MA. Turbulent fluid shear stress induces vascular endothelial cell turnover in vitro. Proc Nat Acad Sci USA. 1986;83: 2114–7.
5. Jones DB, Scholubbers J-G. Evidence that phospholipase C mediates the mechanical stress response in bone. Calc Tiss Int. 1987;41(Suppl. 2):4.
6. Brighton CT, Strafford B, Gross SB, Leatherwood DF, Williams JL, Pollack SR. The proliferative and synthetic response of isolated calvarial bone cells of rats to cyclic biaxial mechanical strain. J Bone Joint Surg. 1991;73A:320–31.
7. Sah RL-Y, Grodizinsky AJ, Plass AHK, Sandy JD. Effects of static and dynamic compression on matrix metabolism in cartilage explants. In: Kuettner K, et al. editors. Articular Cartilage and Osteoarthritis. New York: Raven Press, Ltd; 1992:373–92.
8. Bachrach NM, Valhmu WB, Stazzone E, Ratcliffe A, Lai WM, Mow VC. Changes in proteoglycan synthesis rates of chondrocytes in articular cartilage are associated with the time dependent changes in their mechanical environment. J Biomechanics. 1995;28:1561–9.
9. Guilak F, Sah RL-Y, Setton LA. Physical regulation of cartilage metabolism. In: Mow VC, Hayes WC, editors. Basic orthopaedic biomechanics. Philadelphia: Lippincott-Raven; 1997. p. 179–208.
10. Comper WD editor. Extracellular Matrix. Amsterdam, The Netherlands: Harwood Academic Press 1996, Vols I & II.
11. Sung KLP, Sung LA, Crimmins M, Burakoff SJ, Chien S. Dynamic changes in viscoelastic properties in cytotoxic T-lymphocytes mediating killing. J Cell Sci. 1988;91:179–89.
12. Dong C, Skalak R, Sung KP, Schmid-Schonbien GW, Chien S. Passive deformation analysis of human leukocytes. J Biomech Eng. 1998;110:27–36.
13. Chen Christopher S, Ingber Donald E. Tensegrity and mechanoregulation: from skeleton to cytoskeleton. Osteoarthritis Cartilage. 1999;7:81–94.
14. Viidik A. Tensile strength properties of achilles tendon systems in trained and untrained rabbits. Acta Orthop Scand. 1969;40:261–72.
15. Tipton CM, Matthes RD, Maynard JA, et al. The influence of physical activity on ligaments and tendons. Med Sci Sports. 1975;7:165–75.
16. Lanyon LE, Rubin CT. Static versus dynamic loads as an influence on bone remodelling. J Biomech. 1984;17:897–905.
17. Frost HM. Vital biomechanics. General concepts for structural adaptations to mechanical usage. Calc Tiss Int. 1987;42:145–54.
18. DeLee JC. Tissue remodeling and response to therapeutic exercise [review]. In: Leadbetter WB, Buckwalter JA, Gordon SL, editors. Sports-Induced Inflammation. AAOS: American Orthopaedic Society; 1989. p. 547–54.
19. Forwood MR, Turner CH. Skeletal adaptations to mechanical usage: results from tibial loading studies in rats. Bone. 1995;4(suppl):197S–205S.
20. Deng M, James R, Laurencin CT, Kumbar SG. Nanostructured polymeric scaffolds for orthopaedic regenerative engineering. IEEE Trans Nanobiosci. 2012;11(1):3–14. https://doi.org/10.1109/TNB.2011.2179554 Epub 2012 Jan 23.
21. Ingber DE, Wang N, Stamenovic D. Tensegrity, cellular biophysics, and the mechanics of living systems. Rep Prog Phys. 2014;77(4):046603.
22. Frost HM. A 2003 update of bone physiology and Wolff's Law for clinicians. Angle Orthod. 2004;74(1):3–15.

23. Mow VC, Ratcliffe A, Poole AR. Cartilage and diarthrodial joints as paradigms for hierarchical materials and structures. Biomaterials. 1992;13:67–97.
24. Martin RB, Burr DB. Structure, function, and adaptation of compact bone. New York: Raven Press; 1989.
25. Silbert JE. Advances in the biochemistry of proteoglycans. In: Uitto J, Perejda AJ, editors. Connective tissue disease: molecular pathology of the extracellular matrix. New York: Marcel Dekker; 1987. p. 83–98.
26. Mow VC, Ratcliffe A. Structure and function of articular cartilage. In: Mow VC, Hayes WC, editors. Basic orthopaedic biomechanics. Philadelphia, PA: Lippincott-Raven; 1997. p. 113–77.
27. Guilak F. Compression-induced changes in the shape and volume of the chondrocyte nucleus. J Biomech. 1995;28:1529–41.
28. Guilak F, Ratcliffe A, Mow VC. Chondrocyte deformation and local tissue strains in articular cartilage: a confocal microscopy study. J Orthop Res. 1995;13:410–21.
29. Nimni ME. Collagen: structure, function, and metabolism in normal and fibrotic tissues. Sem Arth Rheum. 1983;13:1–86.
30. Woo SL-Y, Gomez MA, Woo YK, Akeson WH. Mechanical properties of tendons and ligaments. Quasistatic and nonlinear viscoelastic properties. Biorheology 1982;19:385–96.
31. Dickinson RB, Guido S, Tranquillo RT. Biased cell migration of fibroblasts exhibiting contact guidance in oriented collagen gels. Ann Biomed Eng. 1994;22:342–56.
32. Chen BM, Grinnell AD. Integrins and modulation of transmitter release from motor nerve terminals by stretch. Science. 1995;269:1578–80.
33. Hamill OP, McBride DW Jr. The cloning of a mechano-gated membrane ion channel. Trends Neurosci. 1994;17:439–45.
34. Ingber DE, Dike L, Hansen L, Karp S, Liley H, Maniotis A, McNamee H, Mooney D, Plopper G, Sims J, Wang N. Cellular tensegrity: exploring how mechanical changes in the cytoskeleton regulate cell growth, migration, and tissue pattern during morphogenesis. Int Rev Cytol. 1994;150:173–224.
35. Plopper G, McNamee H, Dike L, Bojanowski K, Ingber DE. Convergence of integrin and growth factor receptor signaling pathways within the focal adhesion complex. Mol Biol Cell. 1995;6:1349–65.
36. Miyamoto S, Akiyama S, Yamada KM. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. Science. 1995;267:883–5.
37. Buxbaum RE, Heidemann SR. A thermodynamic model for force integration and microtubule assembly during axonal elongation. J Theor Biol. 1988;134:379–90.
38. Yao L, Bestwick CS, Bestwick LA, Maffulli N, Aspden RM. Phenotypic drift in human tenocyte culture. Tissue Eng. 2006;12(7):1843–9.
39. Ingber DE. Tensegrity-based mechanosensing from macro to micro. Prog Biophys Mol Biol. 2008;97(2–3):163–79. https://doi.org/10.1016/j.pbiomolbio.2008.02.005.
40. Cornelius C, Koverech G, Crupi R, Di Paola R, Koverech A, Lodato F, Scuto M, Salinaro AT, Cuzzocrea S, Calabrese EJ, Calabrese V. Osteoporosis and Alzheimer pathology: role of cellular stress response and hormetic redox signaling in aging and bone remodeling. Front Pharmacol. 2014;5.
41. Barbieri E, Sestili P, Vallorani L, Guescini M, Calcabrini C, Gioacchini AM, Annibalini G, Lucertini F, Piccoli G, Stocchi V. Mitohormesis in muscle cells: a morphological, molecular, and proteomic approach. MLTJ. 2013.
42. Ji LL, Gomez-Cabrera MC, Vina J. Exercise and hormesis: activation of cellular antioxidant signaling pathway. Ann N Y Acad Sci. 2006;1067:425–35.
43. Rattan SI. Targeting the age-related occurrence, removal, and accumulation of molecular damage by hormesis. Ann N Y Acad Sci. 2010;1197:28–32. https://doi.org/10.1111/j.1749-6632.2010.05193.x.
44. Hamel P, Abed E, Brissette L, Moreau R. Characterization of oxidized low-density lipoprotein-induced hormesis-like effects in osteoblastic cells. Am J Physiol Cell Physiol. 2008;294:C1021–33.

45. Delaine-Smith R, Reilly GC. Mesenchymal stem cell responses to mechanical stimuli. MLTJ. 2012.
46. Baksh D, Song L, Tuan RS. Adult mesenchymal stem cells: characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med. 2004;8(3):301–16.
47. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999;284(5411):143–7.
48. Hao W, Dong J, Jiang M, Wu J, Cui F, Zhou D. Enhanced bone formation in large segmental radial defects by combining adipose-derived stem cells expressing bone morphogenetic protein 2 with nHA/RHLC/PLA scaffold. Int Orthop. 2010;34(8):1341–9.
49. Whu SW, Tsai CL, Hsu SH. Evaluation of human bone marrow mesenchymal stem cells seeded into composite scaffolds and cultured in a dynamic culture system for neocartilage regeneration in vitro. J Med Biol Eng. 2009;29(2):52–9.
50. Kratchmarova I, Blagoev B, Haack-Sorensen M, Kassem M, Mann M. Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science. 2005;308 (5727):1472–7.
51. Watanabe T, Sakai D, Yamamoto Y, Iwashina T, Serigano K, Tamura F, Mochida J. Human nucleus pulposus cells significantly enhanced biological properties in a co-culture system with direct cell-to-cell contact with autologous mesenchymal stem cells. J Orthop Res. 2010;28(5):623–30.
52. Yu H, Vandevord PJ, GongW WuB, Song Z, Matthew HW, Wooley PH, Yang SY. Promotion of osteogenesis in tissue-engineered bone by pre-seeding endothelial progenitor cells-derived endothelial cells. J Orthop Res. 2008;26(8):1147–52.
53. Ozturk AM, Cila E, Kanatli U, Isik I, Senkoylu A, Uzunok D, Piskin E. Treatment of segmental bone defects in rats by the stimulation of bonemarrow osteo-progenitor cells with prostaglandin E2. Int Orthop. 2005;29(2):73–7.
54. Chen KY, Chung CM, Kuo SM, Chen YS, Yao CH. Influence of collagen I nanospheres on the growth and osteogenic difference of rat bone marrow stromal cells. J Med Biol Eng. 2009;29(6):284–9.
55. Jung Y, Song J, Shiozawa Y, Wang J, Wang Z, Williams B, Havens A, Schneider A, Ge C, Franceschi RT, McCauley LK, Krebsbach PH, Taichman RS. Hematopoietic stem cells regulate mesenchymal stromal cell induction into osteoblasts thereby participating in the formation of the stem cell niche. Stem Cells. 2008;26(8):2042–51.
56. Borovecki F, Pecina-Slaus N, Vukicevic S. Biological mechanisms of bone and cartilage remodelling-genomic perspective. Int Orthop. 2007;31(6):799–805.
57. Li M, Thompson DD, Paralkar VM. Prostaglandin E-2 receptors in bone formation. Int Orthop. 2007;31(6):767–72.
58. Tsai MT, Lin DJ, Huang S, Lin HT, Chang WH. Osteogenic differentiation is synergistically influenced by osteoinductive treatment and direct cell–cell contact between murine osteoblasts and mesenchymal stem cells. Int Orthop (SICOT). 2012;36:199–205.
59. Kim H, Lee JH, Suh H. Interaction of mesenchymal stem cells and osteoblasts for in vitro osteogenesis. Yonsei Med J. 2003;44(2):187–97.
60. Ahn SE, Kim S, Park KH, Moon SH, Lee HJ, Kim GJ, Lee YJ, Park KH, Cha KY, Chung HM. Primary bone-derived cells induce osteogenic differentiation without exogenous factors in human embryonic stem cells. Biochem Biophys Res Commun. 2006;340(2): 403–8.
61. Delaine-Smith R, Reilly GC. Mesenchymal stem cell responses to mechanical stimuli. MLTJ. 2012.
62. Ytteborg E, Torgersen JS, Pedersen ME, Helland SJ, Grisdale-Helland B, Takle H. Exercise induced mechano-sensing and substance P mediated bone modeling in Atlantic salmon. Bone. 2013;53(1):259–68. https://doi.org/10.1016/j.bone.2012.11.025 Epub 2012 Dec 3.
63. Oliva F, Tarantino U, Maffulli N. Immunohistochemical localization of calcitonin gene-related peptide and substance P in the rat knee cartilage at birth. Physiol Res. 2005;54:549–56.

64. Niissalo S, Hukkanen M, Imai S, Tornwall J, Kottinen YT. Neuropeptides in experimental and degenerative arthritis. Ann N Y Acad Sci. 2002;966:384–99.
65. McDougall JJ, Bray RC, Sharkey KA. Morphological and immunohistochemical examination of nerves in normal and injured collateral ligaments of rat, rabbit, and human knee joints. Anat Rec. 1997;248:29–39.
66. Fortier LA, Nixon AJ. Distributional changes in substance P nociceptive fiber patterns in naturally osteoarthritic articulations. J Rheumatol. 1997;24:524–30.
67. Kashihara Y, Sakaguchi M, Kuno M. Axonal transport and distribution of endogenous calcitonin generelated peptide in rat peripheral nerve. J Neurosci. 1989;9:3796–802.
68. Konttinen YT, Imai S, Suda A. Neuropeptides and the puzzle of bone remodeling. Acta Orthop Scand. 1996;67:632–9.
69. Ackermann PW, Ahmed M, Kreicbergs A. Early nerve regeneration after Achilles tendon rupture—a prerequisite for healing? A study in the rat. J Orthop Res. 2002;20:849–56.
70. Salo PT, Seeratten RA, Erwin WM, Bray RC. Evidence for a neuropathic contribution to the development of spontaneous knee osteoarthrosis in a mouse model. Acta Orthop Scand. 2002;73:77–84.
71. Madsen JE, Hukkanen M, Aspenberg P, Polak J, Nordsletten L. Time-dependent sensory nerve ingrowth into a bone conduction chamber. Acta Orthop Scand. 2000;71:74–9.
72. Modesti A, Oliva F. All is around ECM of tendons!? MLTJ. 2013.
73. Franchi M, Trirè A, Quaranta M, Orsini E, Ottani V. Collagen structure of tendon relates to function. Sci World J. 2007;7:404–20.
74. Bei R, Masuelli L, Palumbo C, Tresoldi I, Scardino A, Modesti A. Long-lasting tissue inflammatory processes trigger autoimmune responses to extracellular matrix molecules. Int Rev Immunol. 2008;27:137–75.
75. Connizzo BK, Yannascoli SM, Soslowsky LJ. Structure-function relationships of postnatal tendon development: a parallel to healing. Matrix Biol. 2013;32:106–16.
76. Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. J Bone Joint Surg Am. 2005;87:187–202.
77. Sharma P, Maffulli N. Biology of tendon injury: healing, modeling and remodeling. J Musculoskelet Neuronal Interact. 2006;6:181–90.
78. Tresoldi I, Oliva F, Benvenuto M, Fantini M, Masuelli L, Bei R, Modesti A. Tendon's ultrastructure. MLTJ. 2013.
79. Proske U, Gandevia SC. The proprioceptive senses: their roles in signaling body shape, body position and movement, and muscle force. Physiol Rev. 2012;92:1651–97.
80. Zimny ML. Mechanoreceptors in articular tissues. Am J Anat. 1988;182:16–32.
81. Abbot LC, Saunders JB, Dec M, et al. Injuries to the ligaments of the knee. J Bone Joint Surg. 1944;26:503–21.
82. Michelson JD, Hutchins C. Mechanoreceptors in human ankle ligaments. J Bone Joint Surg Br. 1995;77:219–24.
83. Hogervorst T, Brand RA. Mechanoreceptors in joint function. J Bone Joint Surg Am. 1998;80:1365–78.
84. Verra WC, van den Boom LG, Jacobs W, Clement DJ, Wymenga AA, et al. Retention versus sacrifice of the posterior cruciate ligament in total knee arthroplasty for treating osteoarthritis. Cochrane Database Syst Rev. 2013;11:10.
85. Raunest J, Sager M, Bürgener E. Proprioception of the cruciate ligaments: receptor mapping in an animal model. Arch Orthop Trauma Surg. 1998;118:159–63.
86. Abbott LC, Saunders JB, Dec M, et al. Injuries to the ligament of the knee joint. J Bone Joint Surg. 1944;26:503–21.
87. Schultz R, Miller D, Kerr C, et al. Mechanoreceptors in human cruciate ligaments. A histological study. J Bone Joint Surg Am. 1984;66:1072–6.
88. Halata Z, Rettig T, Schulze W. The ultrastructure of sensory nerve endings in human knee joint capsule. Anat Embryol. 1985;172:265–76.

89. Kapreli E, Athanasopoulos S, Gliatis J, Papathanasiou M, Peeters R, Strimpakos N, Van Hecke P, Gouliamos A, Sunaert S. Anterior cruciate ligament deficiency causes brain plasticity: a functional MRI study. Am J Sports Med. 2009;37(12):2419–26.
90. Dhillon MS, Bali K, Prabhakar S. Differences among mechanoreceptors in healthy and injured anterior cruciate ligaments and their clinical importance. MLTJ. 2012;2(1):38–43.
91. Scott JJ, Petit J, Davies P. The dynamic response of feline Golgi tendon organs during recovery from nerve injury. Neurosci Lett. 1996;207:179–82.
92. Sha L, Zhao L. Quantitative study on mechanoreceptors in tibial remnants of ruptured anterior cruciate ligament in human knees. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi. 2010;24:1318–22.
93. Haftel VK, Bichler EK, Wang QB, Prather JF, Pinter MJ, et al. Central suppression of regenerated proprioceptive afferents. J Neurosci. 2005;25:4733–42.
94. Jami L. Functional properties of the Golgi tendon organs. Arch Int Physiol Biochim. 1988;96:363–78.
95. Scott JJ, Panesar G. Small-signal sensitivity of muscle spindles reinnervated after long-term denervation. Brain Res. 1995;671:325–8.
96. Kaya D, editor. Proprioception: the forgotten sixth sense. Chapter: muscle, tendon, ligament tear and proprioception. OMICS Group eBooks, 2014.
97. Kumbar SG, James R, Nukavarapu SP, Laurencin CT. Electrospun nanofiber scaffolds: engineering soft tissues. Biomed Mater. 2008;3(3):034002. https://doi.org/10.1088/1748-6041/3/3/034002. Epub 8 Aug 2008.
98. Shanmugasundaram S, Chaudhry H, Arinzeh TL. Microscale versus nanoscale scaffold architecture for mesenchymal stem cell chondrogenesis. Tissue Eng Part A. 2011; 17(5–6):831–40. https://doi.org/10.1089/ten.TEA.2010.0409 Epub 2010 Dec 14.
99. Forman JR, Qamar S, Paci E, Sandford RN, Clarke J. The remarkable mechanical strength of polycystin-1 supports a direct role in mechanotransduction. J Mol Biol. 2005;349(4): 861–71.
100. Wang H, Sun W, Ma J, Pan Y, Wang L, Zhang W. Polycystin-1 mediates mechanical strain-induced osteoblastic mechanoresponses via potentiation of intracellular calcium and Akt/b-catenin pathway. PLOS ONE. March 2014.
101. Bean AC, Tuan RS. Fiber diameter and seeding density influence chondrogenic differentiation of mesenchymal stem cells seeded on electrospun poly(ε-caprolactone) scaffolds. Biomed Mater. 2015;10(1):015018. https://doi.org/10.1088/1748-6041/10/1/015018.
102. Shalumon KT, Chennazhi KP, Tamura H, Kawahara K, Nair SV, Jayakumar R. Fabrication of three-dimensional nano, micro and micro/nano scaffolds of porous poly(lactic acid) by electrospinning and comparison of cell infiltration by Z-stacking/three-dimensional projection technique. IET Nanobiotechnol. 2012;6(1):16–25. https://doi.org/10.1049/iet-nbt.2011.0028.
103. Schagemann JC, Paul S, Casper ME, Rohwedel J, Kramer J, Kaps C, Mittelstaedt H, Fehr M, Reinholz GG. Chondrogenic differentiation of bone marrow-derived mesenchymal stromal cells via biomimetic and bioactive poly-ε-caprolactone scaffolds. J Biomed Mater Res A. 2013;101(6):1620–8. https://doi.org/10.1002/jbm.a.34457 Epub 2012 Nov 27.
104. Spoliti M, Iudicone P, Leone R, De Rosa A, Rossetti FR, Pierelli L. In vitro release and expansion of mesenchymal stem cells by a hyaluronic acid scaffold used in combination with bone marrow. MLTJ. 2012.
105. Guevara-Alvarez A, Schmitt A, Russell RP, Imhoff AB, Buchmann S. Growth factor delivery vehicles for tendon injuries: mesenchymal stem cells and platelet rich plasma. MLTJ. 2014.
106. Della Porta G, Campardelli R, Falco N, Reverchon E. PLGA microdevices for retinoids sustained release produced by supercritical emulsion extraction: continuous versus batch operation layouts. J Pharm Sci. 2011. https://doi.org/10.1002/jps.
107. Della Porta G, Reverchon E. Continuous supercritical emulsions extraction: a new technology for biopolymer microparticles production. Biotechnol Bioeng J. 2011;108 (3):676–86.

108. Cardea S, Pisanti P, Reverchon E. Generation of chitosan nanoporous structures for tissue engineering applications using a supercritical fluid assisted process. J Supercrit Fluids. 2010;54:290–5.
109. Della Porta G, Cavalcanti S, Reverchon E. Supercritical Fluid emulsions extraction: a novel technology for the production of poly-lactic-co-glycolic nanostructured microdevices. In: Proceedings of II Congresso Nazionale di Bioingegneria; 2010 July 8–10; Turin (Italy): Patron, p. 637–8, ISBN 978-88-555-3082-8.
110. Reverchon E, Adami R, Cardea S, Della Porta G. Supercritical fluids processing of polymers for pharmaceutical and medical applications. J Supercrit Fluids. 2009;47:484–92.

Chapter 8
Modular Tissue Engineering: An Artificial Extracellular Matrix to Address and Stimulate Regeneration/ Differentiation

Giovanna Della Porta and Ernesto Reverchon

Abstract Tissue engineering uses living cells as engineering materials cultivated and merged within a tri-dimensional structure in order to develop a tissue-like device; these artificial extracellular matrixes, called *scaffolds*, are important in influencing cells microenvironment and addressing their fate, both ex vivo as well as in vivo. Indeed, tri-dimensional scaffolds allow cell attachment, enable diffusion of vital cell nutrients or catabolites and can also deliver mechanical or biological signals. In the traditional tissue engineering "*top-down*" *approach*, cells are seeded onto biopolymer scaffold and then let them to migrate and colonize the structure; recently, the "*bottom-up*" *approach* has been described to design structural bioengineered microfeatures that can be used as building blocks to create larger tri-dimensional structures and build modular tissues. In some specific applications, a mix of the two approaches is also proposed to generate multiphase matrices. Several technologies for scaffold production are described in this chapter both using conventional organic solvent and dense gas or supercritical fluid; some solutions for artificial extracellular matrices useful for musculoskeletal tissue regeneration are also proposed.

The "Top-Down" or "Bottom-Up" Approach: Which Is the Best Choice?

Traditional tissue engineering strategies involves the so-called "*top-down*" *approach* (see Fig. 8.1), in which cells are seeded on a biodegradable three-dimensional (3D) biopolymer structure [1–3] and it is expected that they will

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populate the scaffold with the aid of dynamic cultivation, often supplemented with growth factors, generating an appropriate extracellular matrix (ECM) [4–6]. These 3D scaffolds are still far from leading to successful tissue reconstruction in a clinical setting because of the absence of specific microarchitecture, a limited mass transport of metabolites or oxygen through the 3D structure, a reduced control over their biodegradation. Moreover, when bio-signals or growth factors are incorporated by simple adsorption onto bulk scaffolds, they normally leads to uncontrolled burst release on implantation with an overdose that may give rise to undesired effects [7, 8].

More recent tissue engineering approach described as "*bottom-up*" *one* or modular tissue engineering aims to address the challenge of recreating bio-mimetic structures by designing structural microfeatures that can be used to build modular tissues such as building blocks to create larger tissues (see Fig. 8.2). These 3D modules can be fabricated in different ways, such as microfabrication of cell-laden hydrogels [9, 10], creation of cell sheets [11], or direct tissue printing [12]. By mimicking native microstructural functional units, these approaches aim to create more bio-mimetic engineered tissues because these modules can be assembled into larger tissues-like structure through a number of methods such as random packing [13, 14], stacking of layers [15], or ordinate assembly [16].

Bottom-up approach is aimed to provide guidance on the cellular level to direct tissue morphogenesis by creating modular tissues with more physiological microarchitectural features [17]. In addition, bio-plotting technology can help this approach by designing a zonal organization into the 3D engineered artificial matric plotted; for example, one strategy may be to spatially pattern cells with specific additives from different zones into stratified layers to better mimic the in vivo zonal organizations of the extracellular matrix. In this sense, bio-plotting additives such as

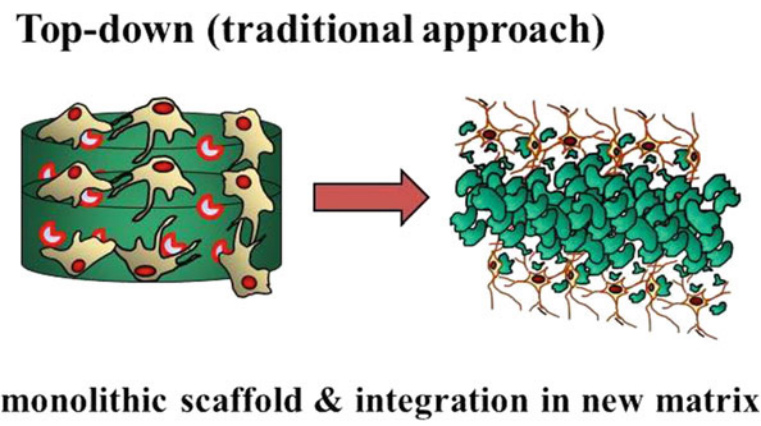

Fig. 8.1 *Top-down approach* involves monolithic scaffold on which the cells are seeded; often, cells do not colonize the interior part of the scaffold

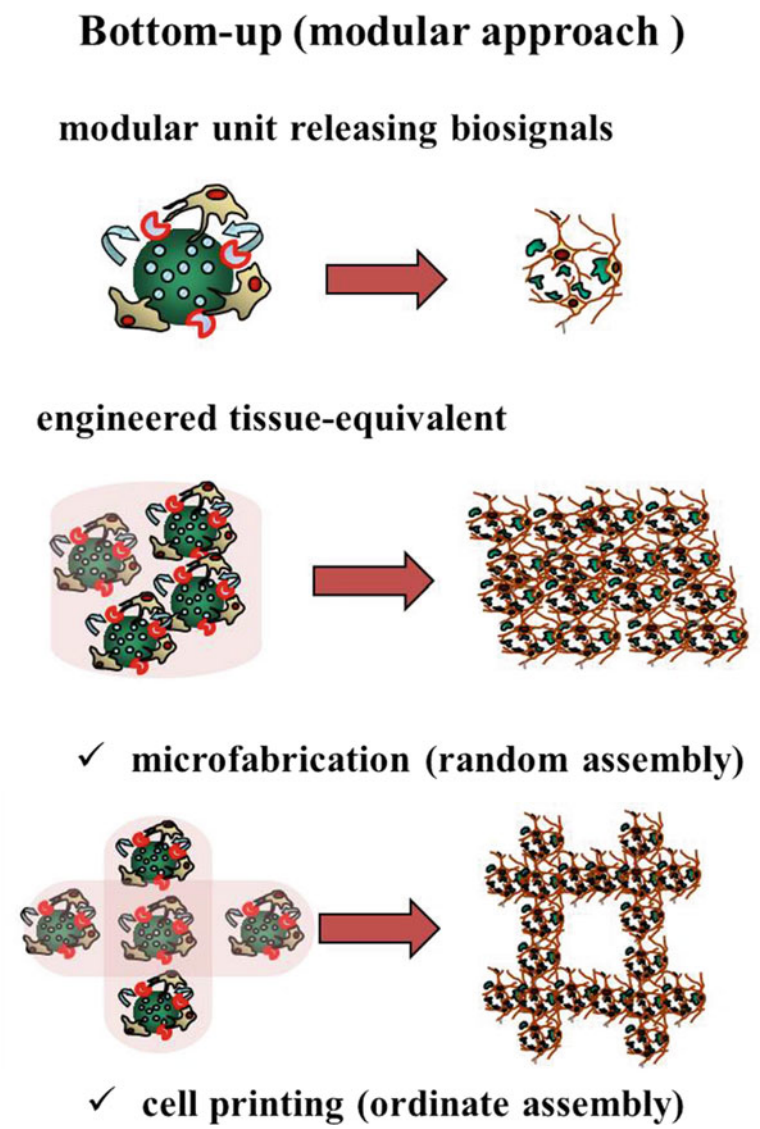

Fig. 8.2 *Bottom-up approach* allows generating scaffolds of higher complexity and functionality that can be designed with several advantages over conventional monolithic scaffolds

biopolymer microcapsules can further be organized into a 3D pre-designed microenvironment where they can deliver biomolecule within the continuous matrix without compromising the properties of the bulk structure; these microdevices allow generating scaffolds of higher complexity and functionality that can be

designed with several advantages over conventional monolithic bulk scaffolds [18, 19]. Moreover, by introducing microspheres, mechanically weak scaffolds can be stabilized or reinforced providing an improved mechanical behaviour [20, 21].

Biopolymer microspheres with peptides immobilized on the surface have been also reported as a mouldable scaffold for cartilage tissue engineering [22, 23], whereas gelatin microspheres with bioactive domains including peptides resulted in cytocompatible microcarriers suitable to deliver live cells such as chondrocytes for in vivo chondrogenesis [24, 25]. During the dynamic cultivation, cells adhered, proliferated and synthesized a thin layer of extracellular matrix (ECM) in and around the macroporous beads allowing a biological sintering via cell–cell and cell–matrix interaction after only a few days of dynamic seeding [26].

Biomaterials for Scaffold Fabrication

Natural polymers are of great importance for tissue engineering, basically due to their intrinsic biocompatibility and biodegradability. This class of polymers is often favoured more than synthetic ones due to their abundant side groups in their molecular chains that allow for further functionalization. Further, natural polymers such as collagen and gelatine contain motifs such as RGD (arginine–glycine–aspartic acid) sequences, which can modulate cell adhesion and improve their behaviour. *Collagen* is the most extensively investigated natural polymer since the ECM of many mesenchymal tissues (including bone and cartilage) is mainly composed of collagen as organic phase. Collagen is an attractive candidate material for bone regeneration due to its excellent biocompatibility, desirable biodegradability and negligible immunogenicity [27, 28]. As a derivative from collagen, *Gelatin* has been also used for biomedical applications; its characteristics include a controllable degradation, and abundant presence of functional groups that allow for further functionalization and modification via chemical derivatization. Specifically, the unique electrical nature of gelatin (commercially available as both positively or negatively charged polymers at neutral pH) enables gelatin to encapsulate bioactive molecules by forming polyion complexes [29, 30].

Fibrin can be prepared by combining fibrinogen with thrombin, which are both derived from the patient's own blood, thereby fabricating an autologous scaffold that does not induce an excessive foreign body reaction. However, the rapid degradation rate and poor mechanical stability of fibrin have been stated as the main limitation for bone tissue engineering. To overcome this problem, composite microspheres (alginate/fibrin, poly-hyaluronic acid/fibrin) were developed to stabilize the fibrin matrix, solving the problems of both alginate shortage of bioactive sequence for cell attachment and fibrin poor capacity for cell encapsulation [31, 32].

Chitosan is a frequently used hydrophilic polysaccharide derived from chitin, which exhibits favourable physicochemical and biological properties for biomedical

applications including biocompatibility and intrinsic antibacterial nature [33], whereas a*lginate* favourable characteristics including its biocompatibility, non-immunogenicity, hydrophilicity and cost-effectiveness make it highly suitable for many applications in drug delivery and tissue engineering. The most pronounced advantage of alginates is related to its gentle gelling behaviour using crosslinking in the presence of divalent cations, such as Ca^{2+} [34, 35].

Poly-(*glycolic acid*) (PGA), *poly*-(*lactic aci*d) (PLA) and their copolymers are a family of linear aliphatic polyesters, which are the most frequently used in tissue engineering because they degrade through hydrolysis of the ester bonds. Because of its relatively hydrophilic nature, PGA degrades rapidly in aqueous solutions or in vivo and loses mechanical integrity between two and four weeks. PLA is also widely used for scaffold fabrication; the extra methyl group in the PLA repeating unit (compared with PGA) makes it more hydrophobic, reduces the molecular affinity to water and leads to a slower hydrolysis rate. It takes many months or even years for a PLA scaffold or implant to lose mechanical integrity. To achieve intermediate degradation rates between PGA and PLA, various lactic and glycolic acid ratios are used to synthesize PLGA. Particularly, *poly-lactic-co-glycolic acid* (PLGA, with a copolymer ratio of 75:25 or 50:50) has received a great interest in the development of injectable microparticulate devices [36]. For example, PLGA microparticles are described for in situ injection to achieve a precise and localized drug delivery which is more effective in chemotherapy, hormone therapy, DNA/protein or vaccine delivery [37–40]. Moreover, PLGA microparticles with a specific size and distribution have also been proposed for the use in tissue engineering as building blocks of implantable 3D scaffolds offering benefits such as good morphology control and better versatility in the release kinetics of specific growth factors for cellular function orienting and directing or biodegradable support for cell culture and/or cell administration [41].

There are other linear aliphatic polyesters, such as *poly*-(*ε-caprolactone*) (PCL) *and poly*-(*hydroxy butyrate*) (PHB), which are also used in tissue engineering research. PCL degrades at a significantly slower rate than PLA, PGA and PLGA. The slow degradation makes PCL less attractive for general tissue engineering applications, but more attractive for long-term implants and controlled release applications [42].

3D Scaffold Fabrication: The Organic Solvent Can Be Replaced by Supercritical Fluid

Conventional technologies for scaffold and microsphere fabrication use organic solvents to solubilize the chosen biopolymer or their blend to generate a 3D scaffold or microcapsules. One largely used method for pre-shaped 3D scaffolds fabrication is the *solvent casting and particulate leaching* (see Fig. 8.3). The protocol involves first mixing water-soluble salt particles in the polymer solution, and then, the

suspension is cast into the mould of a desired shape; after solvent evaporation or lyophilization, the salt particles are leached out to obtain biopolymer porous 3D structure with porosity that can be of 90%. Closed cavities in the resulting matrix and smooth wall are the described drawbacks of these structures as well as complex post-treatments to remove the residual amount of solvents used [43]; moreover, poor metabolites mass transfer within the structure during the cell cultivation and poor cell colonization inside the structure is often described for these scaffolds, even if cultivated in dynamic conditions [44, 45]. *Electrospinning* is a more advanced fabrication technology that uses an electric field to control the formation and deposition of biopolymer microfibres onto a target substrate (see Fig. 8.4). A critical voltage is applied in order to overcome the surface tension of the biopolymer solution and obtain an electrically charged jet; the jet within the electric field is, then, directed towards the ground target, during which time the solvent evaporates forming oriented or random fibres with diameters ranging from several microns down to several hundred nanometres [46]. Limitations described are: low mechanical strength and difficulty in controlling the 3D structure pore shapes and sizes. *Rapid prototyping* is a technology based on the development of computer science and manufacturing industry. The main advantage of these techniques is their ability to produce complex products rapidly from a computer-aided design (CAD) model. One of these rapid prototyping techniques, called 3D printing, has been used to process biodegradable polymer scaffolds for tissue engineering applications [47, 48]. All the described technologies lack biological agents incorporating within the biopolymer structure; for example, peptides such as growth factors cannot be easily loaded into the 3D scaffolds because of the extreme conditions (temperature/organic solvents) used in the fabrication process.

Technologies that replace the use of an organic solvent with dense gases or supercritical fluids are also emerging in fabricating 3D devices for biomedical applications. Supercritical fluids (SCFs) are defined substances at a temperature and pressure above their critical point as described by the pressure vs. temperature diagram for a pure compound (see Fig. 8.5). In a specific pressure and temperature conditions, a substance becomes supercritical and can be described as an homogeneous fluid that can effuse through solids like a gas, but has also the ability to

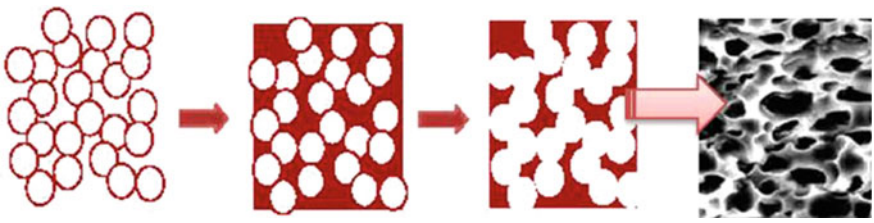

Fig. 8.3 Solvent casting and particulate leaching method and SEM image of the microstructure of the 3D scaffold obtainable

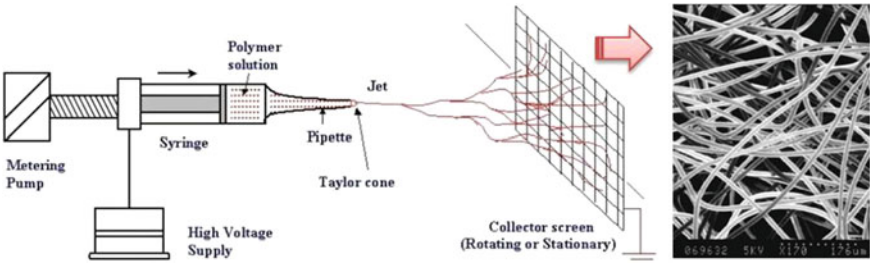

Fig. 8.4 Schematic representation of electrospinning method and SEM image of the microstructure of the 3D scaffold obtainable

dissolve materials like a liquid. In addition, close to the critical point, small changes in pressure or temperature result in large changes in density, allowing a fine tuning of the supercritical fluid solvent power. As a consequence, the supercritical fluids seemed suitable to substitute organic solvents in a wide range of industrial processes [49]. Particularly, its reduced surface tension coupled with higher diffusivity (that is similar to gases and of about two orders of magnitude larger than that of liquid solvents) offers possibilities of fabrication structures not obtainable by using the conventional liquid solvents. Carbon dioxide is the most commonly used supercritical fluids (SC-CO_2) because it is not toxic, not flammable, cheap and with critical parameters of pressure and temperature are readily accessible on the industrial scale (304.1 K and 73.8 bar).

The first use of SC-CO_2 for temporary 3D scaffolds production has been suggested with the g*as-foaming technique*. The process is solventless and very efficient in producing the porous biopolymer structure because the dense gas can foam a

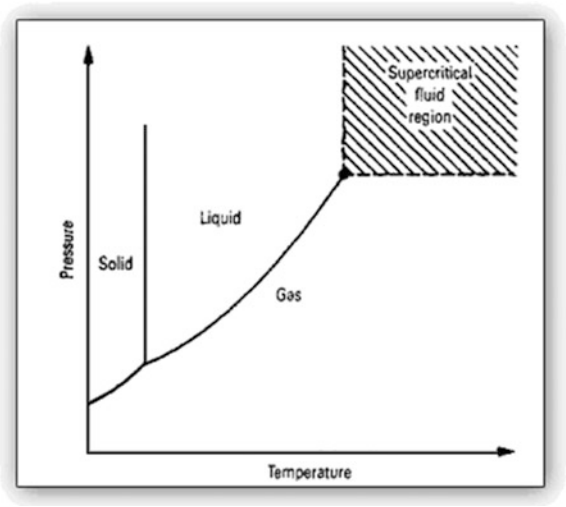

Fig. 8.5 State diagram for a pure component in the PT region

given biopolymer inducing the formation of bubbles within its structure (see Fig. 8.6). It was also described that the morphology of the foams can be varied by controlling the depressurization rate of the foamed biopolymer, such as PLA or PLGA, and the density of the gas; particularly, a rapid depressurization locked in large numbers of spherical pores within the biopolymer structure, whereas a slower depressurization reduced pores elongation. Drawbacks of the gas-foaming process are smooth surfaces and generally closed pores structures [50]. Another technology described for scaffold production by dense gases is the s*upercritical assisted phase inversion method* (*SAPIM*); the process reproduces the traditional phase inversion method (obtained by liquid–liquid diffusion or by varying the system temperature) by using SC-CO_2. Therefore, the dense gas is used to dry the biopolymer structure more rapidly due to an enhanced mass transfer caused by a reduced surface tension and the absence of liquid–vapour interface. In a specific condition of pressure and temperature and system composition, open pores are obtained and no traces of organic solvents are described in the resulted scaffold. Moreover, the use of SC-CO_2 by means of pressure introduces an additional variable to influence the biopolymer/solvent solution de-mixing process and, therefore, the porous structure morphology. Indeed, it was described that it is possible to control the average pore size that can decrease with the increase of SC-CO_2 density (either by increasing the pressure or by decreasing the temperature), whereas the average pore diameter decreased with the increase of the initial polymer concentration [51]. *Reverchon & Cardea* [52] explained this behaviour indicating that the phase separation process can be responsible for the porous cellular structure obtainable in depending on the liquid/liquid de-mixing by nucleation and growth of droplets of a polymer lean phase. The subsequent removal of the solvent produces a dry and stable structure. The proposed process is versatile, and the scaffold characteristics can be continuously modulated varying supercritical CO_2 properties. Limitations are again smooth porous walls and poorly interconnected pores within the 3D structure. To fulfil the necessity of producing interconnected micropores, a supercritical fluid-assisted technique named *supercritical gel drying* has been also proposed and described [53]. The process involves the formation of a biopolymer alcohol gel loaded with a solid porogen that is then dried by SC-CO_2; the final washing with water eliminates the porogen fabricating a porous structure. When the gel drying is performed by supercritical CO_2, the supercritical mixture formed during the process (ethanol plus CO_2) has no surface tension and can be easily eliminated in a single step by the continuous flow of SC-CO_2 in the drying vessel. The major problem during gel drying is the possibility of gel collapse; however, the reduced surface tension of the supercritical mixture used to avoid this problem preserving the nanoporous structure in the fabricated 3D aerogel [54]. These scaffolds were successfully tested for human mesenchymal cells cultivation in a specific dynamic cultivation [55].

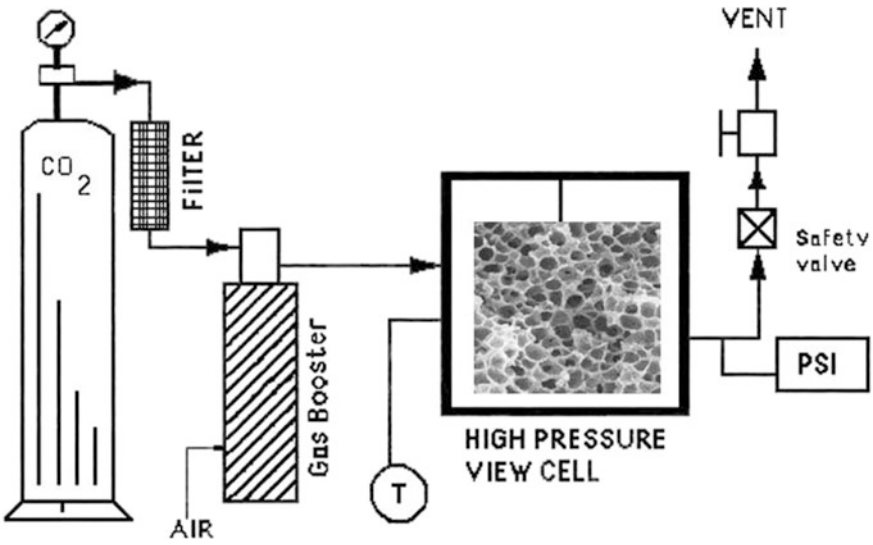

Fig. 8.6 Schematic representation of *supercritical gas-foaming* method with SEM image of the microstructure of the 3D scaffold obtainable

Microdevices and Microcapsules Fabrication: A Challenge for Supercritical Fluid Technology

Several techniques can be used to prepare biopolymer microcarriers, but the solvent evaporation/extraction of emulsion (single, double or multiple) is the most widely used. The process involves single *oil-in-water* (*o/w*) or double *water-in-oil-in-water* (*w/o/w*) emulsions to encapsulate biomolecules [56]. The biopolymer is first dissolved in a water-immiscible organic solvent. The bioactive molecule is then added to the polymer solution to produce a solution or dispersion of the substance; however, more often, a double emulsion is prepared that involves the formulation of a *water-in-oil* (*w/o*) primary emulsion to encapsulate water-soluble molecules like peptides or proteins, unlike the *o/w* formulation which is ideal for water-insoluble compounds [56]. The primary *w/o* emulsion is, then, emulsified (with appropriate stirring and temperature conditions) in a larger volume of water in presence of an emulsifier to yield the final *w/o/w* emulsion. The emulsion is subjected to solvent removal by either evaporation or extraction process to harden the oil droplets [57]. The solid microspheres obtained are, then, washed and collected by filtration or centrifugation and then dried under appropriate conditions or lyophilized. One of the disadvantages of the emulsification method is the poor encapsulation efficiencies of moderately water-soluble and water-soluble compounds. Indeed, these molecules could diffuse out or partition from the dispersed oil phase into the aqueous continuous phase, and their microcrystalline fragments can deposit on the microsphere surface and/or outside of the biopolymer matrix [58].

Moreover, the evaporation has several drawbacks because it requires relatively high temperatures or reduced pressures and shows batch-to-batch reproducibility disturbances, whereas the solvent extraction uses relatively large amounts of a second solvent with the subsequent problem of solvent mixtures recovery. Both processes, also, require quite long processing times (several hours) and, as a consequence, aggregation phenomena may occur between the droplets, producing microspheres with a larger poly-dispersity with respect to the droplets in the starting emulsion [59].

Several supercritical technologies were proposed to produce microparticles, and they are reviewed elsewhere [60]. Recently, the use of SC-CO_2 has also been proposed for biopolymer micro-/nanocarriers manufacturing, replacing the conventional emulsions evaporation/extraction technology. Particularly, SC-CO_2 was proposed as extracting agent of the oily phase of emulsions to lead to solvent-free microparticles [61–65]. The *supercritical extraction of emulsion* (SEE) process was also proposed with a continuous operating mode that allowed greater product uniformity, higher throughput with smaller plant volumes and elimination of batch-to-batch repeatability problems (see Fig. 8.7). Indeed, SC-CO_2 assured a faster oily phase extraction with respect to the traditional evaporation/extraction processes that will have a significant effect on the size distribution of the biopolymer microcapsules which reproduce exactly the size distribution of the originally droplets (with a given shrinkage) because any aggregation phenomena is prevented due to the faster processing [66, 67]. The supercritical technology achieved the fabrication of microcarriers of several biopolymers such as PLGA, PLLA, PCL, PMMA with no solvent residues and higher encapsulation efficiencies with respect to the conventional extraction/evaporation technology, especially when double emulsions were processed [68, 69].

Micro-/nanocapsules entrapping magnetic iron oxide or gold nanoparticles have been also fabricated by SEE technology in order to obtain a remote controlled or triggered drug delivery [70, 71]. SEE-C technology can also generate multiloaded microsystems with different molecules such as hydroxyapatite (HA), gentamicin (GE) and teriparatide hormone (TH), for example, to be used in the bone cement formulation in the treatment of osteoporosis [72].

Micro-/Nanocarriers as Functional Components for a Bottom-Up Approach to Bioengineered Scaffold

Recent tissue engineering strategies suggested the use of 3D scaffold not only to simply support the cells growth but also to interact with the cells on-board providing an additional level of cell environment regulation. In this sense, the locally and controlled release of specific growth factors and/or bio-signals can be a good approach in order to mimic more properly the microenvironment that the extracellular matrix provides to the cells in order to address their behaviour.

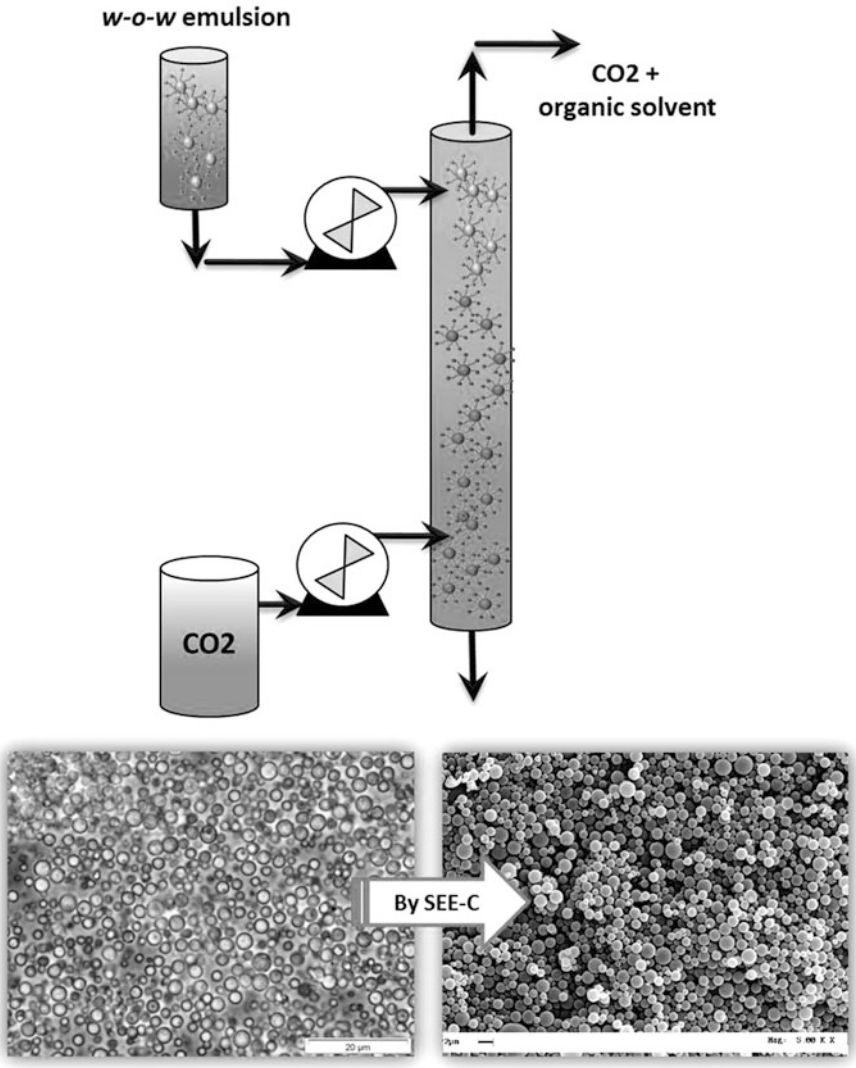

Fig. 8.7 Supercritical emulsion extraction (SEE) process layout description with optical microscope image of an emulsion (left side) and of an SEM image of the microcapsules produced (right side)

Current fabrication methods for GF-loaded scaffolds can be divided into two main categories: attachment of the GF (physical and non-physical) to the scaffold or entrapment of the GF within the scaffold. The attachment may be achieved by adsorption or through chemical crosslinking. Fibrin-based matrices are popular biomaterials for this purpose [73, 74], and the release then occurs passively and through simultaneous fibrin degradation. Entrapment of GFs has been also

proposed in porous solid scaffolds, through solvent casting/particulate leaching and supercritical fluid (SCF) processing [75, 76]. SCF processing seemed to be attractive since it does not require the use of harmful solvents which could adversely affect the GFs. However, the protein denaturation during scaffold fabrication is the main problem associated with the incorporation of proteins in various systems and leads to a considerable loss of their activity. Biomolecules have been also directly incorporated into electrospun nanofibres by mixing the biomolecules into a polymer solution prior to electrospinning the mixture [77] or by using coaxial electrospinning wherein a secondary polymer solution containing the biomolecules is electrospun within the core of the forming nanofibre surrounded by a shell polymer [78–80]. Again severe protein damage may occur during the scaffold production.

Loading GFs into hydrogels is also possible by simply mixing the GF into the hydrogel precursor. As the polymerization occurs, the GFs become entangled in the polymer chains and are physically trapped within the hydrogel network. However, the hydrogels pore size is often larger than the size of the GF, so that it often releases the majority of the contained GF within the first days of cultivation. However, the direct GF loading into scaffolds does not allow a precise control of the release kinetics and often when GFs are exposed to the medium; this may result in loss of bioactivity.

The use of micro- or nanocarriers can overcome this problem by protecting the GF and allowing the use of various populations of particles with different degradation properties and thus different release kinetics of the encapsulated GFs [81, 82]. Indeed, by incorporating GFs into microspheres and then use these microspheres as a dispersed phase surrounded by a continuous matrix (biopolymer or hydrogel) allows to establish a skilled 3D scaffolds able to mimic a surrounding matrix. For example, it has been reported that *h*BMP delivery from PLGA nanospheres incorporated into a PLA scaffold induced significant ectopic bone formation while failure of bone formation was observed with passive adsorption of *h*BMP onto the scaffold, likely due to significant loss of biological activity of the GF and diffusion away from the implantation site [83].

Microspheres embedded into a continuous matrix can provide a temporal and a geometrical controlled release of GFs to surrounding cells; an initial burst release or a sustained release of GFs can be engineered in different part of the bioactive matrix by tailoring the microcarriers size and distribution, as well as, the biopolymer compositions or copolymer ratio loading different molecules and by loading different molecules in different micro-/nanodevices population. In the case of bone engineering, for example, an initial burst release of BMP-2 is actually preferential for promoting new bone formation. This can be explained by the fact that BMP-2 plays an important role during the healing process to promote cell differentiation but is equally important at the early stages in the healing processes for the initial migration of progenitor cells and triggering the fracture healing cascade [20, 84]. Geometrical indications inside a specific scaffold can be also obtained by incorporating different microdevices loaded with different bio-signals in different part of

the structure or by using magnetic micro-/nanodevices to generate a specific molecular/enzyme gradient by their orientation with an external magnetic field.

Bio-plotting technology may also have advantage from microdevices designed for the controlled delivery of bio-signals. Indeed, the bioplotter deposits hydrogel matrix loaded with living cells in small groups using similar technology to traditional printing systems. The advantage of this technique is the high potential level of control in cell and ECM placement and alignment to create engineered tissues with a wide array of properties and geometries. Indeed, by replacing ink with cells, therefore named "*bio-ink*" because suspended in liquid matrix, a specific pattern can be designed and printed onto a substrate that has to be cultured to allow the cells and the produced ECM to integrate into a real tissue structures [85, 86]. However, the bio-plotting technology also allows the loading of additive with the *bio-inks*; these additive can be biopolymer microdevices or microcapsules that may influence not only the mechanical behaviour of the 3D structure but also deliver in a controlled spatio-temporal manner several biomolecules such as growth factor or enzymes in order to better mimic the specific microenvironment of the extracellular matrix and give rise of a faster and more appropriate cell addressing versus a specific fate (see Fig. 8.8). These pre-designed 3D structures may better reproduce the cells microenvironment that encourage cells differentiation and ECM production. Engineered modular tissues, made using these techniques, have often shown insufficient mechanical properties and on the order of native tissues [87]. However, these drawbacks can be overcome by a mixed approach with bio-plotting hydrogel onto a structure with pre-selected mechanical properties.

An example of bioactivated 3D scaffold easy bioplottable can be a continuous hydrogel matrix of Ca-Alginate (2%w/w) loaded with stem cells and additives such PLGA microcapsules carrying growth factors such as VEGF and BMP-2 [88]. PLGA-encapsulated GFs were produced by SEE technology, and they assured a sustained release over 18 days when compared to GFs directly encapsulated into scaffolds that was released in almost 3 days. Osteogenic differentiation pathway of *h*MSCs was clearly observed after few day of cultivation in presence of controlled release of *h*BMP-2 and faster with respect to the control cultivation without the bio-signals delivered within the scaffold structure. Biopolymer micro-/nanocapsules have been also loaded into collagen [89], gelatin [90], fibrin [91], chitosan [92], and by functionalizing each type of spheres separately, the structure of the resulting scaffolds can be precisely controlled at a microscale and/or customized by encapsulation of signalling biomolecules or bioactive minerals.

Recently also emerged a common theme of incorporating biopolymer microcapsules into densely cellular constructs where microcapsules can offer specific microenvironment to chondrogenic cells in high-density culture in order to regulate cell behaviour and tissue formation. As in the case of bone, microspheres may act in a variety of specialized roles to define the three-dimensional arrangement of neocartilage tissue formation, deliver chondrogenic growth factors in a temporally and spatially controlled manner and instruct cell–cell and cell–material interactions. In this particular case, biopolymer microspheres incorporated within densely cellular tissues can also act as spacers, defining 3D space for tissue formation, while

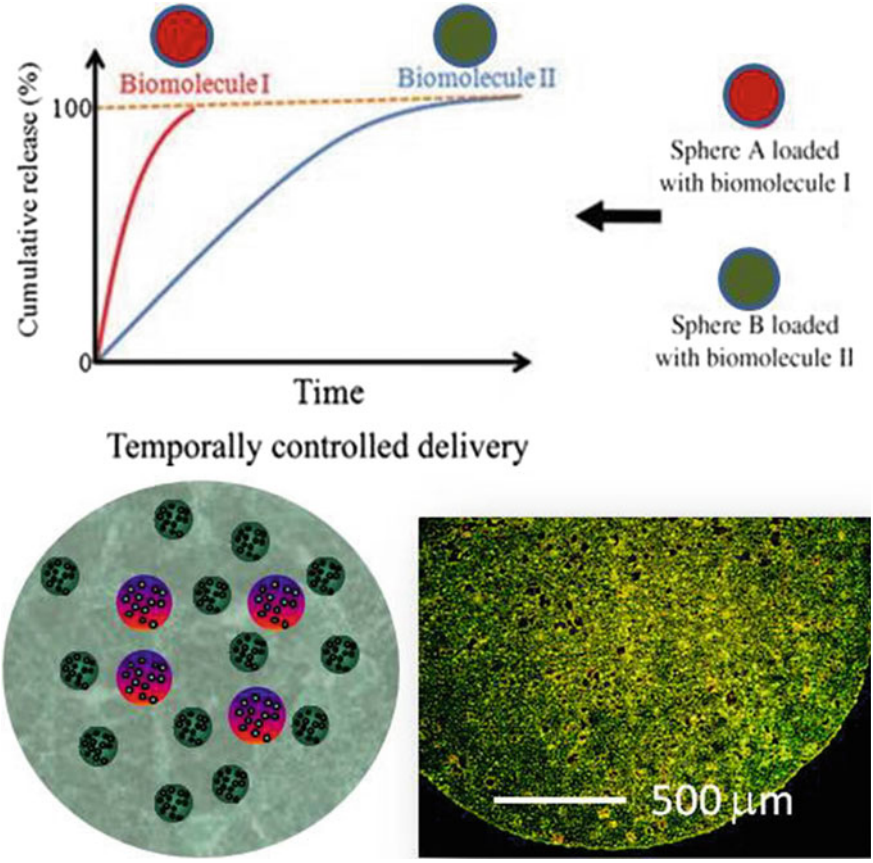

Fig. 8.8 Schematic representation of the use of micro-/nanospheres for spatio/temporal delivery of bioactive molecules. These, pre-designed 3D structure may better reproduce the cells microenvironment that encourage cells differentiation and ECM production

accommodating ECM accumulation and cell infiltration as the microdevice degrades. The inclusion of polymer microspheres into high-density cell cultivation systems enables a more uniform cartilaginous matrix assembly with a morphological structure similar to that of native cartilage tissue when compared to cell-only constructs without microdevices [93, 94]. Moreover, the use of microcapsule with specific properties, such us biopolymer degradation rate and size distribution, can influence cartilage tissue formation, as well as, enabling the formation of a uniform, structurally appropriate cartilage tissue if microspheres are fabricated with a fine-tuned degradation rate proportional to the rate of ECM elaboration [23]. Microdevices may contribute to the mechanical stability of the constructs and give a spacing effect that could be advantageous for creating tissues morphologically similar to native articular cartilage, which consists primarily of an organized ECM containing water, type II collagen and proteoglycans with relatively few, disperse chondrocytes.

Beyond their utility as structural elements, biopolymer microcapsules can delivery chondrogenic growth factors such as TGF-β family. The incorporation of bioactive microspheres in cell culture systems could provide a means to decrease growth factor diffusion distance within densely cellular constructs, improving growth factor delivery to central regions of the tissues. Further, these systems allow growth factor release over a sustained period of time as the microspheres degrade, enabling localized delivery of chondrogenic factors to the surrounding cells at controllable rates over a period of days, weeks or months [23, 95–97]. The sustained delivery of either TGF-β family in implantation models may play a role in maintaining the chondrocytic phenotype of the cells, preventing dedifferentiation or hypertrophic progression, as often occurs after the in vivo implantation of in vitro differentiated MSC pellets [98, 99].

Conclusions and Perspectives

The bottom-up approaches seemed the best tissue engineering approach choice because it is able to fabricate a better reproduction of the 3D microenvironment that interacts with the cells to faster and better address their fate. The modular approach also seemed to rely on the assembly of micron scale structural elements as the basis for providing defined signals to drive the formation of tissues that can be scaled up to create larger tissues. Moreover, the use of biopolymer microcapsules inserted within the 3D scaffold can allow the design of a specific microenvironment able to instruct the cell behaviour and to organize a pre-designed microenvironment with an engineered spatio-temporal delivery of multiple bio-signals.

Biopolymer microspheres are also proposed as regulators of the chondrogenic microenvironment within high-density cell cultures as three-dimensional structural elements for cell expansion and delivery or vehicles for spatiotemporally controlled growth factor delivery, to regulate cell behaviour and/or cell material interactions. These drug-releasing microspheres have the capability not only to overcome diffusion limitations posed by traditional culture techniques, but also to lend tailorable release kinetics for temporal control of differentiation and extended maintenance of differentiation state in vivo.

Furthermore, micro-/nanospheres can serve as reinforcement components or crosslinking agents to provide hydrogels with additional mechanical support. Indeed, microspheres loaded with inorganic materials of high stiffness can reinforce the initial mechanical strength of the composites. For example, biopolymer microspheres can be used as different "*bio-ink additives*" added to a 3D bioplotted hydrogel to give specific mechanical or biochemical indications to the cells in the different layers following the novel concept of 3D organ printing, in which scaffold/cellularized tissues or organ can be fabricated layer by using tissue spheroids as building blocks.

References

1. Van Blitterswijk CA, Thomsen P, Lindahl A, Hubbell J, Williams DF, Cancedda R, de Bruijn J, Sohier J, editors. Tissue engineering textbook. London: Academic Press; 2008. p. 403–54. ISBN 978-0-12-370869-4.
2. Langer R, Vacanti JP. Tissue engineering. Science. 1993;260(5110):920–6.
3. Hillsley M, Frangos J. Review: bone tissue engineering: the role of interstitial fluid flow. Biotech Bioeng. 1994;43:575–81.
4. Martin P. Wound healing–aiming for perfect skin regeneration. Science. 1997;276(5309): 75–81.
5. Caplan AI, Elyaderani M, Mochizuki Y, Wakitani S, Goldberg VM. Principles of cartilage repair and regeneration. Clin Orthop. 1997;342:254–69.
6. Niklason LE, Gao J, Abbott WM, Hirschi KK, Houser S, Marini R, Langer R. Functional arteries grown in vitro. Science. 1999;284(5413):489–93.
7. Gooch KJ, Blunk T, Courter DL, Sieminski AL, Vunjak-Novakovic G, Freed LE. Bone morphogenetic proteins modulate in vitro development of engineered cartilage. Tissue Eng. 2002;8(4):91–601.
8. Schaner PJ, Martin ND, Tulenko TN, Shapiro IM, Tarola N, Leichter RF, Carabasi RA, Dimuzio PJ. Decellularized vein as a potential scaffold for vascular tissue engineering. J Vasc Surg. 2004;40(1):146–53.
9. Dean DM, Napolitano AP, Youssef J, Morgan JR. Rods, tori, and honeycombs: the directed self-assembly of microtissues with prescribed microscale geometries. FASEB. 2007;21: 4005–12.
10. Yeh J, Ling Y, Karp JM, Gantze J, Chandawarkard A, Eng G, Blumling J, Langer R, Khademhosseini A. Micromolding of shape-controlled, harvestable cell-laden hydrogels. Biomaterials. 2006;27:5391–8.
11. L'Heureux N, Paquet S, Labbe R, Germain L, Auger FA. A completely biological tissue-engineered human blood vessel. FASEB J. 1998;12(1):47–56.
12. Mironov V, Boland T, Trusk T, Forgacs G, Markwald RR. Organ printing: computer-aided jet-based 3D tissue engineering. Trends Biotechnol. 2003;21(4):157–61.
13. McGuigan AP, Sefton MV. Design and fabrication of sub-mm-sized modules containing encapsulated cells for modular tissue engineering. Tissue Eng. 2007;13(5):1069–78.
14. McGuigan AP, Sefton MV. The thrombogenicity of human umbilical vein endothelial cell seeded collagen modules. Biomaterials. 2008;29:2453–63.
15. 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.
16. Du Y, Lo E, Ali S, Khademhosseini A. Directed assembly of cell-laden microgels for fabrication of 3D tissue constructs. Proc Natl Acad Sci. 2008;105(28):9522–7.
17. Nichol JW, Khademhosseini A. Modular tissue engineering: engineering biological tissues from the bottom up. Soft Matter. 2009;5(7):1312–9.
18. Narayanan LK, Huebner P, Fisher MB, Spang JT, Starly B, Shirwaiker RA. 3D-bioprinting of polylactic acid (PLA) nanofiber-alginate hydrogel bioink containing human adipose-derived stem cells. ACS Biomater Sci Eng. 2016;2:1732–42.
19. Shen J, Guvendiren M. Recent advances in bioink design for 3D bioprinting of tissues and organs. Front Bioeng Biotechnol. 2017. https://doi.org/10.3389/fbioe.2017.00023.
20. Wang H, Leeuwenburgh SCG, Li Y, Jansen JA. The use of micro- and nanospheres as functional components for bone tissue regeneration. Tissue Eng Part B. 2012;18(1):24–39.
21. Tatard VM, Venier-Julienne MC, Saulnier P, Prechter E, Benoit JP, Menei P, Montero-Menei CN. Pharmacologically active microcarriers: a tool for cell therapy. Biomaterials. 2005; 26(17):3727–37.
22. Mercier NR, Costantino HR, Tracy MA, Bonassar LJ. Poly (lactide-co-glycolide) microspheres as a moldable scaffold for cartilage tissue engineering. Biomaterials. 2005;26: 1945–52.

23. Kang SW, Jeon O, Kim BS. Poly(lactic-co-glycolic acid) microspheres as an injectable scaffold for cartilage tissue engineering. Tissue Eng. 2005;11:438–47.
24. Hong Y, Gao CY, Xie Y, Gong YH, Shen JC. Collagen-coated poly-lactide microspheres as chondrocyte microcarriers. Biomaterials. 2005;26:6305–13.
25. Tan H, Huang D, Lao L, Gao C. RGD modified PLGA/gelatin microspheres as microcarriers for chondrocyte delivery. J Biomed Mater Res Part B Appl Biomater. 2009;91:228–38.
26. Palmiero C, Imparato G, Urciuolo F, Netti P. Engineered dermal equivalent tissue in vitro by assembly of microtissue precursors. Acta Biomater. 2010;6(7):2548–53.
27. Lee CH, Singla A, Lee Y. Biomedical applications of collagen. Int J Pharm. 2001;221:1.
28. Li S-T. Biologic biomaterials: tissue-derived biomaterials (collagen). In: Park JB, Bronzino JD, editors. Biomaterials: principles and applications. Boca Roton, FL: CRC Press; 2003.
29. Schrieber R, Gareis H. Gelatine handbook: theory and industrial practice. Weinheim: Wiley-VCH Verlag; 2007.
30. Yamamoto M, Ikada Y, Tabata Y. Controlled release of growth factors based on biodegradation of gelatin hydrogel. J Biomater Sci Polym Ed. 2001;12:77.
31. Ahmed TAE, Dare EV, Hincke M. Fibrin: a versatile scaffold for tissue engineering applications. Tissue Eng B. 2008;14:199.
32. Perka C, Schultz O, Spitzer R-S, Lindenhayn K, Burmester G-R, Sittinger M. Segmental bone repair by tissue-engineered periosteal cell transplants with bioresorbable fleece and fibrin scaffolds in rabbits. Biomaterials. 2000;21:1145.
33. Di Martino A, Sittinger M, Risbud MV. Chitosan: a versatile biopolymer for orthopaedic tissue-engineering. Biomaterials. 2005;26:5983.
34. Slaughter BV, Khurshid SS, Fisher OZ, Khademhosseini A, Peppas NA. Hydrogels in regenerative medicine. Adv Mater. 2009;21:3307.
35. Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol Biosci. 2006;6:623.
36. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly (D, L lactide-co-glycolide) and its derivatives. J Control Release. 2008;125:193–209.
37. Verrijk R, Smolde IJ, Bosnie N, Begg AC. Reduction of systemic exposure and toxicity of cisplatin by encapsulation in poly(lactide-co-glycolide). Cancer Res. 1992;52:6653–6.
38. Meinel L, Illi OE, Zapf J, Malfanti M, Merkle HP, Gander B. Stabilizing insulin-like grow factor in poly(lactide-co glycolide) microspheres. J Control Release. 2001;70:193–202.
39. Walter E, Dreher D, Kok M, Thiele L, Kiama SG, Gehr P, Merkle HP. Hydrophylic poly (lactide-co-glycolide) microspheres for the delivery of DNA to human derived macrophages and dentritic cells. J Control Release. 2001;76:149–68.
40. Luten J, van Nostrum CF, De Smedt SC, Hennink WE. Biodegradable polymers as non-viral carriers for plasmid DNA delivery. J Control Release. 2008;126:97–110.
41. Jaklenec A, Wan E, Murray ME, Mathiowitz E. Novel scaffolds fabricated from protein-loaded microspheres for tissue engineering. Biomaterials. 2008;9:185–92.
42. Temenoff JS, Mikos AG, editors. Biomaterials. The intersection of biology and materials science. NJ: Pearson Prentice Hall; 2008. ISBN 10-0-13-009710-1.
43. Zhang J, Zhang H, Wu L, Ding J. Fabrication of three dimensional polymeric scaffolds with spherical pores. J Mater Sci. 2006;41:1725–30.
44. Liu X, Ma PX. Polymeric scaffold for bone tissue engineering. Ann Biomed Eng. 2003;32:477–82.
45. Volkmer E, et al. Hypoxia in static and dynamic 3D culture systems for tissue engineering of bone. Tissue Eng Part A. 2008;14(8):1331–40.
46. Shin M, Yoshimoto H, Vacanti JP. In vivo bone tissue engineering using mesenchymal stem cells on a novel electrospun nanofibrous scaffold. Tissue Eng. 2004;10(1–2):33–41.
47. Landers R, Pfister A, Hübner U, John H, Schmelzeisen R, Mülhaupt R. Fabrication of soft tissue engineering scaffolds by means of rapid prototyping techniques. J Mater Sci. 2002; 37(15):3107–16.

48. Hutmacher DW, Sittinger M, Risbud MV. Scaffold-based tissue engineering: rationale for computer-aided design and solid free-form fabrication systems. Trends Biotechnol. 2004; 22(7):354–62.
49. Brunner G. Gas extraction: an introduction to fundamentals of supercritical fluids and the application to separation processes. In: Topics in physical chemistry, vol. 4. Ed. Techniques Ingénieur; 1994. ISBN 3798509441.
50. Mathieu LM, Montjovent MO, Bourban PE, Pioletti DP, Manson JAE. Bioresorbable composites prepared by supercritical fluid foaming. J Biomed Mater Res A. 2005;75:89–94.
51. Tsivintzelis I, Marras SI, Zuburtikudis I, Panayiotou C. Porous poly(lactic acid) nanocomposite scaffolds prepared by phase inversion using supercritical CO_2 as antisolvent. Polymer. 2007;48:6311–3.
52. Reverchon E, Cardea S. Formation of cellulose acetate membranes using a supercritical fluid assisted process. J Membr Sci. 2004;240:187–95.
53. Reverchon E, Cardea S, Rapuano C. A new supercritical fluid-based process to produce scaffolds for tissue replacement. J Supercrit Fluids. 2008;45:365–70.
54. Della Porta G, Del Gaudio P, De Cicco F, Aquino RP, Reverchon E. Supercritical drying of alginate beads for the development of aerogel biomaterials: optimization of process parameters and exchange solvents. Ind Eng Chem Res. 2013;52:12003–9.
55. Pisanti P, Yeatts AB, Cardea S, Fisher JP, Reverchon E. Tubular perfusion system culture of human mesenchymal stem cells on poly-L-lactic acid scaffolds produced using a supercritical carbon dioxide-assisted process. J Biomed Mater Res A. 2012;100(10):2563–72.
56. Li WI, Anderson KW, Deluca PP. Kinetic and thermodynamic modelling of the formation of polymeric microspheres using solvent extraction/evaporation method. J Contr Rel. 1995;37: 187–98.
57. Li M, Rouand O, Poncelet D. Microencapsulation by solvent evaporation: state of the art for process engineering approaches. Int J Pharm. 2008;363:26–39.
58. Mao S, Shi Y, Li L, Xu J, Schaper A, Kissel T. Effects of process and formulation parameters on characteristics and internal morphology of poly(D, L-lactide-co-glycolide) microspheres formed by the solvent evaporation method. Eur J Pharm Biopharm. 2008;68:214–23.
59. Yang YY, Chia HH, Chung TS. Effect of preparation temperature on the characteristics and release profiles of PLGA microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. J Control Release. 2000;69:81–96.
60. Della Porta G, Reverchon E. Supercritical fluid based technologies for particulate drug delivery. In: Ravi Kumar MNV, editor. Handbook of particulate drug delivery. American Scientific Publishers; 2008. p. 35–59. ISBN 978-1-58883-124-8 (Chapter 3).
61. Della Porta G, Reverchon E. Nanostructured microspheres produced by supercritical fluid extraction of emulsions. Biotechnol Bioeng J. 2008;100(5):1020–33.
62. Kluge J, Fusaro F, Casas N, Mazzotti M, Muhrer G. Production of PLGA micro- and nanocomposites by supercritical fluid extraction of emulsions: I. Encapsulation of lysozime. J Supercrit Fluids. 2009;50:327–35.
63. Della Porta G, Falco N, Reverchon E. Antiflammatory drugs release from injectable microspheres produced by supercritical fluid emulsion extraction. J Pharm Sci. 2010;99(3): 1484–99.
64. Della Porta G, Castaldo F, Scognamiglio M, Paciello L, Parascandola P, Reverchon E. Bacteria microencapsulation in PLGA microdevices by supercritical emulsion extraction. J Supercrit Fluids. 2012;63:1–7.
65. Della Porta G, Campardelli R, Falco N, Reverchon E. PLGA microdevices for retinoids sustained release produced by supercritical emulsion extraction: continuous versus batch operation layouts. J Pharm Sci. 2011;100(10):4357–67.
66. Della Porta G, Reverchon E. Continuous supercritical emulsions extraction: a new technology for biopolymer microparticles production. Biotechnol Bioeng J. 2011;108(3):676–86.
67. Falco N, Reverchon E, Della Porta G. Continuous supercritical emulsions extraction: packed tower characterization and application to poly(lactic-co-glycolic acid) + insulin microspheres production. Ind Eng Chem Res. 2012;51:8616–23.

68. Della Porta G, Campardelli R, Reverchon E. Monodisperse biopolymer nanoparticles by continuous supercritical emulsion extraction. J Supercrit Fluids. 2013;76:67–73.
69. Della Porta G, Falco N, Giordano E, Reverchon E. PLGA microspheres by supercritical emulsion extraction: a study on insulin release in myoblast culture. J Biomater Sci Polym Ed. 2013;24(16):1831–47.
70. Campardelli R, Della Porta G, Irusta S, Reverchon E, Santamaria J. Hollow gold nanoshells-PLA nanocomposites for photothermal controlled delivery. J Mater Chem B. 2014;2:409–17.
71. Govoni M, Berardi AC, Muscari C, Campardelli R, Bonafe F, Guarnieri C, Reverchon E, Giordano E, Maffulli N, Della Porta G. 3D engineered multiphase three-dimensional microenvironment to ensure the controlled delivery of cyclic strain and human growth differentiation factor 5 for the tenogenic commitment of human bone marrow mesenchymal stem cells. Tissue Eng Part A. 2017. https://doi.org/10.1089/ten.tea.2016.0407.
72. Della Porta G, Campardelli R, Cricchio V, Oliva F, Maffulli N, Reverchon E. Injectable PLGA/hydroxyapatite/Chitosan microcapsules produced by supercritical emulsion extraction technology: An in vitro study on teriparatide/gentamicin controlled release. J Pharm Sci. 2016;105(7):2164–72.
73. Whitaker MJ, Quirk RA, Howdle SM, Shakesheff KM. Growth factor release from tissue engineering scaffolds. J Pharm Pharmacol. 2001;53(11):1427–37.
74. Sakiyama-Elbert SE, Hubbell JA. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. J Controlled Release. 2000;69(1): 149–58.
75. Kang SW, Kim JS, Park KS, Cha BH, Shimb JH, Kim JY, Cho DW, Rhie JW, Lee SH. Surface modification with fibrin/hyaluronic acid hydrogel on solid-free form-based scaffolds followed by BMP-2 loading to enhance bone regeneration. Bone. 2011;48(2):298–306.
76. Kanczler JM, Barry J, Ginty P, Howdle SM, Shakesheff KM, Oreffo ROC. Supercritical carbon dioxide generated vascular endothelial growth factor encapsulated poly(dl-lactic acid) scaffolds induce angiogenesis in vitro. Biochem Biophys Res Commun. 2007;352(1):135–41.
77. Murphy WL, Peters MC, Kohn DH, Mooney DJ. Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. Biomaterials. 2000;21(24):2521–7.
78. Sanders EH, Kloefkorn R, Bowlin GL, Simpson DG, Wnek GE. Two-phase electrospinning from a single electrified jet: microencapsulation of aqueous reservoirs in poly (ethylene-co-vinyl acetate) fibers. Macromolecules. 2003;36(11):3803–5.
79. Jiang H, Hu Y, Li Y, Zhao P, Zhu K, Chen W. A facile technique to prepare biodegradable coaxial electrospun nanofibers for controlled release of bioactive agents. J Controlled Release. 2005;108(2–3):237–43.
80. Liao IC, Chew SY, Leong KW. Aligned core-shell nanofibers delivering bioactive proteins. Nanomedicine. 2006;1(4):465–71.
81. Ungaro F, Biondi M, d'Angelo I, Indolfi L, Quaglia F, Netti PA. Microsphere-integrated collagen scaffolds for tissue engineering: effect of microsphere formulation and scaffold properties on protein release kinetics. J Controlled Release. 2006;113(2):128–36.
82. Li B, Yoshii T, Hafeman AE, Nyman JS, Wenke JC, Guelcher SA. The effects of rhBMP-2 released from biodegradable polyurethane/microsphere composite scaffolds on new bone formation in rat femora. Biomaterials. 2009;30:6768–79.
83. Wei GB, Jin QM, Giannobile WV, Ma PX. The enhancement of osteogenesis by nano-fibrous scaffolds incorporating rhBMP-7 nanospheres. Biomaterials. 2007;28(12):2087–96.
84. Li B, Yoshii T, Hafeman AE, Nyman JS, Wenke JC, Guelcher SA. The effects of rhBMP-2 released from biodegradable polyurethane/microsphere composite scaffolds on new bone formation in rat femora. Biomaterials. 2009;30(35):6768–79.
85. Federovich NE, Alblas J, DeWijn JR, Hennink WE, Verbout AJ, Dhert WJA. Hydrogels as extracellular matrices for skeletal tissue engineering: state-of-the-art and novel application in organ printing. Tissue Eng. 2007;13(8):1905–25.

86. Roth EA, Xu T, Das M, Gregory C, Hickman JJ, Boland T. Inkjet printing for high-throughput cell patterning. Biomaterials. 2004;25:3707–15.
87. Adam EJ, Alexandra LR, Ramille NS. Advancing the field of 3D biomaterial printing. Biomed Mater. 2016;11:014102. https://doi.org/10.1088/1748-6041/11/1/014102.
88. Della Porta G, Nguyen BN, Campardelli R, Reverchon E, Fisher JP. Synergistic effect of sustained release of growth factors and dynamic culture on osteoblastic differentiation of mesenchymal stem cells. J Biomed Mater Res Part A. 2014. https://doi.org/10.1002/jbm.a.35354.
89. Chan BP, Hui TY, Wong MY, Yip KHK, Chan GCF. Mesenchymal stem cell-encapsulated collagen microspheres for bone tissue engineering. Tissue Eng. 2010;16(2):225–35.
90. Kuroda Y, Akiyama H, Kawanabe K, Tabata Y, Nakamura T. Treatment of experimental osteonecrosis of the hip in adult rabbits with a single local injection of recombinant human FGF-2 microspheres. J Bone Miner Metab. 2010;28:608–16.
91. Ben-Ari A, Rivkin R, Frishman M, Gaberman E, Levdansky L, Gorodetsky R. Isolation and implantation of bone marrow-derived mesenchymal stem cells with fibrin micro beads to repair a critical-size bone defect in mice. Tissue Eng A. 2009;15(9):2537–46.
92. Jayasuriya AC, Bhat A. Fabrication and characterization of novel hybrid organic/inorganic microparticles to apply in bone regeneration. J Biomed Mater Res A. 2010;93(4):1280–8.
93. Liu X, Jin X, Ma PX. Nanofibrous hollow microspheres self-assembled from star-shaped polymers as injectable cell carriers for knee repair. Nat Mater. 2011;10:398–406.
94. Kang SW, La WG, Kim BS. Open macroporous poly(lactic-co-glycolic Acid) microspheres as an injectable scaffold for cartilage tissue engineering. J Biomater Sci Polym Ed. 2009;20:399–409.
95. Solorio LD, Fu AS, Hernandez-Irizarry R, Alsberg E. Chondrogenic differentiation of human mesenchymal stem cell aggregates via controlled release of TGF-beta1 from incorporated polymer microspheres. J Biomed Mater Res A. 2010;92:1139–44.
96. Solorio LD, Dhami CD, Dang PN, Vieregge EL, Alsberg E. Spatiotemporal regulation of chondrogenic differentiation with controlled delivery of transforming growth factor-β1 from gelatin microspheres in mesenchymal stem cell aggregates. Stem Cells Translational Med. 2012;1(8):632–9.
97. Han Y, Wei Y, Wang S, Song Y. Cartilage regeneration using adipose-derived stem cells and the controlled released hybrid microspheres. Joint Bone Spine. 2010;77:27–31.
98. Park JS, Na K, Woo DG, Yang HN, Park KH. Determination of dual delivery for stem cell differentiation using dexamethasone and TGF-beta 3 in/on polymeric microspheres. Biomaterials. 2009;30:4796–805.
99. Park JS, Yang HN, Woo DG, Jeon SY, Park KH. The promotion of chondrogenesis, osteogenesis, and adipogenesis of human mesenchymal stem cells by multiple growth factors incorporated into nanosphere-coated microspheres. Biomaterials. 2011;32:28–38.

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