Regenerative Medicine and Plastic Surgery

Elements, Research Concepts and Emerging Technologies

Dominik Duscher
Melvin A. Shiffman
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To Aura, the love of my life
Dominik Duscher

Foreword

Plastic surgery, by its very nature, takes care of nearly every part of the human body including the skin, muscles, bones, nerves, and blood vessels. As Joseph G. McCarthy, my former Chief at NYU, once said, plastic surgeons are problem-solvers. They are called upon by other practioners to develop creative solutions to unsolvable problems in nearly every anatomic region. Thus, it is not surprising that plastic surgeons have flocked to the burgeoning field of regenerative medicine, which promises even more elegant solutions to clinical problems found everywhere in the human body. Regenerative medicine proposes using cellular and molecular processes to recreate the exact same tissues that plastic surgeons perform long operations to recreate.

It is logical that plastic surgery would be at the forefront of the field of regenerative medicine. And this is indeed the case at stem cell conferences and tissue engineering symposia, where plastic surgeons often outnumber all the other clinical attendees combined. However, up until now, there did not exist an authoritative reference documenting all the ways that plastic surgical practice and regenerative medicine science overlap or provide a road map for the future of both specialties. Drs. Duscher and Shiffman have provided a valuable service by gathering in one place the leading voices in these two fields in clear and concise manner.

Reading through this work, one is impressed by both the breadth of plastic surgery practice and the enormous potential of regenerative medicine to cure a multitude of human diseases. One also sees the potential for regenerative medicine to be integrated into clinical plastic surgery. With beautiful clinical images and artwork, this book will be a central companion to both practicing plastic surgeons who wish to remain abreast of upcoming technological advances and regenerative medicine researchers who wish to understand the current state of the art of surgical reconstruction. The analogies between the two disciplines are clearly laid out, and the possibilities for advances in clinical care leap off the pages.

Ultimately, regenerative medicine may make many operations plastic surgeons perform obsolete. This is familiar territory for plastic surgeons who always need to look for the next clinical arena for innovation. I am confident that clinical plastic surgeons will remain at the forefront and become leaders in this emerging field. In your hands is a comprehensive encyclopedia of two

rapidly converging fields. Drs. Duscher and Shiffman have done an outstanding job of highlighting the interdependent relationship between plastic surgery and regenerative medicine. Ultimately, this is to the benefit of both fields.

Stanford, CA Geoffrey C. Gurtner

Preface

A surgical success will impact the patient in question, but a research success can impact a global population. This thought came to me at the end of medical school and still is the main reason for my fascination for science today. Driven by my desire to create knowledge and discover new things, I found the seemingly endless possibilities of the young field of regenerative medicine particularly enchanting.

Through channelling the power of stem cells to repair or replace damaged tissues, regenerative therapies are making their way into mainstay clinical routine. As both case-based stem cell therapy and global understanding evolve, we are entering an era in which we can design treatments for some of the world's most devastating diseases. Innovative therapeutic concepts borne of the intersection of clinical medicine, engineering, and cell biology have potential to change the way we practice medicine.

The international efforts put into this field have created an unyielding body of literature. Staying abreast of the genetic, epigenetic, cellular, stromal, hematopoietic, and pathologic research emerging each year is critical. A comprehensive, up-to-date reconnaissance of these parameters in the field of regenerative medicine is therefore a valuable tool. The expertise required to generate such a text far exceeded that of its editors, and the roots of this book are nourished in the soul of collaboration. I am indebted to the scores of renowned specialists who have contributed their expertise and ingenuity to this work. This book is intended for surgeons and scientists, for biologists and engineers, and for students of medicine, biomedical engineering, cell biology, and biotechnology, simply for everyone who is interested in the extraordinary potential that regenerative medicine has to offer. The first edition of this book represents an attempt to organize the current knowledge in the field. However, knowledge is of no value unless you put it into practice. Thus, we all need to strive for the clinical translation of the principles presented here to make the dream of tissue and organ regeneration become reality.

You can have results or excuses. Not both.

—Arnold Schwarzenegger

Munich, Germany — Dominik Duscher

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Part I

Elements of Regenerative Medicine

Definitions

1

Dominik Duscher, Matthias M. Aitzetmüller, and Elizabeth A. Brett

1.1 Regenerative Medicine

Regenerative medicine is an area of biomedicine, bridging the gap between life science and engineering [1]. Combining tissue engineering and stem cell biology with a focus on translational aspects, it aims to achieve replacement and engineering/regeneration of cells, tissues, and organs.

1.2 Tissue Engineering

Tissue engineering has the ultimate translational goal to utilize scaffolds, cells, and active molecules to form fully functional tissue. It is an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes. The goal functions include restoration, maintenance, or improvement of tissue or organ [2].

1.3 Stem Cells

Stem cells are undifferentiated cells belonging to multicellular organisms. They are capable of giving rise to identical daughter cells, or alternate phenotypes through differentiation. Stem cells are the building blocks of life, whose un-mutated genetic pool is the hallmark of health. However, stem cells are also the building blocks for molecular medicine in the twenty-first century [3]. Following the principle of regenerative medicine, stem cell-based therapies have the potential to treat countless human diseases.

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History of Regenerative Medicine

2

Maximilian Zaussinger and Dominik Duscher

2.1 History

Regenerative medicine is associated with engineering or regeneration of human cells, tissues, or organs and to restore or establish normal function [1]. Historically, regenerative medicine was first introduced by Kaiser in 1992, who described technologies which would impact the future of medicine [2]. Far earlier, in 1968 the first successful bone marrow transplantation in humans was performed [3]. Subsequently, this development grew and led to achieving further milestones in the fields of stem cells and transplantation.

Although regenerative medicine is considered as a novel target of medical research, the idea of creating artificial organs is not so recent. Already in 1938, Alexis Carrell, a Nobel Prize winner for his work on vascular anastomosis, and Charles Lindbergh, the first pilot who crossed the Atlantic sea alone, published the book *The Culture of New Organs* [4]. In 1954, the kidney was the first organ to be substituted in a human. No rejection reaction occurred due to the factor of identical twins [5].

The regenerative potential of body parts is a common phenomenon in nature; salamanders are able to restore an amputated limb in a few days. Even the human potential of regeneration was well known in ancient times, as described by the myth of the great Titan Prometheus: an eagle was eating his liver during the day and it regenerated itself completely overnight [6]. During the last centuries, regenerative medicine strove to construct artificial organs mimicking natural tissue by combining modulated cells with extracellular matrix-hybridized synthetic polymers that have produced biologically functioning artificial tissues [1]. These developments open new avenues for curing patients with malignant and impaired tissues.

In 1989, a book titled *Tissue Engineering* [7] was published with the first expressive definition of tissue engineering given by Robert Nerem:

> *Tissue engineering is the application of the principles and methods of engineering and the life sciences towards the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions.*

The evolution from tissue engineering into regenerative medicine was driven by intense developments in the financial, research, and political landscape. However, from a financial point of view, the last two decades, anticipated to bring the biotechnological revolution, were characterized by a disconnect between expectations and reality. Current strategies to pursue the objec-

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tives of regenerative medicine are based on three concepts:

- Cell-based therapy
- Either biological or synthetic materials to restore cells and tissues
- Implantation of scaffolds seeded with cells

Understanding innovative technologies is fundamental to developing successful approaches in the biotech sector and hence is influential in developing the field of regenerative medicine [8]. To date, only a multidisciplinary team, including doctors, biologists, bioengineers, surgeons, and chemists, is able to master all key steps in these revolutionary fields of regenerative medicine.

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3 Basic Principles and Current Approach for Soft Tissue Regeneration

Matthias M. Aitzetmüller, Elizabeth A. Brett, Matthias A. Sauter, and Dominik Duscher

3.1 Introduction

Due to the ongoing shift in the distribution of the world's population towards old age, we recently experience a dramatic increase in comorbidities like diabetes or venous and arterial insufficiency. This results in a raising number of chronic wounds which have become not only an individual medical but also a significant economic burden, consuming 2–4% of health care budgets worldwide [1].

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Wound healing is a complex system depending on the timed coordination of several cell types, intra- and extracellular mechanisms, proteins, and pathways, but also on several external factors like infections or mechanical irritation (Fig. 3.1). Defect or dominance of one factor can cause to a sudden breakdown of localized healing capacity, leading to formation of chronic wounds. A famous example for the fragility of the cellular mechanism for tissue homeostasis and repair is the connection between vitamin C deficiency and scurvy resulting in nonhealing wounds and spontaneous bleeding known since the sixteenth century [2, 3]. Mentioned first in journey books of Christopher Columbus as a result of monotone diet, the pathomechanism remained unclear until the twentieth century. Then it could be demonstrated that vitamin C represents a main cofactor for collagen cross-linking and an important factor to reduce oxidative stress [4]. This example shows how impactful minimal alterations in our metabolism can be for tissue regeneration. Therefore, a complete understanding of all molecular and cellular players involved in wound healing is pivotal for developing treatment strategies and effective drugs.

In this chapter, we summarize the most promising recent advances in wound healing therapeutics with the corresponding challenges and shed light on possible solutions for effective application.

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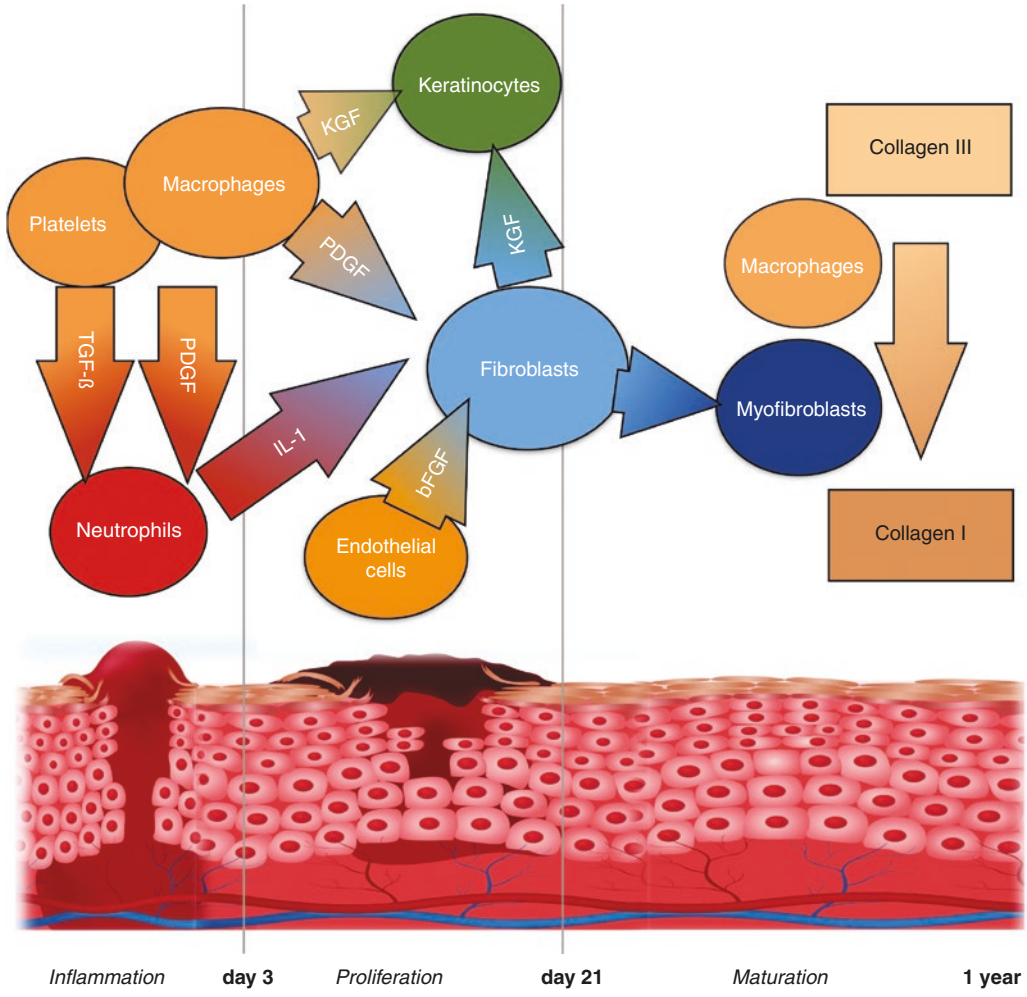

Fig. 3.1 Phases of adult wound healing with main affecting cells and signals. (Left) Coagulation and inflammation—day 0–3. Platelets: formation of platelet plug and secretion of platelet-derived growth factor and transforming growth factor for chemotaxis of neutrophils. Neutrophils: secrete interleukin 1 for the beginning of chemokine-cascade for chemotaxis of inflammatory cells; macrophages: phagocyte bacteria and secrete paracrine factors for keratinocyte-based epithelization and fibroblast activation. (Middle) Proliferation—day 4–21. Beginning of angiogenesis, dependent on endothelial cells: activated by vascular endothelial growth factor. Formation of the extracellular matrix based on fibroblast activation: activated by basic fibroblast growth factor, interleukin 1, and platelet-derived growth factor. Epithelialization: keratinocyte based as a response to keratinocyte growth factor. (Right) Maturation phase—day 21–1 year. Wound contraction: transformation of fibroblasts to myofibroblast. Collagen remodeling: effected by myofibroblasts and macrophages

3.2 Recent Advances in Wound Therapeutics

3.2.1 Growth Factor Therapy

The wound healing promoting effect of growth factors is broadly known. Although they have shown to act beneficial in preclinical studies, large clinical studies supporting this are still missing. A meta-analysis based on the Cochrane database showed a general benefit of growth factor-induced wound healing without any significant general adverse effects [5]. The platelet-derived growth factors (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor

(FGF), and the transforming growth factor-beta (TGF-β) are in current focus of research.

PDGF-BB, administered in hydrogels and available as "Regranex" (Ortho-McNeil, Raritan, NJ), is the only growth factor therapy that is currently approved for treatment of non-healing wounds [6]. Although there has been shown advantage in hypertensive leg ulcers, the application of this gel should be considered only as ultima-ratio treatment due to a higher rate of malignancies in patients treated with PDGF-BB [7].

As another growth factor VEGF was successfully used for accelerated wound closure in diabetic mice. Preclinical study showed less than half of resurfacing time in VEGF-treated group than in a non-treated group [8]. Also non-treated wounds on the contralateral site of animals with VEGF therapy showed an accelerated wound closure time. This leads to the suggestion of an additional systemic effect of local VEGF treatment with the possibility of interacting with tumor growth and of promoting malignant tendencies. There has been only one clinical trial comparing VEGF treatment and placebo showing no significant benefit for VEGF [9].

Several study groups investigated the effect of intralesional EGF injections on wound closure [10–12]. Although first clinical trials have been performed in Cuba in 2006 and have shown accelerated wound healing of high-grade diabetic foot healing and complete wound closure in up to 85% of cases, it is still no treatment option in western countries. A meta-analysis performed by Yang et al. [13] confirmed these first results strengthening the hopes for a new clinical treatment option for diabetic and avascular wounds.

B-FGF is one of the first growth factors that has been investigated. A clinical study by Richard et al. in 1995 involved 17 patients and could find no promoting effect of b-FGF [14]. Up to now the efficacy of b-FGF in wound healing remains unclear. While there exist clinical studies showing a promotion of diabetic wound healing effected by injectable b-FGF, others deny a significant effect of b-FGF releasing sponges while preclinical trials have shown a promising effect [15–17]. Furthermore, not only b-FGF but also acid-FGF has been tested for wound healing and similarly showed inconclusive results [18].

Although the TGF-β family, including TGF-β-1, 2, and 3, has shown to be involved in both promotion of wound healing and scarring, it has not become a possible treatment option in clinical routine yet [19]. The wound healing ability of TGF-β-1 and TGF-β-2 in murine models are well described. Although first clinical phase I and II trials have shown efficacy and safety of a TGF-β-releasing scaffold for treatment of venous ulcers, there exist no supporting phase III study [20].

Despite the fact that growth factor therapy has shown to be effective in preclinical and some clinical trials, only few of these substances hold promise to enter the clinical routine. A therapy containing only one growth factor is most likely not sufficient to efficiently promote wound healing, especially compared to NPWT, which has shown to significantly enhance a plethora of autologous growth factor levels.

3.2.2 Negative-Pressure Wound Therapy

The use of negative-pressure wound therapies (NPWT = vacuum-assisted closure = VAC) provides an effective and elegant way to close wounds, prevent infections, and simultaneously increase local growth factor levels. NPWT has shown benefit on bacterial contamination rate. It also temporarily creates relative hypoxia in the wound region, resulting in significant higher levels of the main growth factors (VEGF, TGF β, and basic FGF), angiopoietin 1 (essential for neoangiogenesis), and bone morphogenetic protein 2 (BMP 2—involved in cartilage and bone metabolism) [21–25]. Several studies suggest that microdeformation of the wound surface leads to accelerated cell migration and matrix production (Fig. 3.2) [23–25]. Interestingly, the temporary hypoxia induces the osteogenetic differentiation of MSCs. Therefore NPWT seems to be the perfect option for treatment of soft tissue defects involving bone defects and infected or potentially infected wounds. Further research has to be carried out to confirm these hypotheses in a clinical setting.

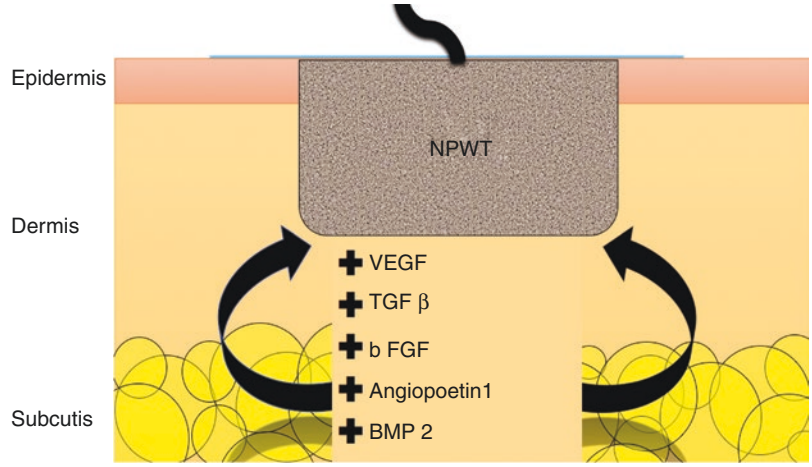

Fig. 3.2 Molecular mechanism of negative-pressure wound therapy

Technical development has led to change in handling of NPWT systems. Dressing changes only three times a week instead of twice daily as recommended in the first trails using NPWT has led to the possibility of a long-term use. By using silver-coated foams, that additionally hinder bacterial growth, NPWT became even more successful [26]. In aggregate, NPWT provides an effective and elegant way to treat difficult wounds by enhancing local growth factor levels and decreasing bacterial contamination of wounds.

3.2.3 Antioxidants and Wnt Modulation

The human skin is constantly exposed to environmental factors such as UV light, radiation, ozone (O_3), or air pollution inducing reactive oxygen species (= ROS). Additionally, cellular metabolism leads to ROS as side products. The causality between free radicals and aging has been described by different groups within the last century [27–31]. By causing accumulation of oxidative toxic products in long-living molecules such as collagen, it leads to peroxidation and dysfunction of these molecules. ROS-induced damaging of the DNA is directly followed by base loss/modification or breakage. ROS can further lead to glycation of proteins followed by degradation. According to this, ROS can significantly inhibit endogenous ability for

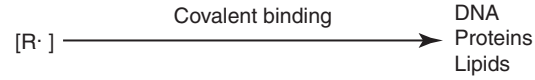

Fig. 3.3 Mechanism of reactive oxygen species (ROS) damage

wound healing by destruction of cells, key proteins, or parts of the ECM (Fig. 3.3).

Antioxidant treatment has been used since several years as a product improving the quality of skin. The most popular and most commonly used antioxidant is a polyphenol, also called aloe vera. As the main component of ointments or gels its therapeutic effect is related to the stimulation of collagen syntheses on the one hand and to anti-oxidative effects on the other hand [32]. But the use of anti-oxidants is not only limited on aesthetic treatment options. Some iron chelators like deferoxamine (= Desferal or more potent, desferriexochelin-772SM (D-Exo)) [33], deferiprone, and deferasirox are already in use in different medical fields [34]. Next to being well known as treatment option for beta-thalassemia [35, 36], anti-oxidant drugs have shown benefits mainly due to their anti-inflammatory potential to increase the retention rate of fat grafts, the survival rate of free flaps, and the healing process of diabetic wounds [37, 38]. This can be explained by the ability of free iron to induce the prolyl-hydroxylation of the hypoxia-inducible factor 1α (HIF-1α), a process leading to inactivation and degradation of HIF-1α [39]. Less free iron results in a higher expression of HIF-1α and thereby

leads to benefits in local neovascularization and tissue regeneration. Harnessing these effects, a transdermal delivery system releasing DFO showed accelerated wound healing in diabetic ulcers. Prophylactic use of this system has a preventive effect on ulcer formation [40].

But not only antioxidative agents, also wnt pathway manipulation is a promising new alley of wound healing research. The wnt pathway represents a sequence of factors that can easily be targeted by nanoparticles. The wnt pathway was found to play an important role in embryonic vertebrae development, in the development of different malignancies, and additionally in tissue repair and scarring [41–44]. Pyrvinium, an antihelminthic drug, inhibits the wnt pathway by promoting the effectivity of the casein kinase 1α (CK1α), leading to accelerated degradation of casein, a factor of wnt pathway [45, 46]. Studies using pyrvinium show a 1.4-fold increase of MSC proliferation by simultaneously inhibiting the osteogenic and chondrogenic differentiation. These changes were initiated by a pyrvinium-releasing sponge. Pyrvinium only affected the proliferation rate of MSCs. Other cell lines like HUVECs have shown no significant changes compared to non-treated cells [47].

Additionally, the wnt pathway has also shown to play a significant role in scar formation. By stimulation of the wnt pathway and the FGF pathway, the regeneration of hair follicles could be induced. Hair follicle secretes bone morphogenetic protein (BMP), which again stimulates myofibroblasts to differentiate into adipocytes. This pathway leads to inhibition of scar formation. Targeting and mimicking these three pathways to either prevent scarring or treat hypertrophic scars or keloids is a future goal of drug development [48–50].

3.2.4 RNA Interference-Based Therapy

Gene expression initially starts in the nucleus with transcription—the production of mRNA (messenger-RNA) followed by an export to the cytoplasm. Translation of mRNA leads to the production of proteins. After this process the mRNA is degraded.

The basic principle of RNA interference (RNAi-based therapy) is based on body's own mechanism for mRNA degradation: By binding to mRNAs, endogenous miRNAs (micro-RNAs) or synthetic siRNAs (small interfering RNAs) lead to mRNA degradation or to formation of double-stranded RNA and as a result to suppression of their translation [51, 52]. Synthetically produced siRNA can be used for knocking down factors which inhibit neo-angiogenesis and inhibit keratinocyte migration followed by re-epithelialization [53–55].

For example, endogenous miRNA-21 is one of the best studied miRNAs. It has been shown to regulate re-epithelialization, cell proliferation, wound contraction, and formation of granulation tissue [54, 56, 57]. RNAi is not limited to wound healing applications, but also offers new possibilities in cancer therapy or treatment of genetic diseases like amyotrophic lateral sclerosis (by targeting and destroying wild-type mRNAs) [58, 59].

Difficulties for the application of this novel therapy include delivery to specific cells and problems with internalization of i-RNA to certain cell types [60, 61]. Additionally, a high degradation rate through RNases leads to a short intracellular half-life. However, further development of this technique may lead to new ways to enhance tissue regeneration and to bring us closer to the holy grail of scarless wound healing [62, 63].

3.2.5 Stem Cell-Based Therapy

Mesenchymal stromal cells (MSCs) can be utilized to treat challenging wounds, such as wounds followed irradiation, ischemic or diabetic wounds. The basic principle is a potential differentiation of stromal cells and a higher local level of growth factors. Although preclinical and clinical studies showed promising results, there still remain several problems. Irregularities are caused by patient's individual factors, such as diabetes or age [64, 65]. These uncertainties still limit the clinical use.

But stem cell-based therapy is not only limited to the regenerative potential of MSCs harvested from bone marrow or adipose tissue. Also peripheral blood cells (PBCs) have shown to secrete a mixture of the pro-angiogenetic factors VEGF and HIF-1, when being temporally conditioned under hypoxic stress [66]. These findings have been used for developing both an implantable and an injectable wound healing system and seem to be a promising approach to accelerate wound healing [67].

3.2.6 Scaffolds and Skin Equivalents

Bioactive dressings are engineered from components that are naturally present in the ECM or composed of polymers to mimic this unique matrix [68]. Biomimetic collagen hydrogels have been shown to accelerate early wound healing by modifying cell recruitment and augmenting granulation tissue formation [69]. Recently biologic matrices have evolved from being simple ECM replacements towards drug and cell delivery vehicles. Novel regenerative matrices are capable of both skin replacement and stimulation of endogenous cells [70]. For example, pullulan (a polysaccharide polymer)-collagen matrices was seeded with mesenchymal stem cells (MSCs) and showed to enhance cell survival [71]. MSCs delivered in such a structured matrix environment have demonstrated enhanced efficacy by increased angiogenic cytokine expression [72]. Therefore, scaffolds can represent an intelligent and efficient drug-delivery vehicle to overcome certain problems of cell-based therapies [73].

In addition to improving wound healing and skin regeneration by increased neovascularization, scaffold-seeded progenitor cells can also enhance tissue repair by inducing a specific immune response. Delivery in the correct niche environment can further enhance the immunemodulatory effects of MSCs and have positive impact on scar formation. ASCs delivered to cutaneous excisional wounds via an ECM patch attenuate wound fibrosis more effectively than ASCs applied without scaffold support [74].

Despite significant scientific advancements and early clinical trials, clinical translation of progenitor cell-seeded biomimetic scaffolds for skin regeneration still remains a challenge. However, innovative therapies based on emerging concepts arising from the intersection of engineering, molecular signaling, and stem cell biology will potentially result in the transformation of fibrotic healing into skin regeneration. Looking ahead, understanding the genetic and epigenetic indicators that might predispose a patient to impaired wound healing or excessive scarring may enhance tissue regenerative approaches further.

3.3 Conclusions

A growing number of patients suffering from chronic wounds have brought soft tissue regeneration into spotlight of current research. The ideal treatment for wound healing is cheap and effective for most wounds. Furthermore, it is essential for upcoming devices to avoid side effect, to provide long-lasting application and should not be impaired by patient-dependent factors such as chronic systemic diseases. Several different approaches have been developed and have shown promising results in preclinical studies. Nevertheless, only NPWT has made the step to clinical routine. Possible reasons for this big gap between basic science and clinical implementation include several uncertain factors such as possible malignancy (growth factor-based therapy), high degradation rate (RNAi, growth factor, and stem cell-based therapy), systemic side effects (pyrvinium), uncertain retention rates, and individual limitations (stem cell-based therapy). Further approached should strive for a holistic attempt to correct the plethora of molecular defects that lead to nonhealing wounds rather than a one-factor replacement therapy.

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4 Sophisticated Biocomposite Scaffolds from Renewable Biomaterials for Bone Tissue Engineering

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4.1 Introduction

Loss or the dysfunction of bone tissue may occur due to trauma, injury, disease, or aging [1]. Currently there are excessive amount of materials to be applied to bone regeneration [2]. In turn, the autograft-, allograft-, or xenograft-based bone regeneration techniques have their disadvantages such as the need for extra surgical procedures, infection, chronic pain, or tissue rejection, which in turn has increased the importance of tissue engineering and regenerative medicine [3]. The main goal of tissue engineering is to assemble isolated functional cells and biodegradable tissue scaffolds made from bioengineered materials with the aim of regenerating diseased or damaged tissue. Many scientists from this multidisciplinary field have focused on designing and generating appropriate scaffolds for various tissues, by primarily overcoming cell-dependent problems in addition to scrutinizing tissue engineering structures in vitro and in vivo [4].

This chapter aims at describing the importance of renewable materials which have great potential for use in bone tissue engineering. In this context, the chapter offers new approaches in the improvement of polymeric composite matrices with the aim of obtaining 3D tissue-engineered scaffolds from renewable biomaterials.

4.2 Biology of Bone Tissue: Structure and Function

Bone tissues are responsible for many crucial assignments, the most notable ones being structural support and protection against external forces in the vertebrates. Its ability to self-repair and rebuild by promoting mechanical requirements makes this tissue very unique in a structural sense. However, healthy bone functions can be influenced by many different pathological situations or diseases. On the other hand, the bone tissue has been established to have limited regenerative capacities depending on patient age, anatomical site, and fracture size since it is hard for the body to repair huge gaps by itself [5, 6]. Critical-sized fractures (~5 mm) do not have the ability to heal on their own and need surgical procedures to ensure the appropriate restoration. Typical fractures seldom give rise to the formation of a hole of critical size, whereas some trau-

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matic defects, cancer, infections of the bone, or age-related degenerations result in areas where the bone cannot renew by itself. Thus, bone tissue transplantation is the second most performed procedure after blood, with over 100 million operations a year, where patients only in the USA pay approximately $800 billion for treating bone diseases annually [6].

Bone, an enduring and extremely vascularized tissue, can keep reconstructing itself throughout a life span. Within its dynamics are different mechanical, biological, and chemical functions which act in controlled harmony. These include structural support, protection and regulation and storage of restorative cells and minerals, in addition to protection and regulation of Ca and P ions by arrangement of crucial electrolyte concentrations in the blood [7]. It actively contributes to the generation of various types of blood cells (known as hematopoiesis) by regulating homeostasis [8]. The bone structure has a complementary role in mobility, through the skeletal structure which has sufficient load-bearing capability and behaves as a protective cover for the sensitive interior organs of the body [9]. For a better understanding of the mechanical features of a compact bone tissue, it is significant in understanding the hierarchical constructional behavior they possess: (1) cancellous and cortical bone; (2) the microstructure (from 10 to 500 μm); Haversian systems, osteons, single trabeculae; (3) the sub-microstructure (1–10 μm); lamellae; (4) the nanostructure (from a few hundred nanometers to 1 micron): molecular structure of constituent elements like fibrillar collagen and embedded mineral; and (5) the sub-nanostructure (less than a few nanometers): molecular structure of component elements such as minerals, collagen, and non-collagenous organic proteins (Fig. 4.1). Thus, the components of bone material are both heterogeneous and anisotropic in nature [10].

The bone ultrastructure is composed of collagen and minerals such as tricalcium phosphate, and hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$. Synthetic HA is one of the most preferred bioc-

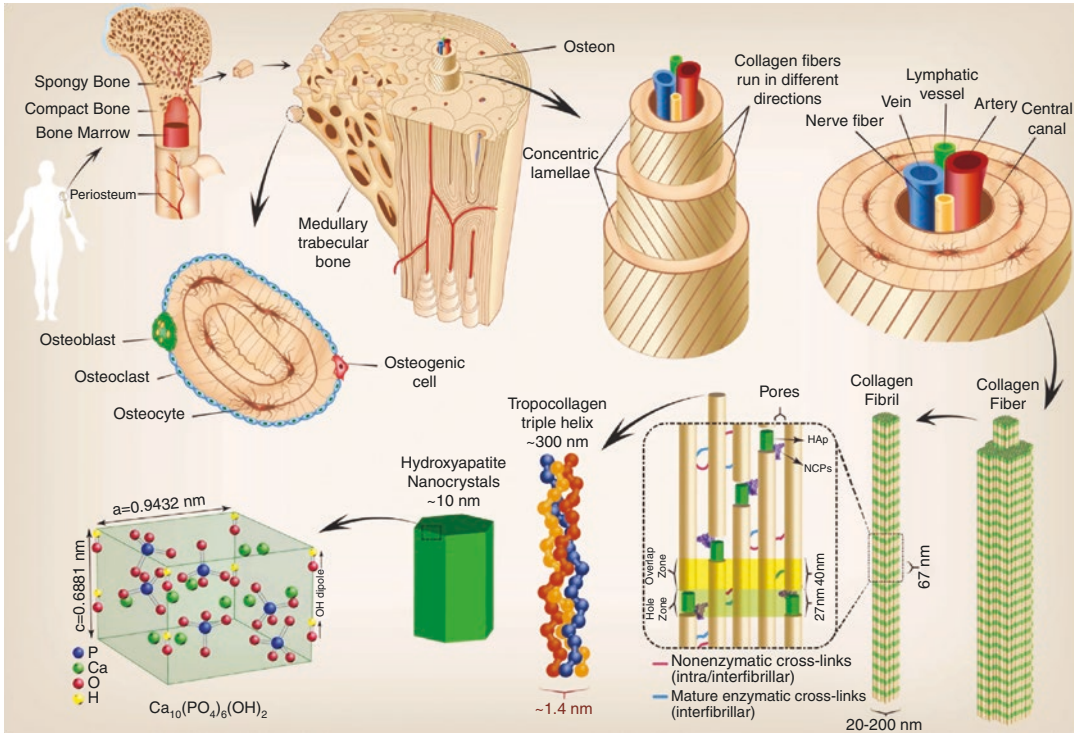

Fig. 4.1 Anatomy of bone tissue: The ultrastructure of compact bone [16]

eramic structures used in the construction of bone substitutes. When examined in detail, bone macromolecules are formed from collagen type I (90%) and over 200 different types of non-collagenous matrix proteins (i.e., osteocalcin, osteonectin, glycoproteins, proteoglycans, and sialoprotein) [11, 12]. These non-collagenous matrix proteins induce intermediate extracellular signals which tend to regulate the homeostasis of various cell types such as osteoblast, osteocyte, and osteoclast. The other crucial section of bone is the mineralized inorganic components (composed of 4-nm-thick plate-like carbonated apatite mineralities). Moreover, the compact structure composed of collagen and HA gives this tissue a unique compressive strength and high fracture toughness [12].

HA is a bioactive, biocompatible, osteoconductive, nontoxic, noninflammatory, and non-immunogenic ceramic for bone tissue engineering and one of the most widely used biomaterials due to its resemblance to the inorganic constituent of the vertebrae, bone and its ability to encourage cell-scaffold adaptation [13]. Hydroxyapatite nanoparticles (HAp) in collagen fibers reach for supporting assistants by activating the production of alkaline phosphatase in bone, resulting in its overwhelming endurance [14]. Nanoscale HAp ($50 \times 25 \times 3$ nm^3) is crucial for appropriate generation of osteocytes in the bone matrix. Naturally produced HAp has a Ca:P ratio of 1.67 which needs to be imitated in the production of HAp to acquire the necessary biological response, solubility, and mechanical sensitivity [15].

Autogenous bone implants are widely selected in bone replacement. Nevertheless, this treatment technique is limited due to insufficiency of donors, infection, veto of implant, etc., especially in wide fractures [17]. Various studies have been conducted since the discovery of the differentiation potential of human adipose-derived mesenchymal stem cells (hAMSCs) into osteogenic lineage, and hence these cells have been considered as an excellent source for bone tissue engineering applications. Even though first practices included the direct implementation of stem cells into fracture locations, nowadays scaffolds combined with stem cells, particularly MScs, are applied, so that they promote cell colonization, immigration, growth, and differentiation [18].

An optimal scaffold for bone tissue engineering practices should permit or enhance cell viability, attachment, proliferation, homing, osteogenic differentiation, vascularization, host integration, and high load-bearing capacity (Fig. 4.2). In addition, it should be simple to apply and susceptible to minimally invasive implant treatment. It should be reproducible on an industrial scale and at the same time be sterile. Eventually, all its features should be practical and meet the demands [19].

4.3 An Overview of Biomaterials in Tissue Engineering

The field of tissue engineering involves chemistry, biology, medicine, and engineering approaches, with the aim of repairing and/or replacing injured tissues and organs with the aid of bioartificial substitutes using biopolymers, cells, and biologically active agents such as growth factors and cytokines (Fig. 4.3). This is a thriving interdisciplinary field presenting new opportunities to scientists [7, 20]. The extracellular matrix comprises a complex combination of structural and functional proteins, glycoproteins and proteoglycans that are organized in a unique tissue-specific three-dimensional structure. They play a vital role in morphogenesis, composition, and function of tissues as well as organs [21].

Providing a suitable microenvironment, that is to say, fabricating scaffolds or decellularized extracellular matrices for cell growth, migration, and proliferation is crucial in tissue engineering (Fig. 4.4). This is due to the fact that scaffolds which include growth factors or other signaling molecules serve as a so-called niche for cells [7, 23, 24]. In essence, big progress in the fabrication of novel three-dimensional (3D) tissue-engineered scaffolds, using biodegradable polymers for the purpose of therapy, has been achieved. An extensive number of attempts at developing new scaffold technologies using both polymers and cells, including stem and/or

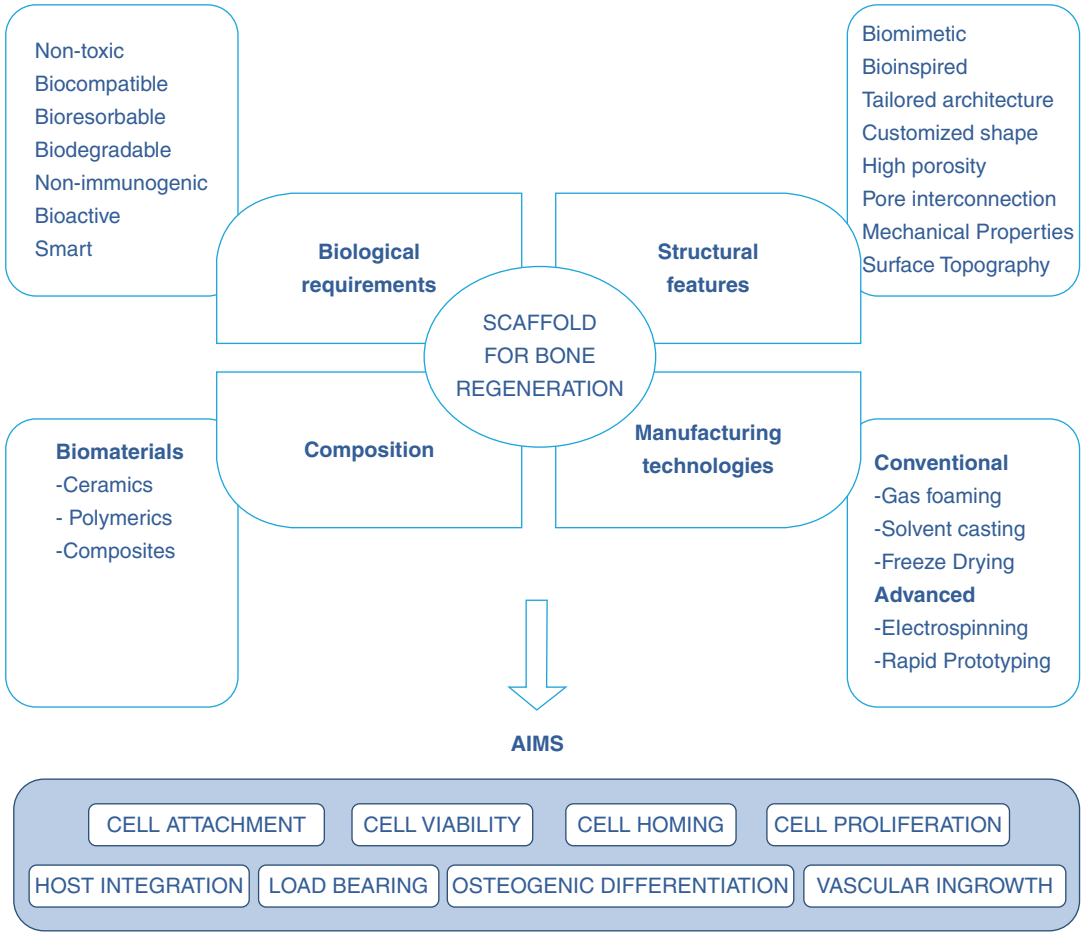

Fig. 4.2 General overview of scaffold construction for bone regeneration [19]

somatic cells, isolated from various tissues have been made. Polymers used in the fabrication of scaffolds in regenerative medicine can usually be categorized as synthetic or natural, where the commonly used polysaccharides (starch, alginate, chitosan, hyaluronic acid derivatives, etc.) and proteins (collagen, fibrin gels, silk, keratin, etc.) are examples for natural polymers (Table 4.1). On the other hand, synthetic polymers such as polylactic acid (PLA), poly(L-lactic acid) (PLLA), poly(D,L-lactic-co-glycolic acid) (PLGA), polyglycolic acid (PGA), and polycaprolactone (PCL), approved by U.S. Food & Drug Administration (FDA), can be easily processed and handled in contrast to natural polymers which is their superiority (Table 4.2) [25]. Major advances seen in biomaterials technology in recent years have led to the development of sophisticated materials [26]. Ideally, functionalized biomaterials like ceramics and natural/synthetic biodegradable polymers can be utilized for the production of 3D scaffolds which tend to supply not only mechanical support but also microscale architecture for neo-tissue construction allowing in vitro and in vivo cell growth, attachment, migration, and proliferation [24, 27, 28]. These biomaterials are seen to have a wide range of applications, including replacement of biological tissues and development of instruments for injury and surgical applications, and medical diagnosis has led to a revolution in biomaterial science [26].

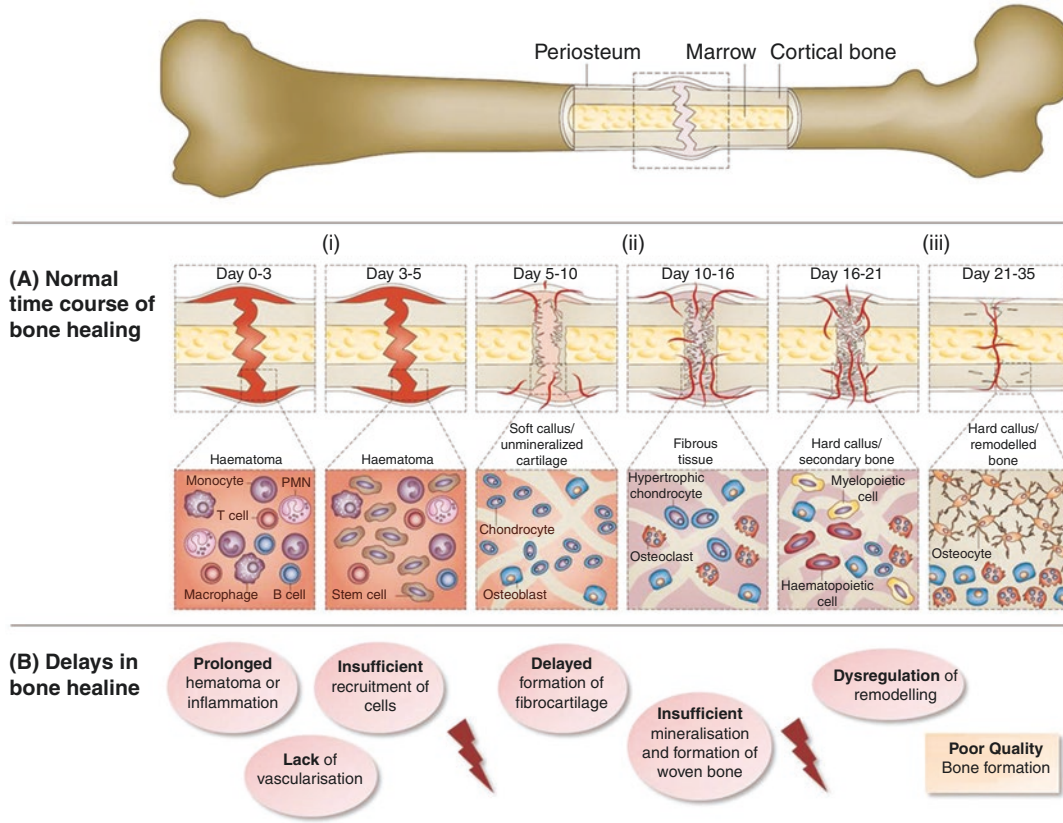

Fig. 4.3 The repairing mechanism of femur fractures and common complications that may occur [9]

4.4 The Importance of Popular Renewable Materials for Regenerative Medicine

The applicability of native materials containing polysaccharides and proteins in the structure of hydrogels has been well studied. These materials, including ECM proteins such as collagen, elastin, fibrin, keratin, hydroxyapatite, and hyaluronic acid, show significant bioactivity in biomedical applications [30].

Bone is a complicated material consisting of mostly collagen, proteins, with hydroxyapatite in organic component. Although HA is the essential inorganic constituent of bone, it does not have the ability to be applied as bone healing material alone because of its delicate and brittle nature. At present, many researchers have devoted themselves to the development of durable hybrid biomaterials of hydroxyapatite with proteins and alternative synthetic polymers [31–35]. For many years, HA ceramics that can improve bone mass and formation of the implant and the bone interface have become quite important as bone grafting material, due to their great mechanical properties, corrosion resistance, biocompatibility, bioactive properties, and perfect osteoconductive features [17, 36, 37]. Using an enhanced hygienic, nontoxic and in addition to an environmentally friendly approach, HA powders have been obtained utilizing bioproducts such as corals, cuttlefish shells, natural gypsum, natural calcite, bovine bone, sea urchin, starfish, and eggshell [38–41]. Chemical studies have demonstrated that these bio-wastes, contrary to popular opinion, are rich in calcium

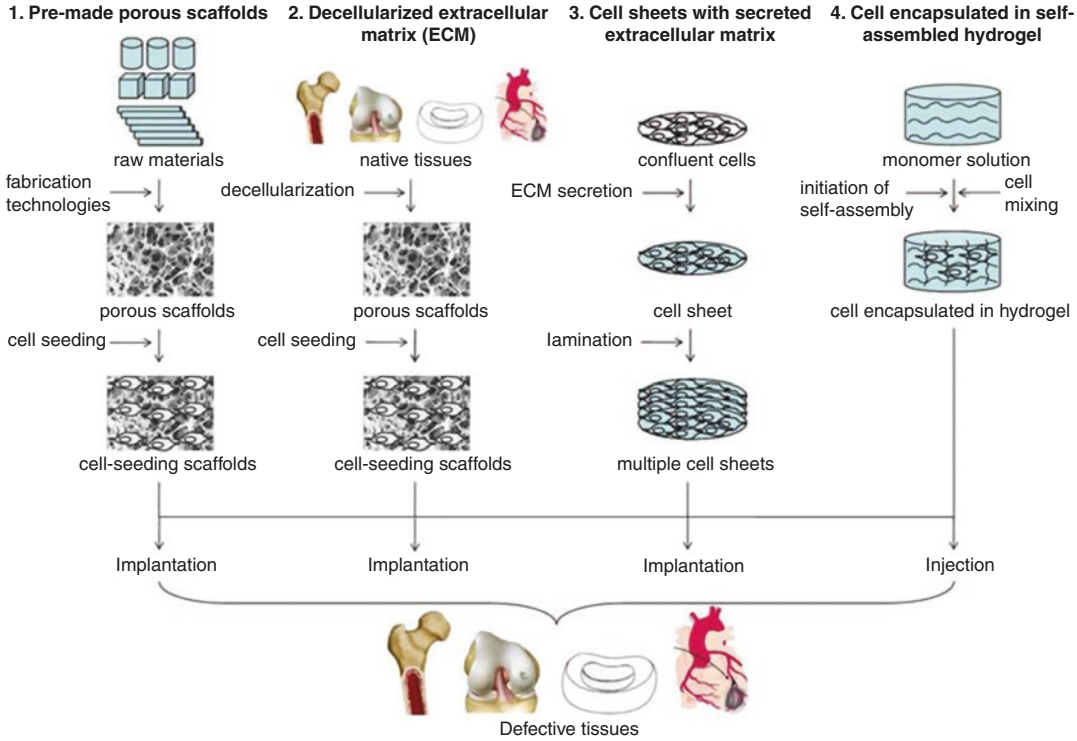

Fig. 4.4 Different scaffold fabrication techniques in tissue engineering and regenerative medicine [22]

in the form of carbonates and oxides. Eggshells are one of the best examples for bio-waste. Millions of tons of eggshells are produced by people as bio-waste on daily basis throughout the world. The eggshell constitutes ~11% of the whole weight of an egg and consisted of calcium carbonate (~94%), calcium phosphate (~1%), and organic matter (~4%) [42]. In addition, eggshells are inexpensive, abundant in nature, biocompatible, yet not osteoconductive. Therefore, transforming these powders in HA before implantation is favorable [43].

Keratins are structural proteins that display high mechanical resistance owing to numerous intra- and intermolecular disulfide bonds containing a fair amount of cysteine [44]. Keratin is mostly consisting of ß-sheets, a small number of α-helices, and loops [45, 46]. Waste keratins are generally obtained from human hair (Fig. 4.5), animal nails, horns, hoofs, wool, and feathers [47]. Additionally, about 300,000 tons of hair is wasted in hair salons, hospitals, and similar places each year [48]. Keratin obtained from renewable sources is highly biocompatible, possesses cellular interaction sites, and exhibits enhanced biodegradability. In contrast to alternative natural materials, human hair keratins have different benefits like being abundant, bioactive, having a powerful capacity to self-assemble inside hydrogels, and being an exact source of autologous proteins [49, 50]. Likewise, in addition to enhancing mechanical properties, this autologous protein has some signaling patterns like Leucine-Aspartic Acid-Valine (LDV) and Glutamic Acid-Aspartic Acid-Serine (EDS) peptide regions which increase the adhesion characteristics of cells [47, 51]. Nonetheless, new improvements have been made to obtain keratin easily from human hair which has resulted in good tissue engineering applications [52].

Collagen is the most widespread protein in the body and provides endurance and constructional stability to tissues containing skin, blood vessels, tendons, cartilage, and bone [27]. The characterizing property of the collagen is its molecular form that is defined by a unique

Table 4.1 Well-known naturally derived polymers used in tissue engineering and regenerative medicine [29]

Polymer	Biocompatibility	Disadvantage	Biodegradability	Application
Collagen	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Proteolytic removal of small non-helical telopeptides	Bulk, controllable	Skin, cartilage, bone, tendons, ligaments, vessels, nerves, bladder, liver
Hyaluronic acid	Minimal foreign body reaction, no inflammation	Highly viscous solution, many purification steps after chemical modification	Bulk, 1 h to 1 month	Skin, cartilage, nerves ligaments, vessels, liver
Alginic acid	Minimal foreign body reaction, no inflammation	Uncontrollable dissolution of hydrogel	Bulk, 1 day to 3 months	Skin, cartilage, bone, nerves, muscle, pancreas
Chitosan	Minimal foreign body reaction, no inflammation	Uncontrollable deacetylation and molecular weight	Bulk, 3 days to 6 months	Skin, cartilage, bone, nerves, muscle, pancreas
Gelatin	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Weak mechanical property	Bulk, controllable	Skin, bone, cartilage, breast ligaments
Fibrin	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Weak mechanical property	Bulk, controllable	Skin, bone, cartilage, liver, tendons, vessels ligaments
Poly(hydroxyalkanoate)	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Pyrogen removed	Bulk, controllable	Skin, bone, tendons, nerves cartilage, ligaments, heart; vessels, muscle
Silk	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Inflammation of sericin	Bulk, controllable	Skin, ligaments, bone, cartilage, tympanic membrane, vessels, tendons

Table 4.2 Well-known synthetic polymers used in tissue engineering and regenerative medicine [29]

Polymer	Biocompatibility	Disadvantage	Biodegradability	Application
Poly(lactic acid)	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Local inflammation, random chain hydrolysis	Bulk, 24 months	Skin, cartilage, bone ligaments, tendons, vessels, nerves, bladder, liver
Poly(glycolic acid)	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Local inflammation, random chain hydrolysis	Bulk, 6–12 months	Skin, cartilage, bone ligaments, tendons, vessels nerves, bladder, liver
Poly(lactic-co-glycolic acid)	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Local inflammation, random chain hydrolysis	Bulk, 1–6 months	Skin, cartilage, bone ligaments, tendons, vessels, nerves, bladder, liver
Poly(caprolactone)	Minimal cytotoxicity, mild foreign body reaction, minimal inflammation	Hydrophobic	Bulk, 3 years	Skin, cartilage, bone ligaments, tendons, vessels, nerves
Poly(ethylene oxide)	Mild foreign body reaction, no inflammation	Complex biodegradability	Bulk, 1 month-5 years	Skin, cartilage, bone, muscles
Polyanhydrides	Minimal foreign body reaction, minimal inflammation, minimal cytotoxicity	Limited mechanical property	Surface erosion, controllable	Bone
Poly(propylene fumarate)	Mild foreign body reaction, minimal inflammation	Weak mechanical property	Surface erosion, 1 week–16 months	Bone
Poly(orthoester)s	Mild inflammation, mild foreign body reaction	Weak mechanical property	Bulk ~ several months	Ear, bone, cartilage
Polyphosphazene	Minimal foreign body reaction, minimal inflammation	Wide molecular weight distribution	Surface erosion, 1 week–3 years	Skin, cartilage, bone, nerves, ligaments

conformation which is a three α-polypeptide chain of one or more spaces formed in a triple-helical structure of $[Gly–X–Y]_n$ arrangement in one of the main sorts of constructional ECM proteins [30, 53]. This design comprises a supercoiled triple helix that consists of three left-handed polyproline-like chains twisted together into a right-handed triple-helix. Hydroxyapatite and collagen, the most important structural protein present in bone, are two main constituents of bone. They compose 89% of the organic matrix and 32% of the volumetric constituent of bone. Therefore, it is a special protein that promises to produce bone from cultured cells [54]. Collagen is one of the most frequently used materials due to its superior biocompatibility, biodegradability, weak immunogenicity, and cell-adhesive properties in tissue engineering [55, 56]. Although collagen can be produced from different organisms, generally, bovine skin, tendon, and porcine skin-derived collagens for tissue engineering practices are preferred. Yet, collagen obtained from bovine sources includes the risk of infection with illnesses such as bovine sponge-like encephalopathy. Additionally, particularly porcine-derived mammalian collagens are refused for religious reasons [57]. Marine living creatures are also a native origin of collagen and, probably, are more secure source than

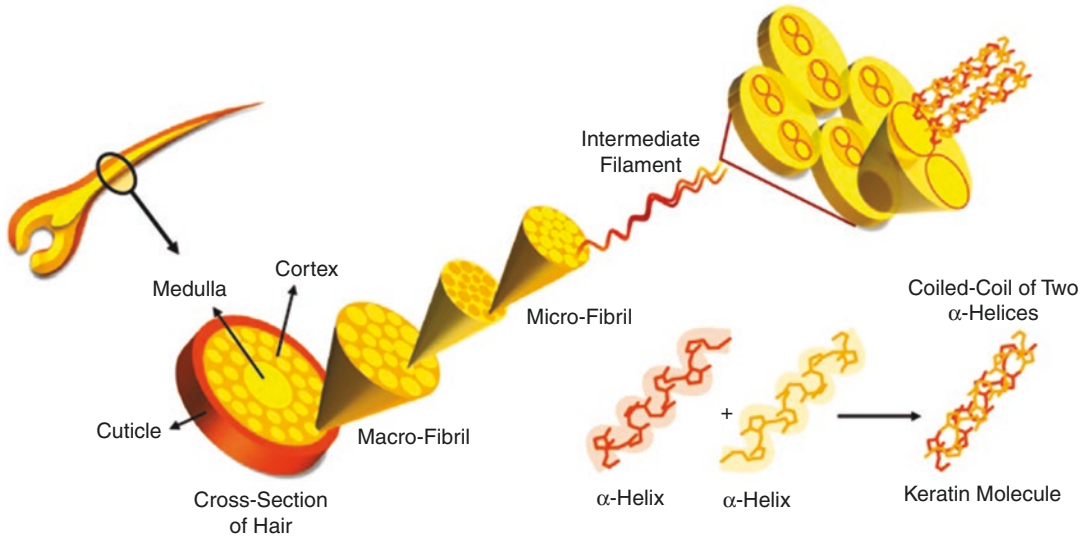

Fig. 4.5 The microscopic structure of hair [52]

mammals. Recent studies focus primarily on the extraction and characterization of collagen from various fish types like salmon, shark or deep-sea redfish and marine sponges. Jellyfish, which is also of marine origin, is another alternative charming source of collagen [58–61]. The worldwide growth of the jellyfish population has caused great concern in the ecological environment. Their potential for utilization in tissue engineering, in addition to the food industry and medicine, we believe, will assist in the preservation of the jellyfish population. Jellyfish has more than 60% collagen, thus the potential to become a perfect source for in biomedical applications [62–64].

4.5 Fabrication of 3D Scaffolds from Keratin-Collagen-nHA for Bone Tissue Engineering

Keratin is insoluble in several prevalent solvents like dilute acids, alkalines, water, and organic solvents. Soluble hair keratins can directly be obtained from human hair utilizing reducing assistant solutions in alkaline or acidic media (Fig. 4.6) [49, 50]. A common way of obtaining keratin includes the utilization of reducing assistants because the natural structure is difficult to extract, owing to its extremely cross-linked status with disulfide bonds [65–67].

Hydroxyapatite is usually obtained through chemical methods by way of calcium hydroxide or nitrate as pioneers [69]. Recently the synthesis of nanostructures using native resources or waste like eggshell, fish scale, or bovine bone has become an outstanding issue. Eggshell, one of the main residual outputs of the food industry, is a great resource of calcium carbonate (95%) enabling its use in the synthesis of HA. There are many different studies related to the synthesis of HA utilizing eggshells [70, 71]. Nanostructured HA has been obtained via various techniques, like homogeneous precipitation, hydrothermal synthesis, combination of electrospinning and thermal treatment, and application of fibrous β-$Ca(PO_3)_2$ crystalline as pioneer [72–74]. Derkus et al. [31] have demonstrated a significantly novel method, the sonochemical synthesis technique, which is a more applicable, homogenous, and cheap method for the synthesis of nanostructured HA (nHA) utilizing various resources. This technique was implemented in the synthesis of nHA using eggshells as the resource (Fig. 4.7), for the design and application of an aptasensor, which has emerged as an interesting application in literature [31].

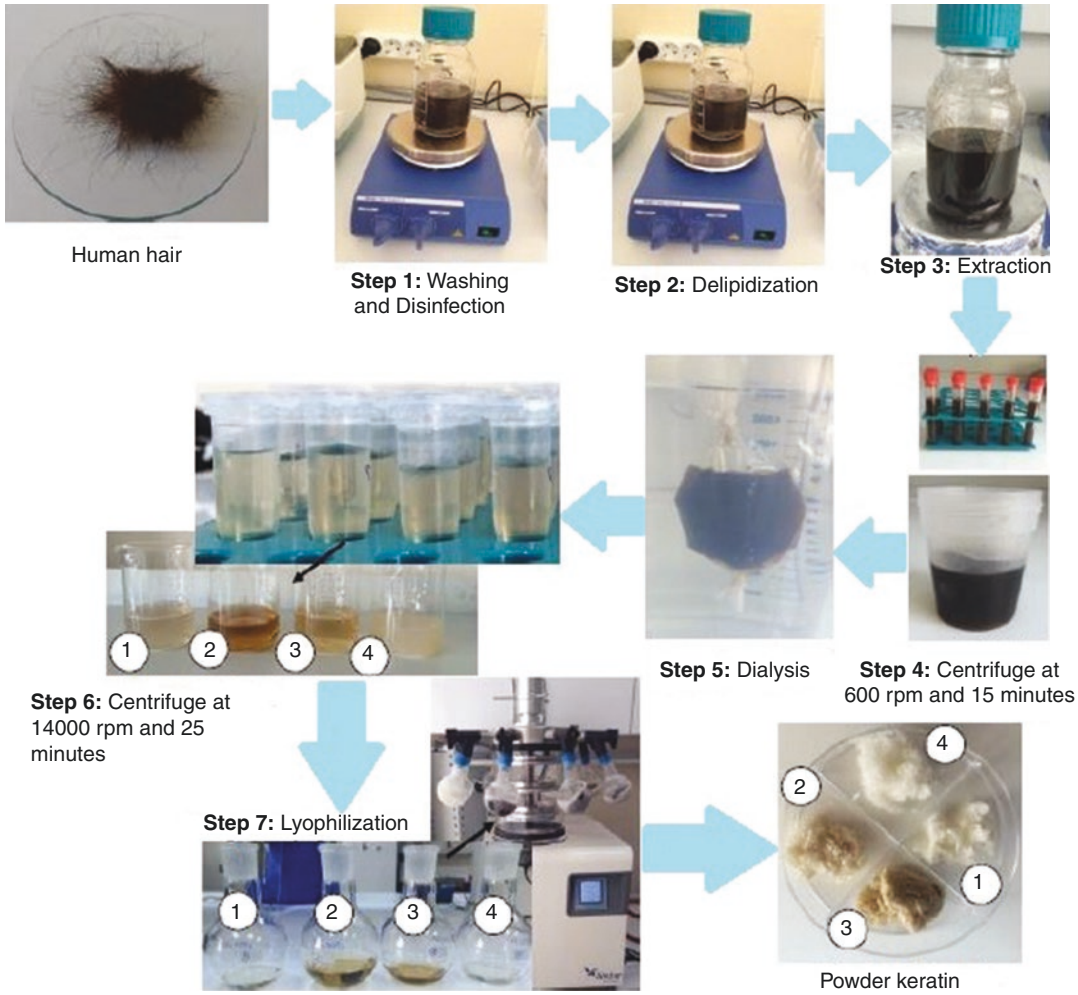

Fig. 4.6 Keratin extraction process from human hair [68]

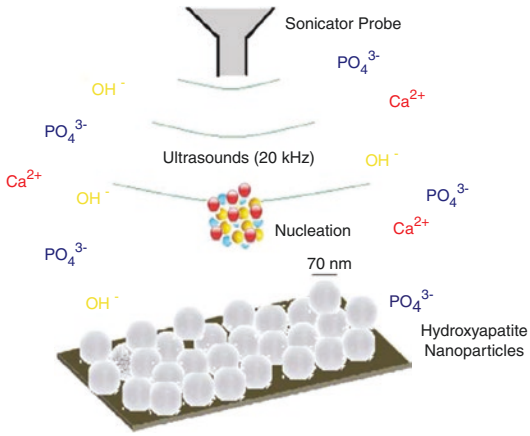

Fig. 4.7 nHA synthesis from eggshell by sonochemical method [77]

Collagen-originated biomaterials are actually based on three basic techniques and sub-techniques of these. The first one is to decellularize the collagen matrix protecting the primary tissue form and ECM architecture, whereas the second method is based on extraction, purification, and polymerization of collagen and its various constituents in order to create a handy scaffold and finally to obtain a collagen solution from different biomolecules. All methods could be applied to several cross-linking techniques and protocols that can be applicable to a large arena of tissue resources [75, 76].

The collagen matrix or ECM could be produced through decellularization methods. Gilbert

et al. [76] have discussed the three ways for tissue decellularization: physical, chemical, and enzymatic. Physical techniques include snap freezing, which disturbs cells by forming ice crystals, leading to high pressure that explodes cells and in turn agitates and stimulates cell lysis. The chemical processes of decellularization involve multiple reagents that remove the cellular ingredient of ECM. These materials range from acids to alkaline tests, which are as good as chelating agents like EDTA, ionic or non-ionic detergents and solutions of excessive osmolarity. Enzymatic therapies like trypsin, which particularly separates proteins and nucleases, evacuating DNA and RNA, are usually utilized to fabricate decellularized scaffolds as well. Nevertheless, all of these methods are unable to fabricate an ECM exactly free of cellular waste on their own; therefore a combination of different techniques is frequently necessary for this purpose [75].

The alternative source for collagen-originated biomaterials are actually marine resources as previously defined. Various ways were applied and enhanced to obtain collagen from jellyfish so as to be able to fabricate collagen-originated biomaterials (Fig. 4.8). Advanced isolation techniques were asserted on three major bases of solubility: in acid solutions, in inactive salt solutions, and in proteolytic solutions. Proteolytic extraction changes collagen molecular architecture by separating the terminal telopeptide areas resulting in the proportional decrease of tropocollagen self-assembled fibrils. In order to prevent this effect, endogenous proteases could be inhibited during acid solubilization. Nevertheless, acid ejection which utilizes light pepsin solubilization is the most efficient technique in terms of yield, although some telopeptides do separate or are partly denatured [77].

There are a limited number of studies concerning the application of bioengineered keratin, jellyfish collagen, and nHA scaffolds to bone tissue engineering. Arslan et al. [17] fabricated 3D tissue-engineered osteoinductive biocomposite scaffolds utilizing human hair keratin, jellyfish collagen, and eggshell-derived nanostructured spherical HA (Fig. 4.9). Two different osteoinductive scaffolds, collagen-nHA and collagen-keratin-nHA, were produced utilizing the freeze-drying method. hAMSCs were then seeded into these scaffolds and the early osteogenic differentiation markers were evaluated. The collagen-keratin-nHA osteoinductive biocomposite scaffolds were observed to have the potential of being used in bone tissue engineering.

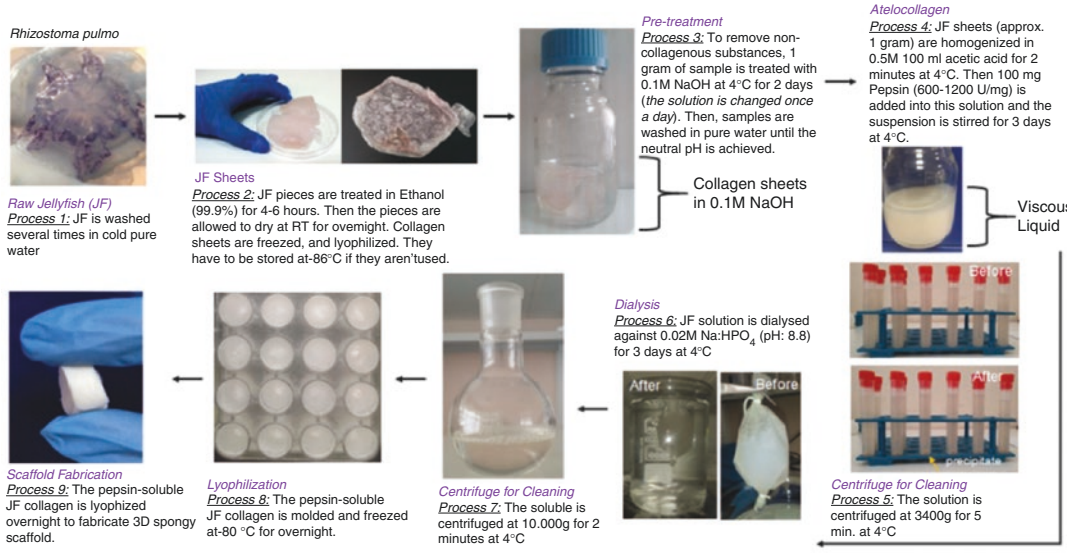

Fig. 4.8 Process steps of jellyfish collagen isolation [31]

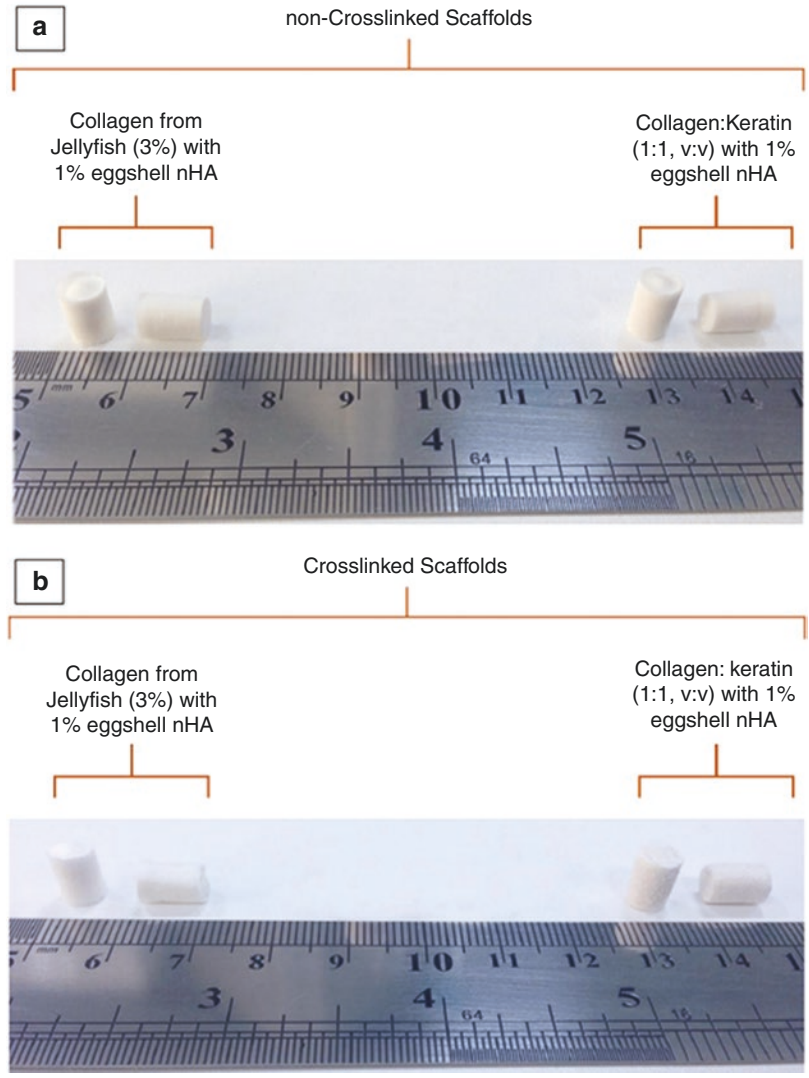

Fig. 4.9 Keratin-collagen-nHA 3D osteoinductive biocomposite scaffolds [17]

4.6 Conclusions

The field of tissue engineering and, in particular, bone tissue engineering has been studied extensively. Polymeric products, in combination with mineral based nanostructures, have been used by various research groups in order to trigger the osteogenic differentiation. Recently, natural resources have become popular due to their cost efficiency, nontoxic nature and easy-to-produce materials suitable for bone tissue engineering. Different research groups have focused on the synthesis of hydroxyapatite bioceramics, which constitute the inorganic phase of bone, using various waste material like mussel shells, flue gas desulfurization gypsum, fish bones, and eggshells. Likely, some research groups have been focused on the isolation of collagen with low immunogenicity and high purity from different kind of species such as jellyfish instead of the traditionally used skin or rodent tail. In our opinion, adaptation to this approach is like "killing two birds with one stone." Firstly, waste is evaluated as a renewable material resource of unlimited volume and chemical diversity. Secondly, it will have a posi-

tive effect on waste accumulation in the environment. Provided that biomaterials obtained from waste resources have low immunogenic response and toxicity, this technology can be expected to become available for clinical use in the next few years.

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Part II

Research Concepts

5 Mechanotransduction in Wound Healing and Scar Formation

Dominik Duscher

5.1 Introduction

Scar formation belongs to the most complex biological processes and represents a substantial source of morbidity worldwide. In humans, scarring is the typical response to tissue injuries. The process of fibrotic repair, providing early restoration of tissue integrity rather than functional regeneration, offers a survival advantage and is therefore evolutionary highly preserved [1, 2]. Despite extensive research efforts dedicated to the expansion of our understanding of the mechanisms underlying scar formation, effective clinical therapies for scar mitigation are only beginning to be developed. A detailed understanding of the numerous signaling pathways involved is essential to develop remedies for fibrosis and scarring. Initial research efforts concentrated on the biochemical mechanisms involved in scar formation, however, evidence begins to emerge that mechanical forces play a previously underestimated role in the modulation of these pathways. The impact of mechanical forces on cutaneous scarring has been first observed as early as the nineteenth century [3], but only recently the underlying signaling mechanisms begin to be elucidated. Mechanotransduction, which refers to the mechanisms by which mechanical forces are converted to biochemical stimuli, has been closely linked to inflammation and is believed to play a pivotal role in cutaneous fibrosis [4]. There is increasing evidence that all phases of wound healing are influenced by mechanical forces [5], but the field of wound mechanobiology is still in its infancy. However, utilizing the recent insights into how mechanotransduction of environmental cues effects the behavior of cells and tissues will help us to formulate effective therapeutics and may lead to the achievement of the ultimate goal, to transform fibrotic healing into tissue regeneration.

5.2 Molecular Biomechanics of Scar Formation

The field of mechanobiology continues to advance rapidly. The application of innovative in vitro and in vivo models leads to a more thorough understanding of the effects of mechanical forces on biological processes [6]. It could be demonstrated that cells are able to convert mechanical stimuli into biochemical or transcriptional changes via the process of mechanotransduction [7]. Signal transduction from the microenvironment involves numerous proteins and molecules of the ECM, the cytoplasmic membrane, the cytoskeleton, and the nuclear membrane, which transport mechanical cues

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down to the nuclear chromatin to alter cellular programs at the genetic and epigenetic level [8].

Several attempts to define the role of mechanical influences in molecular biology have been made. The most widely accepted system linking the different levels of mechanotransduction is known as tensional integrity or "tensegrity" [9]. First described as an architectural concept [10], this principle was adapted and developed by Ingber et al. to explain how cellular structures and processes are influenced by mechanical force. However, a complete understanding of the complex mechanotransduction pathways in living organisms remains elusive. Nevertheless, the observations made in small and large animal studies implicate a significant involvement of mechanical influences in the development of cutaneous scarring. Translating these findings into clinical therapies must be our principal goal.

5.3 Extracellular Mechanotransduction

The extracellular matrix (ECM) is much more than just an inert three-dimensional network passively offering structural support. It is a dynamic and living tissue responsible for numerous functions. The ECM governs cell adhesion, migration, differentiation, proliferation, and apoptosis and is highly involved in the complex processes of mechanotransduction (Fig. 5.1) [11, 12]. Mechanical cues transported through the ECM to cells can directly affect gene expression, because

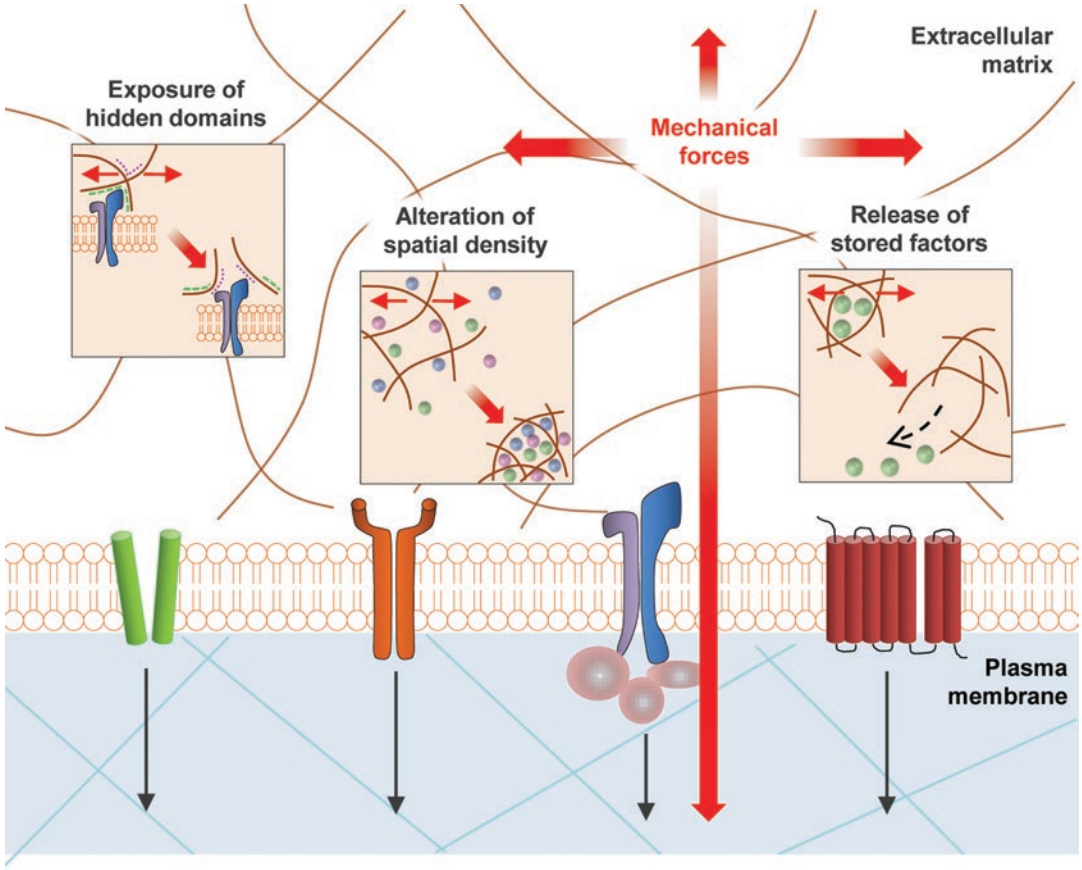

Fig. 5.1 Extracellular mechanotransduction. Biomechanical cues directly affect the extracellular matrix (ECM), which is a dynamic structure with multiple functions. Mechanical stimuli can expose hidden domains and alter spatial concentration of growth factors within the ECM, resulting in changes of cellular behavior and phenotype. Additionally, stored factors within the ECM can be released based on the effects of mechanical force. Reproduced with permission [44]

of a direct link between structural proteins of the ECM to nuclear chromatin [13].

Additional evidence supporting the theories how ECM can alter cell functionality and phenotype is provided by the fact that tissue stiffness and rigidity could be linked to tumor growth and malignancy [14]. The extracellular environment can function in both ways, being pro-oncogenic [15], but also reverse malignant behavior if corrected [16]. Similarly, microenvironmental signals can influence scarring and fibrosis [17–19]. It could be demonstrated that scar progression results from a positive feedback loop connecting the accumulation of ECM and increased matrix stiffness to enhancement of fibroblast proliferation and collagen production via mechanoresponsive mechanisms [20, 21].

In addition to direct effects on cell behavior via ECM-cell membrane/cytoskeletal interfaces, mechanical cues can also execute indirect affects. Specifically, alterations in ECM structure can expose normally hidden domains and binding sites that have regulatory capabilities [22]. The ECM can also alter the spatiotemporal composition of the microenvironment by changing the concentrations of soluble and matrix-bound effector molecules and growth factors, such as transforming growth factor beta (TGF-β), resulting in considerable impact on biological functions [23].

5.4 Intracellular Mechanotransduction

The complexity of the mechanisms by which cells "feel" and interact with their environment is incompletely understood. However, recent efforts have led to the identification of key signaling pathways involved in intracellular mechanotransduction. The central mediators of mechanotransduction include mechanoresponsive ion channels (e.g., Ca^{2+}), growth factor and cytokine receptors (e.g., for TGF-β and SDF-1), integrin-matrix interactions, and G protein-coupled receptors (GPCRs) (Fig. 5.2) [24, 25].

The fact that conformational alterations of ion channels govern numerous cellular functions is

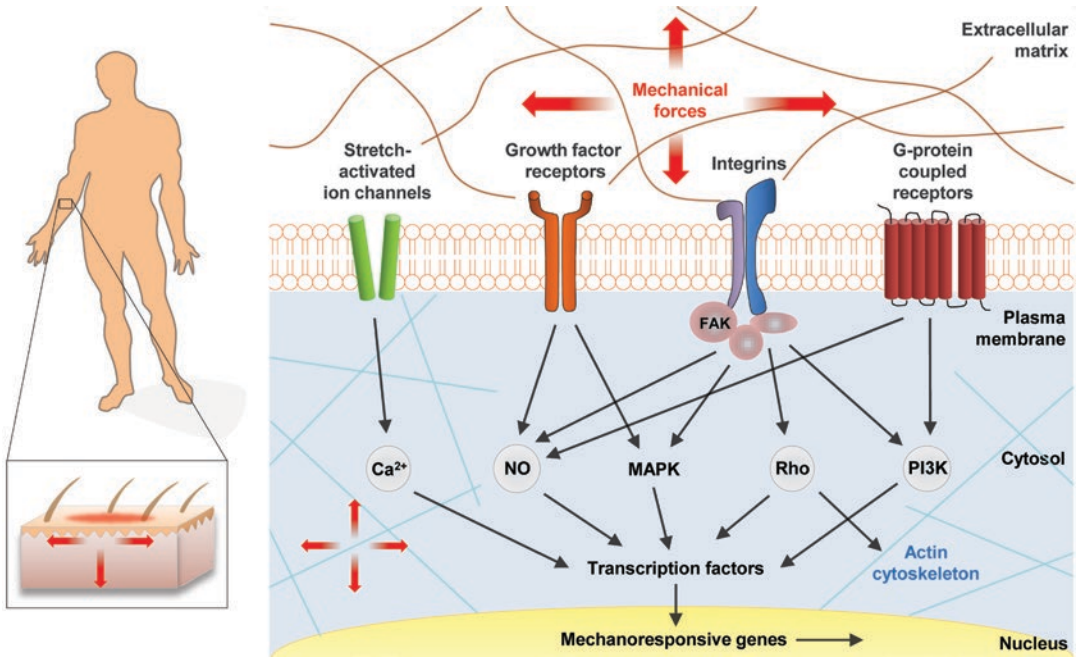

Fig. 5.2 Intracellular mechanotransduction. Key players of mechanotransduction on the cellular level are mechanoresponsive ion channels (e.g., Ca^{2+}), growth factor and cytokine receptors (e.g., for TGF-β or SDF-1), integrin-matrix interactions, and G protein-coupled receptors (GPCRs). Reproduced with permission [44]

an established biological concept. However, an understanding of how mechanoresponsive calcium channels influence fibrotic pathways only recently begins to emerge [26]. Specifically, calcium-dependent ion channels have been demonstrated to be heavily involved in the arrangement of elements of the cytoskeleton, which are associated with mechanotransduction [27]. Additionally, Ca^{2+} influx caused by mechanical stimulation of the cell membrane activates mitogen-activated kinases (MAPKs), which enhance pro-fibrotic gene expression [28].

Growth factors and cytokines together with their receptors are implicated in all stages of wound healing and the development of cutaneous scarring. Recent work has shown promising results regarding scar formation and appearance when the cytokine system is manipulated [29]. Specifically, modulating the ratios of subsets of transforming growth factor beta in the wound microenvironment has received significant attention (see below). Mechanical stimuli result in the release of TGF-β from its reservoir in the extracellular latent complexes [30–32]. This growth factor is associated with numerous fibrotic diseases, and acts mainly via the transforming growth factor-β receptor 2 and its downstream effector proteins Smad 2 and 3. The TGF-β signaling cascade controls numerous pro-fibrotic mechanisms, such as collagen production and fibroblast to myofibroblast differentiation [33, 34]. Another mechanoresponsive signaling molecule responsible for the regulation of cutaneous healing and fibrosis is stromal cell-derived factor 1 (SDF-1 or CXCL12). It has been demonstrated that mechanical stretching can upregulate SDF-1a in skin which directly leads to the recruitment of circulating pro-regenerative MSCs through the SDF-1a/CXCR4 axis [35]. However, somewhat contradictory results regarding the influence of SDF-1 on wound healing and scar formation have been reported. Whereas hypertrophic burn scars could be associated with increased SDF-1a/CXCR4 signaling [36], it could also be demonstrated that the therapeutic application of SDF-1 to cutaneous wounds of mice and pigs leads to enhanced healing with decreased fibrosis [37, 38]. Additionally, a local increase of SDF-1 has been identified as the potential underlying mechanism of noncontact, low-frequency ultrasound therapy, which has been shown to have beneficial effects in the treatment of chronic wounds [39]. This further corroborates the pivotal role of cytokines at the intersection of mechanotransduction and tissue healing, which strongly merits further investigation.

Although ion channels and cytokines certainly play an important role in the mechanobiology of scar formation, the most extensively studied cellular elements in this context are integrins. The members of this family of heterodimeric transmembrane receptor proteins possess a cytoplasmic domain, which communicates with the actin cytoskeleton, and an extracellular domain-binding molecules of the ECM [40]. Integrins carry out both "outside-in" communication of environmental mechanical cues to the cell and "inside-out" interactions via forces produced by the cytoskeleton [40]. This two-way signaling process is influenced by intracellular proteins, which bind to integrins to form large macromolecular structures, the so-called focal adhesions [41, 42]. The most prominent of these intracellular binding proteins is the focal adhesion kinase (FAK). Conformational changes in the complex macrostructure of the focal adhesions caused by mechanical stimuli lead to an activation of the non-receptor protein tyrosine kinase FAK via autophosphorylation. While integrins have no intrinsic enzymatic activity, they can affect downstream signaling pathways via FAK. Specifically, FAK signaling has been heavily linked to wound healing aberrations [4, 43]. While mechanical stimuli influence all cell types involved in wound healing [44], the effects of FAK activation demonstrate how mechanical cues can change biological mechanisms in different cell types in diametrically opposed directions. Our laboratory has previously demonstrated that FAK is a key regulator of mechanosensing in cutaneous fibroblasts and that a fibroblast-specific deletion of FAK leads to reduced fibrosis after injury in a mouse model of scar formation [4]. In stark contrast, the loss of FAK in cutaneous keratinocytes leads to a significant delay in wound healing and dermal proteolysis in mice

[43], suggesting a skin layer-specific effect of FAK signaling with a complex influence on extracellular matrix repair.

Further highlighting the important role that FAK plays in the mechanical regulation of tissue repair, our laboratory recently demonstrated how mechanically activated pathways link scar formation with extracellular-related kinase (Erk, part of the family of mitogen activated kinases [MAPKs]). Erk could be identified as a key mediator in the FAK-related response to wound tension, leading to the overproduction of collagen and the pro-fibrotic chemokine monocyte chemoattractant protein-1 (MCP-1) [4, 45]. Moreover, other groups have shown involvement of other MAPKs in tension-induced fibrotic reactions, namely c-Jun N-terminal kinase (JNK) and p38 isoforms [46, 47]. However, their specific roles in the mechanobiology of wound healing and scar formation need further clarification.

FAK also executes its effects via the Rho family of GTPases. The activity of RhoGTPases could be linked to cell motility, adherence, and cytoskeletal dynamics, as well as to the stimulation of myofibroblast differentiation [48, 49], demonstrating their extensive mechanosensing utility. Moreover, evidence is accumulating that FAK-RhoGTPases signaling influences mechanobiology via two downstream effectors of the mammalian Hippo pathway. The Hippo pathway is an evolutionary highly conserved signaling pathway involved in cell proliferation, apoptosis, differentiation, stem cell function, and malignant transformation (Fig. 5.3) [50, 51]. Specifically, the mechanoresponsive Hippo-effectors YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif, also known as WWTR1) have been identified as key factors of tissue regeneration [52–54], and have pivotal roles in cutaneous wound healing [55].

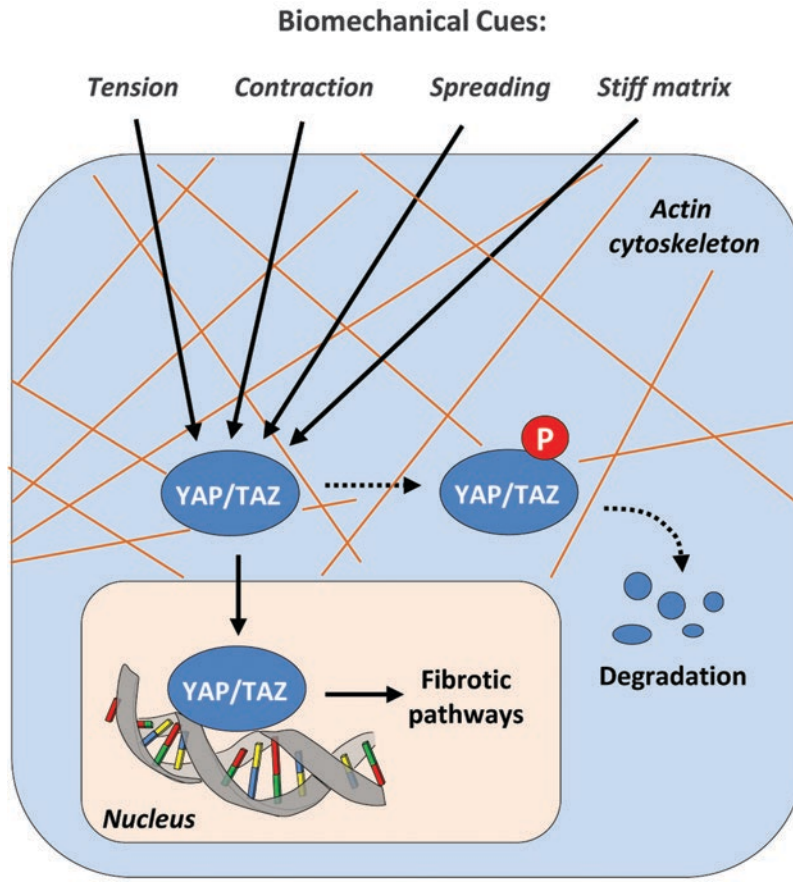

Fig. 5.3 The Hippo pathway in mechanotransduction. The two main downstream Hippo-effectors YAP and TAZ have recently been identified to orchestrate biomechanical influences to alter cell behavior via the transcription of pro-fibrotic signaling molecules such as connective tissue growth factor (CTGF) and TGF-β. This predisposes YAP and TAZ as potential targets for anti-fibrotic therapy. Reproduced with permission [86]

Stimulated via biomechanical cues, YAP and TAZ are stabilized and trigger the transcription of pro-fibrotic targets such as connective tissue growth factor (CTGF) and TGF-β [54, 56]. YAP and TAZ have recently been characterized to be GPCR regulated. Specifically, G12/13 and Gs-coupled receptors act upstream of these transcriptional coactivators to modulate the Hippo pathway [57]. Remarkably, GPCRs function generally as cell surface mechanoreceptors and have been shown to influence similar intracellular pathways as the focal adhesion complexes [24, 25]. Despite the need for further research to fully understand the involvement of the Hippo pathway in biomechanics of wound healing and scar formation, it is likely that YAP and TAZ signaling is an attractive target for anti-fibrotic treatment approaches.

5.5 Modulation of Cutaneous Biomechanics to Reduce Scarring

5.5.1 Pharmacological Approaches

Excessive scarring often results from aberrant signaling in response to injury. Deregulation of signaling pathways at the intersection of mechanotransduction and inflammation leads to a disruption of homeostasis between collagen production and collagen degradation. Therapies targeting fibrosis consequently seek to modulate these pathways for a shift from fibrosis towards regenerative healing [58–61].

Transforming growth factor beta (TGF-β) controls collagen synthesis, and consequently fibrosis, which suggests its suitability as a target in anti-fibrotic therapy. TGF-β exists in at least three isoforms. While TGF-β1 and 2 have pro-fibrotic effects, TGF-β3 acts as an anti-fibrotic [62]. Based on the diverse effects of the TGF-β family on tissue fibrosis, strategies for therapeutic application include neutralizing antibodies to TGF-β1 and 2, or alternatively, means to raise TGF-β3 levels. Surprisingly, the promising preclinical results of TGF-β antibodies have not been able to be translated from bench to bedside. Clinical trials evaluating anti-TGF-β antibodies for systemic sclerosis [63] and scleroderma [64] concluded with disappointing results. Similarly, TGF-β3 had no clinically significant anti-fibrotic effect despite promising results in experimental studies and failed in an international Phase III clinical trial [65].

In addition to the TGF-β family, alternative therapeutic targets for the treatment of fibrosis are under current investigation. Promising areas of research include the hedgehog pathway [66], extracellular cross-linking enzymes (transglutaminases, lysyl oxidases, and prolyl hydroxylases) [67–69], early growth response gene-1 [58], canonical Wnt [59], heat shock protein 90 [61], histone deacetylase [60], and IL-10 [70–75].

Despite remarkable results in preclinical models, translation of substances influencing mechanoresponsive pro-fibrotic pathways has been lagging behind expectations and further innovation and refinement is needed before the clinical application of such therapies. The key to any mitigation of scar formation is the coordinated modulation of numerous cellular and molecular processes. Targeting a single element of a process as complex as scar formation is unlikely to yield clinically significant results and it is likely that only therapeutic approaches tackling multiple effectors in the aberrant physiology of problem wounds will be successful.

5.5.2 Mechanomodulatory Approaches

Evidence is accumulating that a mitigation of fibrosis in the setting of cutaneous injury can be achieved by modulating traction forces on wounds

via mechanical off-loading. Environmental cues play a critical role in scar formation and development and studies suggest that mechanical tension is a driver of fibrosis [4, 76]. Based on this theory it is not surprising that trials utilizing compression dressings [77–81] or just paper tape for stable wound approximation [82] have revealed moderate efficacy in scar reduction.

Building upon these findings, approaches utilizing active mechanical off-loading in contrast to passive approximation have recently been developed. Minimizing tension across healing wounds has proven effective in both preclinical and clinical studies resulting in decreased scarring via influencing numerous mechanoresponsive signaling pathways [4, 83]. Moving these insights from bench to bedside, a phase I clinical trial [83] as well as two multicenter randomized controlled trials [84, 85] have demonstrated that stress-shielding of surgical incisions leads to a significant reduction of scar formation (Fig. 5.4). Lim AF et al. [84] showed that utilizing the principles of mechanomodulation significantly improves aesthetic outcomes following scar revision surgery. Similarly, Longaker MT et al. [85] observed a significant reduction of scarring following abdominoplasty surgery in a 12-month, prospective, open-label, randomized, multicenter clinical trial providing the first level I evidence for postoperative scar reduction. Collectively, these findings suggest that the complexity of the pathways involved in fibrosis and the limitations of our current understanding necessitate mechanomodulatory rather than pharmacological approaches to mitigate fibrosis, at least in the cutaneous setting.

5.6 Conclusions and Future Perspectives

A profound understanding of the biomechanical principles influencing scarring and fibrosis is imperative to combat this significant pathology that represents a substantial healthcare burden worldwide. Elucidating how mechanoresponsive signaling pathways affect scar development is the only way how effective strategies to mitigate fibrotic processes can be formulated. Consequently, advances at the intersection of biology and material science already lead to novel therapeutics for hypertrophic scarring recently beginning to enter the clinical realm. However, a complete understanding of the mechanobiology of scar formation has yet to be achieved to potentially ultimately transform tissue fibrosis into regenerative healing.

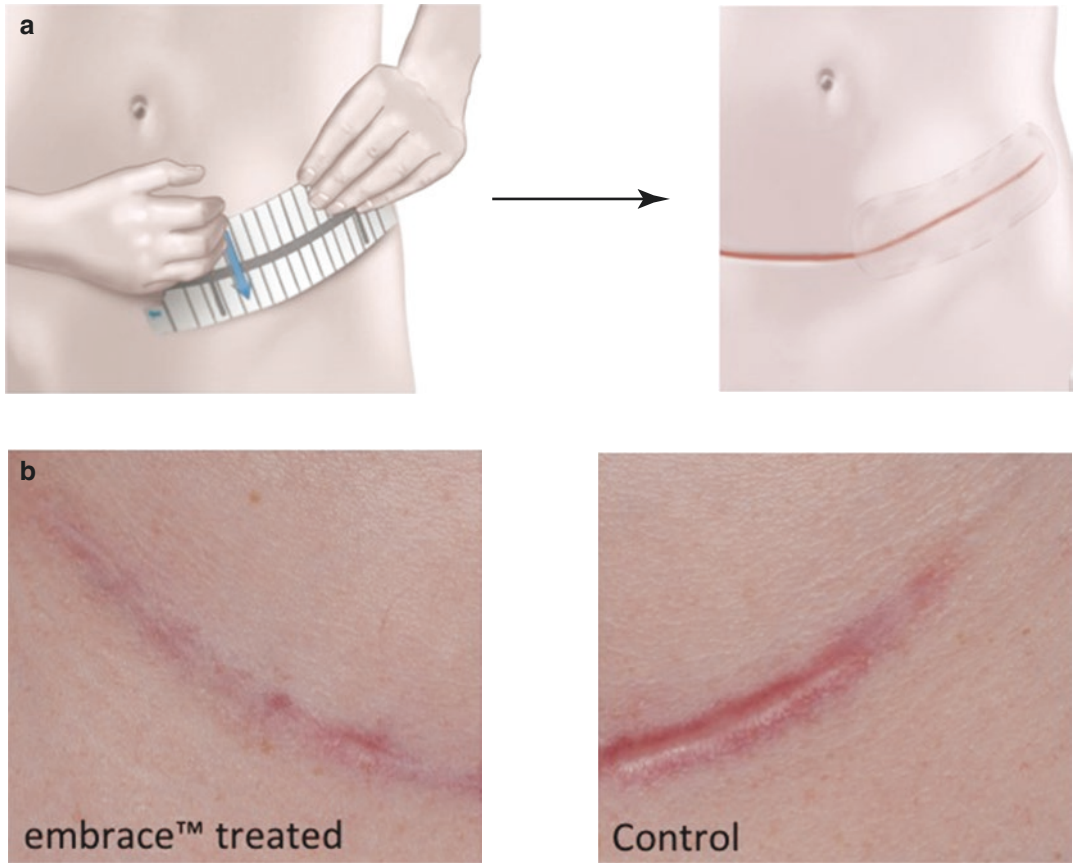

Fig. 5.4 Mechanomodulation therapy to reduce cutaneous scarring. The embrace™ device is an elastomeric silicone dressing. The user applies the device using an applicator that pre-strains the dressing. The dressing is applied directly over the center of the closed incision to mechanically off-load the wound. Randomized controlled clinical trials have demonstrated that mechanomodulation of the wound environment using embrace™ significantly reduces scar development. Adapted with permission [84, 85]

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6 Stromal Cell-Derived Factor 1 (SDF-1) Signaling and Tissue Homeostasis

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6.1 Introduction

Stromal-derived factor 1 (SDF-1) is a highly potent chemoattractant protein that critically regulates cell migration and behavior during embryogenesis, hematopoiesis, tissue repair, and regeneration. Through similar molecular mechanisms, SDF-1 may also regulate the migration and behavior of cancer stem cells. Impaired SDF-1 signaling, on the other hand, is a feature of aging and certain disease states such as diabetes. This chapter outlines the structure of SDF-1 and its function in development, homeostasis, and disease.

6.2 SDF-1 Structure and Function

SDF-1, also known as CXC chemokine ligand-12 (CXCL12), is a small molecular weight protein belonging to the CXC motif family of chemokines with several isoforms. SDF-1 exists as two alternative splice variants, SDF-1α and SDF-1β, which are identical except for the addition of 4 amino acids at the carboxyl-terminus of SDF-1β. The functional significance of this difference is not known [1]. SDF-1 is located on chromosome 10, unlike the remainder of the CXC subfamily, which cluster on chromosome 4, or the CC chemokines which cluster on chromosome 17. Also unique among chemokines, SDF-1 has remained evolutionarily conserved, with 99% homology between mouse and human SDF-1 [1–3]. SDF-1 is constitutively and ubiquitously expressed in many tissues and cell types, including the stromal and endothelial cells located in bone marrow [4], cardiac tissue [5–7], skeletal muscle [8, 9], liver [10], neural dendritic tissue [11, 12], and the kidney [7, 13].

Studies at the molecular level demonstrate that the promoter region of the SDF-1 gene contains hypoxia inducible factor-1 (HIF-1) binding sites, as revealed by chromatin immunoprecipitation analysis, and that SDF-1 expression is upregulated in endothelial cells by HIF-1α [14, 15]. HIF-1 consists of an oxygen-sensitive α-subunit, and a constitutive β-subunit [16]. In normoxia propyl hydroxylases (PHDs) prepare the α-subunit for degradation [17, 18]. In hypoxia, HIF-1a is stabilized, and can accumulate, translocate to the nucleus [19], and bind to the hypoxia-responsive region of the SDF-1 promoter, upregulating SDF-1 expression [15]. SDF-1 expression is normally directly proportional to

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decreased local tissue oxygen tensions but can also be activated under normoxic conditions by HIF-1 mimetics [15, 20].

The biologic effects of SDF-1 are mediated by the chemokine receptors CXCR4 and CXCR7. CXCR4 is the primary receptor of SDF-1. Unusual in chemokine signaling, SDF-1 is the only ligand of CXCR4 [21]. CXCR4 (aka fusin and CD184) is a 352 amino acid rhodopsin-like transmembrane-specific G protein-coupled receptor (GPCR).Various cell lines express CXCR4, including muscle cells, endothelial cells, leucocytes, and progenitor cells. The expression of CXCR4 is upregulated by HIF-1a and nuclear factor-kb (NF-kb) [22, 23]. Numerous stem cells express functional CXCR4 and follow SDF-1 gradients, including hematopoietic cells [24], embryonic pluripotent stem cells (PSC) [25, 26], primordial germ stem cells (PGC) [27–29], and various tissue-committed stem cells including neural [30, 31], skeletal/smooth muscle [8, 9], cardiac [5, 32], hepatic [10, 33], nervous [34], endothelial [15, 35], renal tubular- [13], and retina pigment-epithelial cells [36]. The SDF-1-CXCR4 axis has a central role in chemotactic responses, cell mobility, and paracrine signaling. SDF-1-CXCR4 binding can alter cellular expression of adhesion molecules, metalloproteinases, and angiogenic factors including vascular endothelial growth factor (VEGF) [25]. By activating and/or modulating the function of several cell surface integrins, SDF-1 increases cellular adhesion in response to VCAM-1, intercellular adhesion molecule-1, fibronectin, and fibrinogen [37]. The SDF-1/CXCR4 axis is also involved in maintaining hemostasis of the bone marrow niche [38, 39], and hematopoiesis [40]. Additionally, SDF-1 may directly affect cell proliferation and survival [41, 42], including myeloid progenitor cells [43]. CXCR7 (RDCI) has more recently been identified as the second SDF-1 receptor, regulating distinct physiological processes [39, 44].These cellular and molecular mechanisms underpin the actions of SDF-1 in the developing embryo, as well as in homeostasis, regeneration, and repair in the adult.

6.3 Embryological Development

During embryological development, SDF-1 is expressed in parallel with its receptor CXCR4 in numerous adjacent tissue pairs, including the ectoderm/mesoderm in gastrulation and the mesoderm/endothelium in neuronal, cardiac, vascular, thyroid hematopoietic, and craniofacial development [45]. Transgenic mice, deficient in either CXCR4 or SDF-1, have lethal embryonic phenotypes with multiple congenital malformations, including defects in intestinal vasculature, cardiac ventricular septum, lymphoid and myeloid hematopoiesis, neuronal migration of cerebellar neurons, and hematopoietic colonization of embryonic bone marrow (BM) [4, 30, 31, 46, 47]. Rapid tissue growth during embryonic development continually outpaces the supportive vasculature, creating localized areas of hypoxia. Hypoxia upregulates the expression of SDF-1, which in turn acts to guide the migration of CXCR4-expressing embryonic stem cells to hypoxic areas where they can contribute to tissue regeneration and neovascularization [45]. Embryonic hematopoietic stem cells (HSCs), for example, migrate from the liver to the fetal BM in the third trimester along SDF-1 gradients [48], and BM colonization is disrupted in both SDF-1 and CXCR4 knockout mice [4].

Likewise, aortopulmonary septal cells express CXCR4 and migrate towards the SDF-1-rich regions in the cardiac outflow tract during conotruncal development [4]. In the cerebellum SDF signaling prevents premature ventral migration of external granular layer (EGL) cells. In early development CXCR4-expressing EGL cells are bound to pia mater cells on the dorsal edge of the cerebellum, which in turn express SDF-1. This chemotactic attraction is thought to hold EGL cells in position until they are ready to differentiate. CXCR4- or SDF-1-deficient mice have EGL cells that exhibit early pathological ventral migration [30, 31, 45]. In summary, SDF-1 gradients orchestrate complex cellular migration during embryogenesis.

6.4 BM Maintenance

In addition to its embryologic role, SDF-1 also regulates the bone marrow microenvironment postnatally [24, 49–55]. Bone marrow is physiologically hypoxic [15] and SDF-1 is constitutively expressed by BM endothelial cells and mesenchymal stromal cells, including osteoblasts [4, 45, 56]. A subset of BM cells, known as "CXCL12 abundant reticular cells" (CAR), highly express SDF-1 and form networks within the BM surrounding all sinusoidal endothelial cells [57]. SDF-1 expression by BM stromal cells and CAR cells serves as a potent chemoattractant for CXCR4-expressing immature and mature hematopoietic stem and progenitor cells [3, 58, 59]. This generates a unique hypoxia-induced microenvironment in which SDF-1 maintains HSC niche homeostasis [57]. Proteolytic degradation of bone marrow SDF-1 or disruption of SDF-1–CXCR4 binding by granulocyte colony-stimulating factor (G-CSF) mobilizes and disrupts the quiescence of hematopoietic progenitor cells within the bone marrow, leading to differentiation and egress into the peripheral circulation [54, 60]. This mobilization is mediated through the metalloproteinase-9 (MMP-9) release of Kit-ligand, evidenced by the observation that HSC mobilization is suppressed in MMP9-deficient mice [50].

SDF-1 expression by BM cells also facilitates the homing of transplanted HSCs [24]. HSCs injected into the peripheral bloodstream, in both clinical and experimental data, migrate to the bone marrow and repopulate it with myeloid and lymphoid cell lines [61]. BM-derived progenitors virally transfected to express a modified SDF-1-intrakine, which has altered structure and function, blocked the expression and function of CXCR4 and impaired B lymphopoiesis and myelopoiesis, compared to BM-derived progenitors transduced to express SDF-1 [62]. Cytokines such as Kit-ligand (stem cell factor, SCF) and interleukin-6 (IL-6) upregulate CXCR4 expression on mouse CD34+ HSCs and improve HSC engraftment [24, 63]. BM SDF-1 levels increase after irradiation or with treatment using other cytotoxic DNA-damaging agents, including Cy or 5-fluorouracil (5-FU). The increase in SDF-1 correlates with increases in BM-homing and/or repopulation by primitive human HSCs [56].

SDF-1 mediates homing of CXCR4-expressing hematopoietic stem and progenitor cells by inducing integrin-mediated arrest under shear stress on BM endothelium [21, 24, 37, 64]. SDF-1 rapidly and transiently upregulates CD34+ HSC adhesion to both CS-1/fibronectin and vascular cell adhesion molecule-1 (VCAM-1) expressed by BM stromal cells, and enhances very late antigen-4 (VLA-4)-dependent cell adhesion in primitive LTC-IC and committed CD34 cells [65]. Activation of lymphocyte function-associated antigen-1 (LFA-1), α4 and α5β1 (VLA-5) converts the rolling of CD34+ cells into a stable arrest on the BM endothelium [37]. Blocking CXCR4 inhibits the homing and engraftment of CD34+ human progenitor cells in NOD/SCID mice (Fig. 6.1) [24].

The SDF-1-mediated niche microenvironment within the BM maintains HSCs in an undifferentiated quiescent state [50, 66]. Induced deletions of CXCR4 in mice result in large numbers of HSCs entering the cell cycle [56, 67, 68]. The CXCR4/SDF-1 axis is associated with differentiation of both pre-B cells and the megakaryocytic progenitors [69, 70], which has led to SDF-1 being called "pre–B-cell growth-stimulating factor." Synergistic action of SDF-1 with other cytokines may also enhance survival [71, 72]. SDF-1 acts with thrombopoietin or KitL, to suppress apoptosis and trigger CD34+ cells to progress from G0 into the S and G2/M phases of cell cycle [67]. In summary, the SDF1/CXCR4 signaling pathway plays critical roles in supporting HSC mobilization, migration, engraftment, proliferation, and survival.

6.5 Tissue Repair and Regeneration

The SDF-1/CXCR4 axis is essential for the trafficking of mature and immature hematopoietic stem and progenitor cells from the bone marrow

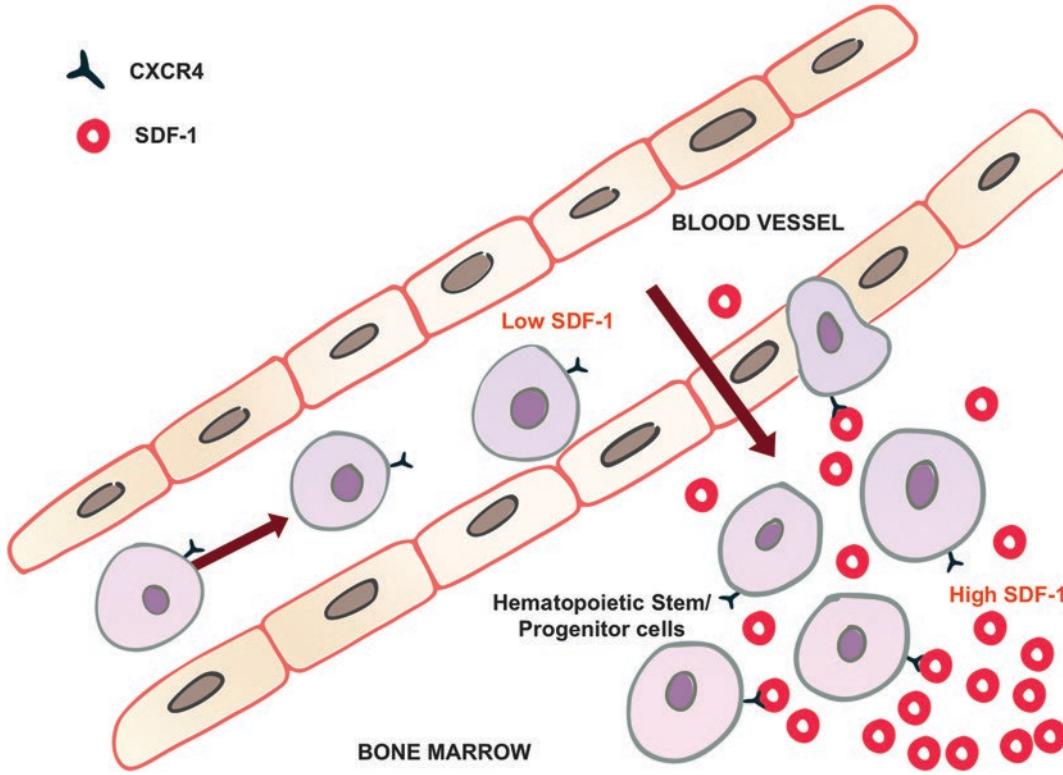

Fig. 6.1 Homing of the CXCR4-expressing hematopoietic stem/progenitor cells to the bone marrow. SDF-1 enhances the adherence and arrest of hematopoietic stem/progenitor cells to endothelial cells by increasing VCAM-1, VLA-4, VLA5, LFA-1, and VL1

to areas of tissue injury. HIF-1a is stabilized in the endothelial cells of ischemic tissue and induces expression of SDF-1. This results in elevated plasma SDF-1 and decreased bone marrow SDF-1 levels. This has been observed across a variety of injury models including following cardiac infarction [6], limb ischemia [73, 74], toxic liver damage [10, 33], excessive bleeding [6], and total body irradiation or chemotherapy [56]. Disrupting the BM-to-peripheral blood SDF-1 gradients stimulates CXCR4-expressing cells to egress from the BM into the circulation and towards sites of increased SDF-1 expression (Fig. 6.2). Mobilized cells include lymphocytes, monocytes, neutrophils, megakaryocytes, hematopoietic stem/progenitor cells (HSPCs), as well as non-hematopoietic stem/progenitor cells (NSPCs) with repopulating potential (CFU-S) [3, 4, 59, 75–77]. These SDF-1-responsive cells populate the peripheral blood in tandem with increasing plasma SDF-levels [78–80].

SDF-1 gradients direct the CXCR4+ HSCs and NSPCs to the respective sites of injury, where they proliferate and assemble for tissue regeneration [15, 25, 33, 58, 81]. SDF-1 signaling is localized to endothelial cells, providing a luminal signal to facilitate adhesion and egression of CXCR4+ cells from the circulation into ischemic tissue, similar to what has been reported in the BM (2, 64). The binding of SDF-1 to the CXCR4 receptor initiates a cascade of signaling processes within the CXCR4+ cells, initiating adhesion, transgression across the endothelial basal lamina, paracrine activity, and cell retention at the target organ [15, 32, 64]. SDF-1 activates lymphocyte function-associated antigen-1 (LFA-1), very late antigen-4 (VLA-4), and VLA-5, which enable cells to firmly adhere to vascular endothelium and begin to migrate out of the circulation [37]. When the CXCR4+ cells encounter the extra cellular matrix (ECM)-rich basal lamina membrane, SDF-1 induces the secretion of matrix metalloproteinases

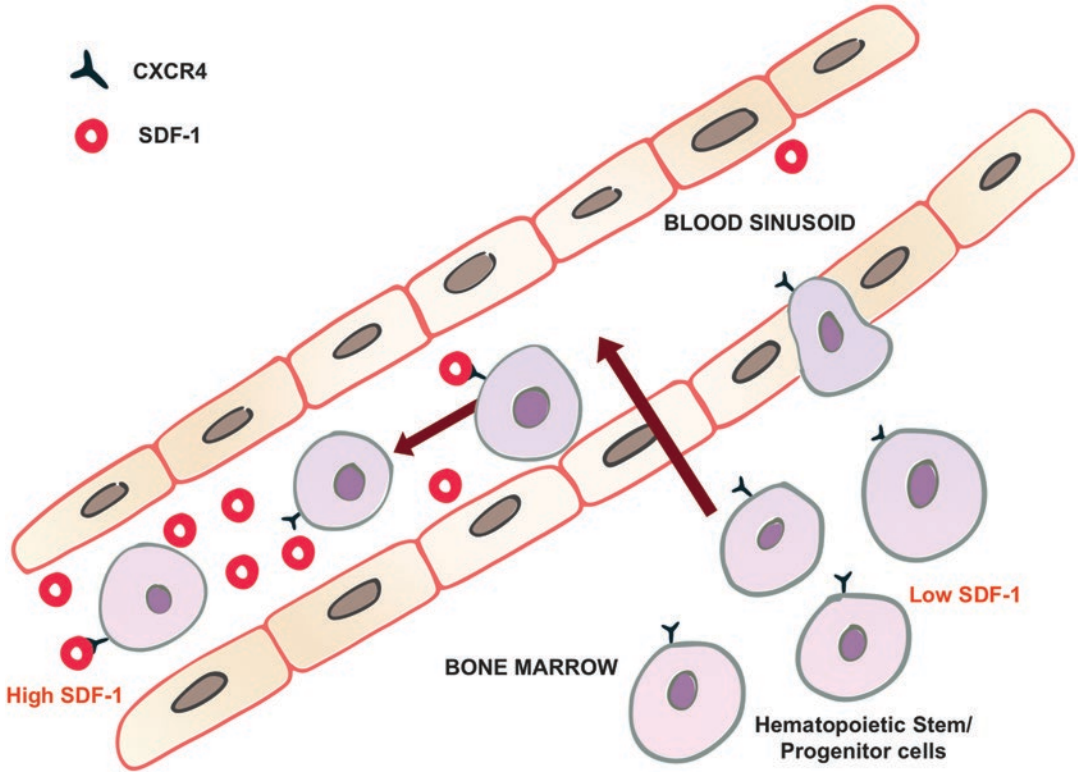

Fig. 6.2 Egression of the CXCR4-expressing hematopoietic stem/progenitor cells from the bone marrow into the bloodstream as a result of disrupting the resting SDF-1 gradient

(MMPs) MMP-2 and MMP-9, enzymatically degrading the ECM basal lamina membrane [33, 82]. Interestingly, MMP-9 inhibitors completely inhibit SDF-1-mediated cell migration [83]. Neural progenitor cells may also secrete MMP-3 in response to SDF-1 [84].

A critical component of tissue regeneration is neovascularization, the formation of new blood vessels. SDF-1 induces the secretion of nitric oxide NO and angiogenic factors, including VEGF, in resident endothelial cells and lympho-hematopoietic cells, promoting neovascularization [41, 85]. In addition, SDF-1 recruits circulating progenitor cells, which contribute to neovascularization through either paracrine stimulation of resident cells, incorporation into newly forming blood vessels, or vasculogenesis [86], the de novo formation of blood vessels distinct from angiogenesis, the sprouting of vessels from existing vascular structures. Vasculogenesis is largely attributed to putative BM-derived endothelial progenitor cells (EPCs), which are recruited to hypoxic tissue by SDF-1 [15]. Vasculogenesis is abundant throughout embryogenesis, and is believed by some to contributes to post-ischemic vascular regeneration by similar regulatory pathways in the adult [87], though this is controversial (Fig. 6.3). Transplanted BM-derived progenitor cells have been detected in ischemic tissue as proliferative clusters outside existing blood vessels [88]. These clusters eventually form cords aligned in the direction of hypoxic gradients and have been co-stained with von Willebrand factor, believed to confirm their endothelial phenotype. However, these results have been questioned and many believe that putative EPCs instead serve a largely paracrine function that enhances the local angiogenic process. Regardless, ischemic tissue recruits EPCs and other BM-derived progenitor cells via SDF-1, promoting neovascularization and enhancing tissue regeneration [89]. Restoration of normal tissue oxygenation levels eventually decreases SDF-1 to baseline levels.

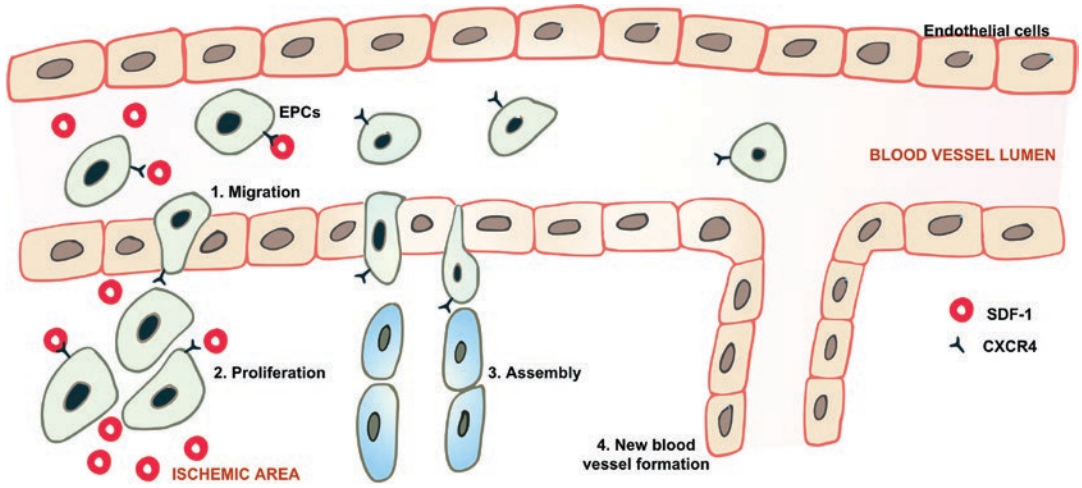

Fig. 6.3 Vasculogenesis: endothelial progenitor cells (EPCs) home to the area of ischemic tissue by SDF-1 gradients, proliferate, and assemble to form new blood vessels

6.6 Disease States

6.6.1 Underexpression

Impaired HIF/SDF-1function, present in certain disease states, limits the capacity for neovascularization and tissue repair. In the setting of aging, HIF-1, and subsequently SDF-1, expression is reduced during wound healing [PMID: 19182665]. Aged mice also have reduced numbers of CXCR4-expressing mesenchymal stem cells (MSCs) and stromal cells within their bone marrow and fewer CXCR transcripts within those cells that are CXCR4+. HSCs from young mice transplanted into the SDF-1 deficient bone marrow of aged mice demonstrated higher cycling, reduced engraftment, and myeloid biased differentiation, all of which are features of aging, suggesting the effects of aging are intrinsic to both the HSCs and their supportive stromal cells. Interestingly, aged stroma demonstrated increased reactive oxygen species (ROS), and treatment with N-acetyl-cysteine (NAC) for 1 week improved SDF-1 expression and niche supporting activity in terms of proliferative potential and attenuated the HSC aging phenotype [90].

Diabetes impairs the synthesis and stabilization of HIF-1α, hastens its degradation, and hyperglycemia-induced increases in ROS prevent HIF-1α from binding to the SDF-1 promoter [91–93]. In diabetic mice, wound HIF-1α protein levels are reduced [93], and myocardial cell HIF-1α activity is reduced, which is associated with an increased myocardial infarction size after an ischemic insult [94]. Hyperglycemia impairs HIF-1α activity in hypoxic aortic smooth muscle cells, which accelerates smooth muscle cell proliferation and atherosclerotic disease progression [95]. Human dermal fibroblasts (HDFs) and dermal microvascular endothelial cells (d-HMVECs) biopsied from diabetic ulcers have decreased HIF-1α compared to the same cells biopsied from nondiabetic venous ulcers [96]. Reduced HIF-1α leads to reduced expression of SDF-1 and impaired neovascularization. Reduced SDF-1 levels in diabetic cutaneous wounds have been associated with impaired progenitor cell recruitment [97, 98], further impacting tissue repair. Diabetic mice also demonstrate fewer circulating CXCR4+ cells circulating in the cerebral circulation following middle cerebral artery (MCA) occlusion [99].

6.6.2 Overexpression

Hypoxia-driven SDF-1 expression also appears to mediate both tumor progression and metastasis [14] and the recruitment of progenitor cells by tumor vasculature [100]. Tumors are thought to

depend on a small population of cancer stem cells (CSCs) for their continuous growth. CSCs possess tumor initiation and self-renewal capacity and can give rise to bulk populations of non-tumorigenic cancer cell progeny through differentiation [101]. SDF-1 signaling acts in CSCs through both the CXCR4 [102] and CXCR7 axes [102]. CSCs, like NSPCs, are responsive to an SDF-1 gradient [103]. Thus, the SDF-1–CXCR4 axis mediates numerous other neoplastic processes which enhance and facilitate cancer survival. CXCR4+ tumor cells are guided towards organs with high levels of SDF-1 expression, including the lymph nodes, lungs, liver, or bones. Indeed, a number of CXCR4+ cancers, such as breast, ovarian, and prostate cancer, rhabdomyosarcoma and neuroblastoma, have been shown to metastasize through the blood to the bones and lymph nodes in an SDF-1-dependent manner [11, 104]. Additionally, tumors continually outgrow their blood supply, creating a hypoxic microenvironment, which in turn is associated with chronically elevated SDF-1. This seemingly constitutive expression of SDF-1 recruits EPCs and BM-derived CXCR4 responsive stromal cells, promoting neovascularization [105], and differentiation of recruited fibroblasts into tumor-associated myofibroblasts [106]. Together these cells provide a supportive stromal environment that promotes tumor growth.

SDF-1 signaling may also facilitate cancer survival via other mechanisms, dependent on the cancer subtype. For example, the interaction between SDF-1 and CXCR4 plays a key role in retaining acute lymphoid leukemia and acute myeloid leukemia (AML) cells in the BM and protecting these cancer cells from apoptosis [107, 108]. In AML cells, the SDF-1–CXCR4 axis mediates VLA-4–VCAM-1 interactions, which promotes their survival and drug resistance [109, 110].

6.7 Future/Therapy

As our understanding of SDF-1 signaling across various biological processes has increased, so have the opportunities to therapeutically manipulate this pathway [111]. We discuss a few examples of therapeutic strategies manipulating different aspects of this pathway.

For successful BM transplantation host HSCs must be mobilized into the peripheral blood to facilitate extraction. Disrupting the SDF-1/CXCR4 binding by blocking the CXCR4 receptor with ADM3100 [51], by MMP-mediated enzymatic cleavage using GCSF [54], or blocking SDF-1 using diprotin [112] successfully mobilizes HSCs into the peripheral circulation.

Numerous approaches have been attempted to augment the deficient SDF-1/CXCR4 signaling in diabetes. Sitagliptin inhibits dipeptidylpeptidase-IV (DPP-IV), the enzyme which usually catabolizes SDF-1, and results in increased SDF-1 signaling. In a non-randomized clinical trial of type 2 diabetes Sitagliptin was able to mobilize progenitor cells [113]. Direct administration of SDF-1 into diabetic wounds can also promote homing of circulating progenitors and enhance wound healing [98]. HIF-1α and SDF-1 have been applied to diabetic wounds using plasmid-based and viral methods for delivery, and both were successful in improving neovascularization and accelerating wound healing [1–3, 114, 115].

BM fibroblasts and adipose derived stromal cells (ASCs) made to overexpress SDF-1 in cell-based therapies promote wound healing [116, 117] and improve survival of ischemic skin flaps on diabetic mice [118]. Autologous BM progenitor cells from diabetic mice treated with SDF-1 promote diabetic murine wound healing and neovascularization [119], and this approach circumvents the difficulties of using allogeneic cells.

In cancer therapy, in contrast, the aim is to block or inhibit SDF-1/CXCR4 signaling to inhibit growth and metastasis of tumors. Small molecule CXCR4 antagonists (e.g., T140) have been employed to inhibit growth and metastasis of experimental tumors in animal models [120, 121]. Chetomin has been identified as a small molecule that inhibits the transcriptional co-activation of HIF-1a. Systemic administration of chetomin-inhibited hypoxia inducible transcription of HIF-1a regulated genes within tumors and inhibited tumor growth in mice [122]. The emergence of more targeted and efficient approaches to modulating SDF-1 signaling will

permit the augmentation of endogenous pathways of tissue repair and regeneration while preventing them from being hijacked by neoplastic processes.

6.8 Conclusions

SDF-1 critically regulates chemotaxis and plays a pivotal role in the response to ischemic insult. SDF-1 expression by injured tissue is mediated by hypoxia and HIF-1 and stimulates mobilization and recruitment of circulating progenitor cells. Through a similar mechanism, stem cells selectively home to the bone marrow compartment after intravenous infusion. Reduced expression of SDF-1 has been linked with impaired tissue repair in the setting of disease, while overexpression has been linked with the unregulated tissue growth of cancer. The SDF-1/CXCR4 axis is also significantly associated with several diseases which have not been a focus of this chapter, including HIV, cancer, WHIM syndrome, rheumatoid arthritis, pulmonary fibrosis, and lupus.

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Stem Cell Differentiation Directed by Material and Mechanical Cues

7

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7.1 Introduction

Stem cells self-renew and have the capacity to differentiate into specialized cell types under certain physiologic or experimental conditions, making them critical for tissue regeneration. The breast is home to both adipose stem cells (ASCs) and mammary epithelial stem cells. These cells are responsible for maintaining the glandular and adipose networks of the breast, respectively. Adipose tissue is primarily responsible for breast volume [1]. The mammary gland is composed of multiple systems of branched ducts that connect functional glandular units called acini to the nipple, allowing milk to be produced and released from the breast [2].

Ideally, for regenerative medicine applications, adult stem cells should be abundantly available from harvesting through minimally invasive procedures. Furthermore, adult stem cells can differentiate into multiple cell lineages in a manner that is both reproducible and able to be regulated, safely and effectively transplanted, and manufactured in accordance with Good Manufacturing Practice guidelines [3]. Hence, these aspects must be considered when determining the utility of a particular stem cell in a tissue engineering application, especially for eventual translation into clinic.

Stem cell properties are regulated and maintained using various approaches that could modulate them to suit the application. The desired outcomes include, but are not limited to, genetic regulation, soluble factors, and interactions with the extracellular matrix (ECM). Recently, ECM has been found to contribute significantly to alterations in cell phenotype and behavior, providing cues to ensure specific structure, biochemical, and mechanical properties [4]. For instance, fiber alignment, pore size, matrix density, matrix composition, and material stiffness serve as environmental signals from ECM that are transduced into downstream gene expression and stem cell fate [5]. Although there are several critical design parameters that must be assessed in tissue engineering, it is of the utmost importance to consider mechanical and material properties of scaffolds because, as a substitute for native ECM, scaffolds are an important player in regulation of cell behavior. As a result, understanding the role of such cues on stem cell maintenance and differentiation has grown concomitantly with advances in three-dimensional (3D) culture systems and biomaterial scaffolds.

Due to the high global prevalence of breast cancer and increasing incidence of breast

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reconstruction procedures following mastectomy, there is a necessity to develop approaches to regenerate breast tissue de novo and restore healthy tissue appearance and function. Since the breast is home to both ASCs and mammary epithelial stem cells, this chapter will focus on the mechanical and material cues that control differentiation of stem cells in mammary tissues. We will also discuss current approaches in breast tissue engineering that aim to restore healthy tissue.

7.2 Technique

7.2.1 Breast Reconstruction

Mastectomy is commonly performed for women who have been diagnosed with breast cancer or who are at elevated risk of developing breast cancer, as in the case of individuals with the BRCA1 mutation. This procedure involves removal of all breast tissue. Following mastectomy, patients have the opportunity to undergo breast reconstruction surgery in which a plastic surgeon recreates a breast shape using an artificial implant, a flap of autologous tissue, or both simultaneously. Breast tissue must be properly reconstituted after mastectomy to recover the aesthetic and, if possible, some functional properties of the breast. Donor site morbidity, inadequate supply of donor tissue, patient comorbidities, and patient choice may lead surgeons to perform prosthetic reconstruction using acellular dermal matrices (ADMs) rather than performing an autologous reconstruction [6].

Human ADMs are widely used in conjunction with breast implants, with many advantages. ADMs are derived from full-thickness skin that has been physically or chemically treated to remove cells and cellular components (by repeated freezing and thawing, osmotic solution, enzyme digestion) and retain the native structure of the dermal fiber meshwork. It is mainly composed of collagen I, a structural protein with a stable triple helix structure that conceal amino acid differences from the host immune system, and once transplanted into the host, the ADM degrades over time and the triple helix collagen structure collapses [7]. Bacterial collagenases are used to further degrade the ADM [8]. Their availability and quality depend on the ability of a tissue bank to recover suitable dermal tissue, process and decontaminate the tissue, and release the tissue that meets appropriate sterility standards [9]. The process of decellularization is very important as studies have shown that extracellular components in cell-free dermal matrices or ADM are critical for success in biomedical applications as scaffolds.

ADMs are advantageous for this indication due to improved aesthetic outcomes, reduction in postoperative pain, decreased operative time, and improved structural strength and vascular ingrowth [6, 10–12]. Further, ADMs have been reported to provide better control of the mastectomy space, optimize implant positioning, allow for increased intraoperative expansion, and prevent migration of the implant [11]. Despite the many benefits, literature is accumulating in which ADMs are associated with increased incidence of postoperative complications, such as infection or seroma formation [6].

Past generations of implanted materials were designed with a focus on establishing a natural-looking breast. More recent generations utilize tissue engineering techniques in which biodegradable scaffolds containing appropriate cells and factors are implanted into the defect area to stimulate cells de novo tissue regeneration (Fig. 7.1). Alternative methods are being investigated to regenerate breast tissue while addressing the limitations of currently utilized techniques. Tissue engineering scaffolds that successfully integrate with host tissue, support growth, and biodegrade in a controlled manner to be replaced by new tissue, all while achieving ease of use and low price-points, directly address these limitations. Advancements in our understanding of the breast and the influence of materials on cell behavior have enabled for significant improvements toward regenerative constructs. Fabrication and optimization of such scaffolds would drastically improve standard of care for breast reconstruction patients.

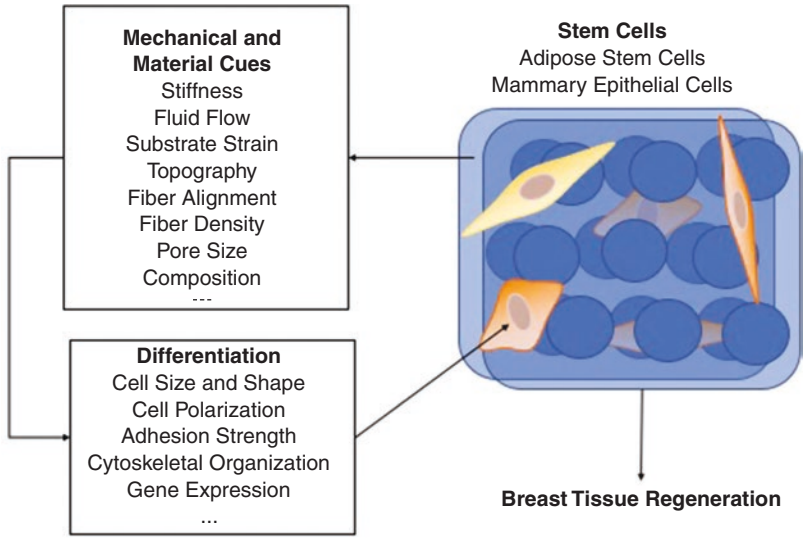

Fig. 7.1 Summary of breast tissue engineering approaches. Relevant types of stem cells are seeded on a scaffold. The mechanical and material properties of the scaffold provide signals to the stem cells that contribute to changes in cell size, shape, polarization, adhesion strength, cytoskeletal organization and stiffness, and other characteristics. These changes trigger downstream gene expression changes that lead stem cells to differentiate into various progenitor cells in order to regenerate the breast tissue

7.2.2 Mechanical Cues

Stiffness, or elasticity, is the key mechanical factor that dictates stem cell behavior. Cells can "feel" the softness of a material based on the distribution of focal contacts and their ability to pull against the ECM, triggering cellular mechanotransducers to produce downstream signals based on the magnitude of force needed to deform the matrix [13]. Therefore, mechanical cues imparted on stem cells cannot be ignored by tissue engineering and regenerative medicine.

Depending on the niche, the mechanical properties of the tissue can vary widely. For instance, brain tissue stiffness is about 0.1 kPa, whereas that of calcified bone is greater than 30 kPa [14]. Natural variations in ECM stiffness manifest during development to guide stem cell migration and differentiation into various tissues [15–17]. The concept that cells migrate preferentially onto stiffer surfaces, otherwise known as durotaxis, is a fundamental process during embryonic morphogenesis [18]. Moreover, mesenchymal stem cells (MSCs) will either differentiate into bone or fat when exposed to a stiff or compliant matrix, respectively, mimicking the mechanical properties of the natural tissue [19, 20]. Particularly, in terms of breast tissue, mammary cells within compliant matrices demonstrate growth control, organization of glandular architecture, expression of proteins consistent with more "differentiated" phenotype, and support cell polarization [21, 22]. In contrast, mammary cells on stiff matrices are more proliferative and experience increased proliferative signals, characteristics that are not exhibited in normal mammary tissue [23–26].

Mechanical regulation within stem cells has been proposed to occur through three avenues:

1. Force-sensitive protein conformational changes in focal adhesions or in matrix.
2. Changes in Rho activity.
3. Stretch-activated calcium channels [5, 18].

These regulatory pathways are susceptible to dysfunction if the microenvironment becomes abnormally rigid, as is frequently seen in malignant tissues. With rigid ECM, cell-generated forces are dissipated within the cells themselves, likely altering the conformation of proteins that connect cytoskeleton and ECM [4, 27]. It has been shown that stiff ECM induces differentiation

of mesenchymal stem cells (MSCs) into cancer-associated fibroblasts (CAFs), supporting carcinoma progression [28].

Other important mechanical cues include fluid flow and substrate strain. These external mechanical forces stimulate stem cell differentiation through enhancement of adhesion strength, cytoskeletal stiffness and organization, and mechanotransductive signals [29, 30].

7.2.3 Material Cues

ECM and 3D scaffolds also provide structural and biochemical cues to stem cells, such as topography, fiber alignment, density, pore size, and component composition [31, 32]. These aspects communicate more intimate details about the niche to the stem cells. Although pore size provides a direct physical constraint on cell size and shape, which is known to determine intracellular downstream signaling, ECM topography relays important biophysical signals critical to stem cell differentiation [20, 33, 34]. Instead of directly affecting cytoskeletal tension, such topographical cues appear to directly modulate the molecular arrangement, dynamic organization, and signaling of alpha- and beta-integrins [5, 14, 18]. Upon binding of stem cells to ECM, integrins cluster to form dynamic adhesion structures called focal adhesions (FAs) [35]. On the cytoplasmic side of FAs, cytoplasmic tails of integrins can interact with different adaptor and signaling proteins that provide direct physical linkage to the actin cytoskeleton [5, 35]. Also, ECM-integrin binding can activate tyrosine kinase and phosphatase signaling which elicits downstream biochemical signals important to gene expression and stem cell fate regulation [36].

Glandular breast tissue is revered for its ability to involute and regenerate, regulating milk production based on hormonal control. Since a functional unit of the mammary gland is an epithelial cell and adjacent ECM, it is logical that responsiveness of mammary epithelial cells to hormones is facilitated by concomitant modification of the ECM [37, 38]. Hence, ECM tensile requirements change in order to accommodate the distinct demands required for different stages of breast tissue regeneration. This is exemplified by the fact that fibronectin (FN) and a5b1-integrin, two highly prevalent mammary tissue components, are under endocrine control [39, 40]. FN is responsible for modifying the mechanics and structure of collagen fibers; the more FN in the ECM, the higher the fraction of linear collagen fibers relative to cross-linked collagen, resulting in decreased tissue elasticity [32, 41] Additionally, a5b1-integrin binding is required for assembly of secreted FN into fibrils which is thought to be a mechanism for precise temporal-spatial integration between FN assembly, local tissue tension, and specific cell or tissue requirements [42, 43].

7.3 Discussion

7.3.1 Tissue Engineering Constructs for Breast Reconstruction

7.3.1.1 Mammary Adipose Tissue

When fat grafting is performed, the lipoaspirate incorporates terminally differentiated mature adipocytes and stromal vascular fraction (SVF), which includes preadipocytes and multipotent adipose-derived stem cells (ADSCs) [44]. The proliferation and differentiation of SVF-derived cells is key for graft survival. ADSCs and preadipocytes cooperate to encourage angiogenesis and adipogenesis through growth factor release and differentiation into mature adipose cells [45]. Major limitations of fat grafting are resorption, volume loss, and necrosis that may lead to long-term inflammation and progressive calcification [46, 47]. To address such undesirable outcomes, experimental work using laminin-alginate beads as carriers of preadipocytes has proven effective in vitro and in vivo [48].

Engineered adipose tissue approaches generally utilize natural and synthetic polymer constructs. Synthetic scaffolds include those fabricated with PLA, PGA, PLGA, PET, PTFE, and PEGDA scaffolds [49]. Natural polymers utilized mainly consist of collagen, hyaluronic acid, natural ECM, and Matrigel [50]. Synthetic and natural hydrogel biomaterials, in particular, are well suited for this

application because the polymers are porous and deform easily, mimicking the properties of the native ECM [51]. Conversely, rather than replicating the physical structure of the native tissue, solid scaffolds aim to guide the regeneration process by designing a scaffold architecture to guide tissue formation [52, 53]. A critical obstacle in design of solid biomaterials is their potential for interference with diagnostic imaging for early stage breast cancer detection.

7.3.1.2 Mammary Epithelial Tissue

Tissue-engineered mammary epithelium has been developed to meet the increasing clinical need for breast tissue regeneration that is not achieved through addressing adipose tissue loss alone. Currently, they primarily serve as useful models of mammary gland development, regeneration, and tumorigenesis, enabling better understanding of healthy and unhealthy breast epithelial tissue [2, 54–57].

Several studies have successfully modeled acinar and ductal structures in vitro. A 3D culture system was fabricated in which hormone action on human breast epithelium can be suitably studied [57]. This model is responsive to major mammotropic hormones and the influence of those hormones on epithelial morphogenesis can be observed in vitro. Similarly, specialized 3D hydrogels can be fabricated by incorporating ECM proteins with relevant growth factors to grow primary breast cells [58]. Such scaffolds can recapitulate the endogenous morphology and development, allowing for creation of a life-like in vitro system with which to study the mammary gland. Further, the advent of 3D bioprinting technology allows for high precision control of cellular and structural deposition when creating tissue-engineered solutions [59]. This approach has been recently utilized for the reconstruction of the nipple-areola complex [60].

7.4 Conclusions

Substantial evidence has accumulated regarding the response of stem cells to mechanical and material cues in both healthy and pathological microenvironments. Similarly, cancer stem cells, which share many properties of healthy stem cells, are implicated in cancer recurrence and are sustained by cues from the microenvironment. Therefore, it is critical to evaluate the degree to which tissue-engineered constructs for breast reconstruction may potentially cultivate cancer resurgence. This increases the importance of deliberately designing scaffolds that support regeneration while deterring cancer regrowth.

Tissue engineering approaches to breast reconstruction offer promising alternatives to current techniques, addressing limitations that impact patient outcomes. Future directions focusing on mechanical and vascular support, regeneration-inducing factors, dynamic composite biomaterial scaffolds, and high-precision fabrication techniques will offer improved control over the mechanical and material parameters of scaffolds. With these improvements, scaffolds can relay more appropriate signals to the stem and progenitor cells within, leading to more regulated and reproducible tissue regeneration. Advancements will positively change the landscape of the field of breast reconstruction and, importantly, will drastically enhance patient quality of life.

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Bacteriophages: A New (Yet Old) Weapon Against Infections

8

Stephen K. Mathew and Reba Kanungo

8.1 Introduction

The alarming spread of antimicrobial resistance, identified by the WHO as a global threat, is drawing healthcare into the post-antibiotic era [1, 2]. Healthcare-associated infections (HAIs) are among the top five leading causes of morbidity and mortality in industrialized countries [3]. Infections by extensively drug-resistant bacteria are being increasingly reported: just one, methicillin-resistant *Staphylococcus aureus* (MRSA), kills more Americans every year than emphysema, HIV/AIDS, Parkinson's disease and homicide combined [4, 5].

Bacteria are extremely adept at developing mechanisms to survive hostile environments. This is underscored by the isolation of *Escherichia coli*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* strains resistant to even silver salts present in antibacterial preparations [6].

Development of new antibiotics has been hampered by rising costs of drug development coupled with relatively low returns of investment due to the rapid development of resistance to the new agent [7, 8]. In the face of ever-increasing resistance, this dearth of research and development has been called "the perfect storm" [9]. With only a few large multinational pharmaceutical companies involved in antibiotic discovery, the Infectious Diseases Society of America (IDSA) launched the "10 × '20 Initiative" with the aim of supporting the development of ten new systemic antibiotics by 2020, which was successful in identifying seven novel agents targeting Gram-negative bacilli [10, 11]. However, resistance against agents such as ceftolozane-tazobactam has already been observed [10, 12].

Surgical site infection (SSI) currently ranks as the most common cause of nosocomial infection, accounting for 31% of all hospital-acquired infections, and is associated with a mortality rate of 3% [13–16]. The additional cost of managing an SSI exceeds $20,000 per admission, and more than $90,000 per infection where an antimicrobial-resistant organism is responsible [14, 17]. The economic burden of antibiotic-resistant infections to the US healthcare system is estimated to be more than $20 billion each year [5].

Postoperative infection, though rare following plastic surgery, can significantly affect the cosmetic outcome, which also increases the risk of malpractice suits [13, 18]. It complicated approximately 1% of clean surgeries and 4% of clean contaminated surgeries [19]. As cosmetic surgery becomes increasingly popular, SSIs, particularly

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those caused by MDR bacterial strains and are difficult to treat, become a pressing issue [18].

The "perfect storm" threatening to derail much of the progress in medicine could also be an opportunity for alternate antimicrobial modalities to emerge [20]. One of these modalities currently generating significant interest was discovered more than a century ago, in the pre-antibiotic era. Felix d'Herelle discovered bacteriophages and also realized their potential as antibacterial agents, following which it was used to contain human infections in several countries. The new world of antibiotics that was soon discovered, and the promise it brought—the end of infectious diseases—however, dwarfed much of the interest in phage therapy. Acceptance of bacteriophages as a therapeutic modality was further hampered by poorly designed studies generating conflicting results. Bacteriophage preparations available in the early twentieth century, apart from being of questionable quality, were also being marketed for pathologies not necessarily caused by bacterial infections [20]. While phage research died out in the western world, it remained an active area of research and use in parts of Eastern Europe and former Soviet Union.

Following the rediscovery of phages by the western world, the first randomized controlled trial (RCT) which was published in 2009 saw researchers treat chronic otitis and venous leg with bacteriophage-based preparations [21–23].

8.2 Current Status of Skin Infections in Plastic Surgery

Between 2006 and 2009, a conservative estimate of 1.9% of surgical procedures in the United States was complicated by surgical site infections (SSIs) [17]. In 2006, the Surgical Care Improvement Project (SCIP) drafted nine measures to reduce surgical complications; of these, six focussed on prevention of postoperative infections [24]. However, a decade later, despite a high level of compliance with the core measures, infection rates remain largely unaffected and have only been further complicated by resistance to commonly used antimicrobial agents. SSIs now reportedly complicate over a tenth of inpatient and outpatient procedures [25–27].

The incidence of wound infections following breast plastic surgery, considered a "clean surgery", ranges from 3% to 30%, and is more than 50% among women undergoing reconstruction after treatment for breast cancer [13, 28]. Wound care is particularly problematic in burn patients, in whom 50% of all deaths are due to resultant infections [29, 30].

A rise in infective complications has been accompanied by a dramatic increase in the use of antibiotics. In plastic surgery alone, there has been up to a 200% increase since 1975 [31]. Prophylactic antibiotics are widely used even in procedures, such as rhinoplasties, that are rarely complicated by postoperative infections [32]. The overuse of antibiotics, due to lack of consensus, specific guidelines and a fear of litigation, has further contributed to antimicrobial resistance, and could paradoxically make empirical prophylactic antibiotics ineffective [32, 33].

8.3 History of Bacteriophage Therapy

While anecdotes of river waters possessing the ability to cure infectious diseases can be found in historical and religious texts, the idea of bacteriophages and their action as an antibacterial can be traced back to 1896, when British bacteriologist Ernest Hankin suggested the presence of an unidentified, heat-labile, filterable substance in the rivers Ganga and Yamuna in India with antibacterial activity against *Vibrio cholerae* which possibly helped to limit the spread of cholera [34].

Frederick Twort [34], a bacteriologist from England, reported a similar phenomenon almost 20 years later and advanced the possibility of this being due to a virus. Twort, however, did not pursue this finding and it was another 2 years before Felix d'Herelle, a microbiologist at the Institut Pasteur in Paris, France, officially discovered bacteriophages. He observed the bacteriophage phenomenon in 1910, in Mexico, while studying methods of controlling an epi-

zootic among locusts. D'Herelle, who a few years later was called to investigate an outbreak of severe dysentery among French troops, stationed on the outskirts of Paris, observed the appearance of small, clear areas on agar cultures when Shigella strains isolated from the patients were incubated with bacterium-free filtrates from the faecal samples. He termed these clear areas as "plaques", and proposed the name "bacteriophage" for a "virus parasitic on bacteria" [34]. Not long after, d'Herelle carried out what could be labelled a phase I trial when he along with his family members ingested phage preparations to demonstrate their safety before administering it to children with dysentery at the Hopital Des Enfants-Malades, Paris, all of whom exhibited signs of recovery [5]. However, the results of these studies were not immediately published and, therefore, the first reported use of phages to treat infectious diseases in humans came from Bruynoghe and Maisin in 1921, who used bacteriophages to treat staphylococcal skin disease [34, 35].

D'Herelle continued his studies on phages and some of his most sensational work was carried out in India, where he visited in 1927. There were reportedly no deaths among cholera patients in Calcutta and Lahore who received d'Herelle's phage preparations orally and intravenously, in contrast to a mortality rate of 40% among patients who received conventional injections of fluids and salts [36, 37].

Contrasting these successes, several scientists highlighted d'Herelle's failure to meet scientific standards for research. Combined with the introduction of penicillin to medical practice, this led to dwindling interest in d'Herelle's research [38].

Commercial phage preparations began with d'Herelle as well, whose laboratory produced at least five phage preparations: Bacté-coli-phage, Bacté-rhino-phage, Bacté-intesti-phage, Bacté-pyo-phage, Bacté-staphy-phage—marketed by Societé Francaise de Teintures Inoffensives pour Cheveux (now, L'Oreal). Therapeutic phage preparations began to be available in the United States since the 1930s, with companies such as Eli Lilly and Abbott Laboratories taking an interest. Commercial production, however, was plagued with quality control issues: d'Herelle also reported that some preparations being marketed contained no detectable biologically active phage [37]. Though commercial production in the Western world declined with the advent of antibiotics, phage preparations were available in France till 1978 at d'Herelle's company, and at the Institut Pasteur till the 1990s [5, 37]. Phages continued to be used therapeutically in Eastern Europe and the former Soviet Union, centred around the Eliava Institute of Bacteriophages, Microbiology and Virology in Tbilisi, Georgia, and the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw, Poland [34]. The former was focussed on phage cocktail formulation and production (the Eliava Institute had a production capacity of up to two tons per week), and the work at the latter has been extensively documented [37].

8.4 What Is a Bacteriophage?

Bacteriophages are essentially viruses; as obligate parasites, they infect, replicate within and finally lyse the bacterium [20]. Over 6000 different bacteriophages have been discovered, which have been classified into 13 families depending on morphology, type of nucleic acid, and presence or absence of an envelope. About 96% of the discovered phages are "tailed", possessing an icosahedral head and a double-stranded DNA genome. Tailed phages, which comprise the order Caudovirales, are classified into 3 families based on the morphological features of the tail: Myoviridae (contractile tail), Siphoviridae (long, non-contractile tail) and Podoviridae (extremely short tail). The remaining 4% of the phages, classified into 10 families, may contain single-stranded or double-stranded RNA or DNA. These phages are cubic, filamentous or pleomorphic. Most therapeutic phages are tailed; some cubic and filamentous phages have also been used for therapy [21, 39].

Bacteriophages attach to receptors on the bacterial surface via tail fibres or base plate spikes, following which they inject their genome into the cell [40]. The nature of the receptor, its chemical

composition and spatial configuration, along with the structure of viral-receptor binding proteins play a major role in stabilizing the bacterial cell-bacteriophage interaction [41]. These receptors might be the same antigens determining the serotype of the bacteria, or transport channel proteins, or pili [7]. Importantly, receptor binding confers specificity on the bacteriophages. Termed the host range, this specificity is typically narrow—limited to a single bacterial species, or to a few strains within a species, or even a single strain.

Phages can also be divided roughly into two groups, according to their life cycle: lytic or lysogenic. In the lytic cycle, the bacterial cell machinery is hijacked to assemble and package progeny phages, which are released following death of the host cell and its rapid lysis with the help of holins and lysins [40]. Phages with a large burst size—the number of progeny phages released from each infected bacterial cell—are preferred for use in therapeutics [7]. Temperate phages undergo lysogeny, where the phage genome integrates with the bacterial genome and are transmitted vertically through successive generations of the bacteria. The genome of temperate phages may encode transmissible bacterial virulence factors, as seen with Corynebacterium diphtheriae where only isolates that harbour tox^+ phages produce diphtheria toxin [42]. At the same time, host genes for virulence and toxin production may be packaged into the bacteriophages during replication, which may in turn be transferred to other bacteria. As a result, temperate phages are thought to be less suitable than lytic phages for use in therapeutic preparations. However, it may be possible to inactivate genes responsible for lysogenicity and toxin production by genetic modifications, overcoming a disadvantage of lysogenic phages [39].

8.5 Why Should We Consider Bacteriophage Therapy?

Bacteriophages are a potent, natural antibacterial capable of inducing rapid bacterial cell lysis [21]. They are also ubiquitous, with up to 1×10^8 particles per gram of soil, and can be readily isolated from various environments. It is estimated that they destroy one-half of the bacterial population worldwide every 48 h [40]. Billions of years of this co-evolutionary predator-prey relationship have made bacteriophages a potentially rich source of antibacterial agents [29, 40].

Strain specificity, briefly mentioned earlier, allows for targeted therapy, limiting the deleterious effect on the normal microbial flora. This can help prevent adverse effects associated with antibiotic use, such as *Clostridium difficile* colitis, a leading cause of nosocomial diarrhoea particularly associated with the use of cephalosporins and clindamycin [33]. Bacteriophages also have little or no effect on eukaryotic cells, thus staving off more of the adverse effects associated with antibiotic use [21, 29]. Application in the nose and sinuses in an animal model did not alter the normal architecture of the mucosa [43]. Oral administration in patients with diarrhoea did not lead to adverse events [44, 45].

An added advantage, in contrast to antibiotics, is that the concentration of bacteriophages increases after reaching the site of infection due to self-replication [46]. As a result, the required dose of phages would generally be much less than that of antibiotics [47]. Economic considerations also favour bacteriophage therapy over conventional antibiotics, as the cost and complexity of developing a phage system is less than that of developing a new antibiotic [8]. While it is unlikely that bacteriophages will replace antibiotics, phage therapy could decrease antibiotic resistance by reducing the need for antibiotics [29]. Phages can also find use in situations where the necessary antibiotics are contraindicated, such as nephrotoxic antibiotics in patients with impaired renal function [37].

8.6 What Is the Evidence that Bacteriophages Work?

While bacteriophages as a therapeutic option failed to take off in the United States and Western Europe particularly following the discovery of antibiotics, clinical research with bacteriophages

continued in the former Soviet Union and Eastern Europe. These studies were published primarily in non-English languages, and as a result, not readily available to the global scientific community [34]. Interest in phages in the Western world was partly rekindled by the work of Smith et al. who demonstrated the effectiveness of a single intramuscular dose of phage in potentially lethal infections in animals by *Escherichia coli*. This was in contrast to the need for multiple doses of antibiotics such as tetracycline, ampicillin and chloramphenicol to control the infection. The emergence of phage-resistant strains of *E. coli* over the course of the experiments was however noted [48, 49].

Numerous experiments studying various infection models (bacteremia, central nervous system infection, sinus infection, lung infection, urinary tract infection, osteomyelitis, skin and wound infection, including burns) caused by bacterial pathogens such as *Pseudomonas aeruginosa*, *E. coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* have since been conducted. Bacteriophage therapy decreased mortality in several studies. Where it was compared to antibiotics as controls, a more positive outcome was observed. No adverse effects were observed in mice following inoculation with high doses [21, 29, 30, 39, 43]. Phage treatment was shown to improve survival in mice infected with methicillin-resistant *Staphylococcus aureus* (MRSA), and lethal doses of *Vibrio vulnificus* [29, 50, 51]. Bacteriophages have also been shown to be effective against *Yersinia pestis*, responsible for plague, and against Burkholderia pseudomallei, a Category B bioterrorism agent that causes melioidosis [52, 53].

There is currently a lack of consensus on the most effective route of administration to target specific infections. Where some studies suggest that aerosolized formulations of phages are effective against respiratory infections, others have found systemic administrations to yield better access to bacteria in the lungs [54, 55]. This is also an important consideration in severe skin and soft tissue infections with a propensity to progress to septicaemia. Phages administered systemically offered better protection than when administered locally in a mouse burn wound model [30]. Orally delivered phages were effective against gastrointestinal infections caused by *E. coli* and *Campylobacter jejuni*, but concerns of phage inactivation due to gastric acidity need to be addressed [54].

Bacteriophages can also help to tackle biofilms which prove to be a significant challenge to conventional treatment. Biofilms are commonly associated with chronic, refractory infections, due to indwelling medical devices, and can be a thousand times more resistant to antibiotics than free-floating bacteria [29]. Treatment of silicone catheters with bacteriophage significantly reduced biofilm formation by *Staphylococcus epidermidis*, *P. aeruginosa*, *E. coli* and *Proteus mirabilis* [54, 56]. Bacteriophages have several advantages over antibiotics in treating infections caused by biofilms. Replication at the site of infection allows for a high concentration of phages on the biofilm; they are able to infect dormant cells within the biofilm; and phages may possess or induce the bacterial cells to express enzymes capable of dissolving the biofilm matrix [20]. Phage treatment has been shown to significantly reduce biofilm biomass and cell density in experimental models [29].

Lysin, a phage genome-encoded protein expressed by tailed phages, which enables liberation of progeny phages from infected bacteria, is also a candidate therapeutic agent against Gram-positive bacteria because of its ability to destroy peptidoglycan, a vital component of the cell wall [39].

Bacteriophages have complex pharmacokinetics that are yet to be fully elucidated. Most researchers observed that phages afforded best protection when given within a few hours of bacterial inoculation, but computerized models have predicted that inoculations given too early could either be less effective or fail completely [51, 57, 58]. Paradoxically, some antibiotics can even diminish the effectiveness of phages [58]. Available data from animal experiments suggests that phages enter the bloodstream following a single oral dose within 2–4 h and are found in the internal organs within 10 h. Phages were

preferentially compartmentalized to the liver and spleen, irrespective of the route of administration [29]. In the human body, administered phages can remain for up to several days [5]. A better, if not complete, understanding of the behaviour of bacteriophages in vivo is necessary to achieve consistent and predictable results with bacteriophage therapy.

8.7 Concerns with Bacteriophage Therapy

Since phages capable of infecting across bacterial species or genera are few in number, rapid and precise identification of the pathogenic bacteria is necessary in order to select an appropriate bacteriophage from an established phage library [39]. Such an individualized approach has been by and large successfully followed in Poland [37]. Use of phage cocktails targeting commonly encountered bacterial species and strains can potentially tackle this shortcoming. However, these cocktails would have to be re-formulated regularly taking into account prevalent species and strains [21]. Even when mixed to form cocktails, the host range can remain relatively narrow [37]. This also limits the role of bacteriophages in empirical therapy [7]. Experiments have extended the host range of phages through genetic modifications that allow them to overcome barriers to adsorption and infection [59]. There is also much to be understood of the interaction between phages and the target bacterial hosts at the site of infection, as opposed to under laboratory conditions [60].

Phages administered intravenously can activate the immune system. Subsequently, phage titres may fall due to innate immunity and phagocytosis in the blood and liver. While non-neutralizing antibodies have been observed following certain phage injections, clinical and animal trials have not demonstrated serious immunological reactions. Long-term intrasinus application of phages did not alter the local profile of immune cells in an animal model [43]. The immunological response against every phage considered for parenteral therapy, however, would need to be studied [7]. The large size of the phage particles, when compared to antibiotic molecules, also limits the concentrations that can be achieved in therapeutic preparations—solutions may become viscous at high concentrations, more than 10^{13} phages per mL [7]. Models created to calculate dosage requirements would need to take into account the complex pharmacokinetics of bacteriophages [47].

Some bacteriophages, though mostly temperate phages, enhance virulence by transferring genetic elements vital to the bacteria. The ability to produce exotoxin in *Vibrio cholerae* is carried and transferred by phages, as is Shiga toxin production in *E. coli*, as well as virulence determinants in *P. aeruginosa*, *Shigellae* and *S. aureus* [20, 61]. This potential problem with therapeutic bacteriophages may be overcome by selection of phages incapable of such transfer, or by genetically modifying them. The genome sequence of therapeutic phages needs to be characterized, which would also help to confirm the absence of undesirable genetic elements. The safety and efficacy of phages should also be demonstrated [20, 39, 40, 46].

A possible side effect of phage therapy, also seen with bactericidal antibiotics, is the release of cell wall components which are mediators of septicaemia, such as endotoxins from Gram-negative bacteria [62]. Patients receiving phages have occasionally experienced right hypochondrial pain and fever a few days into treatment, possibly due to the release of endotoxins [63]. Genetically modified, non-replicating phages designed to digest bacterial genomic DNA kill bacteria with minimal release of endotoxin. The survival rate of mice infected with *P. aeruginosa* was significantly higher with non-replicating phages that do not cause endotoxin release, than with lytic phages; this was also correlated with lower levels of inflammation [62].

As with antibiotics, the development of resistance by the bacterial targets could blunt the efficacy of phages. Resistance to phages is often due to changes, as a result of mutations or acquisition of genes, in the receptors on the bacterial surface [30]. However, phages rapidly co-evolve with bacteria and bacteriophages capable of overcoming protective bacterial systems have

been isolated [7, 39]. Phage cocktails effective against various bacterial strains and possible mutants arising during therapy could pre-empt the rise of resistance [54]. Bacteriophages can multiply within bacteria only if their density exceeds a threshold [47]. On the other hand, a higher-than-necessary concentration would lyse the target bacteria before secondary phage multiplication can be initiated, necessitating multiple doses to eradicate infection [47]. Dosage would also depend on duration of infection at initiation of therapy. Phage preparations administered up to 10 days after infection have been successful [54].

Therapeutic preparations will need to be stable and viable during transportation and storage [40]. Purified phages remained stable for up to 2 years when maintained at 4 °C [52, 53, 64]. Further research is required on phage delivery formulations, and on long-term stability of the phages within formulations [54].

The pharmaceutical industry has largely stayed away from phage therapy probably because it does not see large investments being profitable [60].The risk of mutations developing during the course of therapy also challenges large-scale production as it would require rigorous monitoring [46]. Institutes such as the Eliava Institute, Georgia, and Queen Astrid Military Hospital, Brussels, Belgium, have shown that small-scale production of bacteriophage cocktails, following strict quality-control protocols, is possible [64].

Current regulations requiring full clinical trials for each therapeutic bacteriophage make it difficult for bacteriophages to find their way to routine clinical use. While stringent legislation is necessary for any therapeutic product licensed for human use, factors unique to phages need to be taken into account. Regulatory authorities would need to discuss and consider whether phage therapy merits a distinct set of rules [7, 46].

8.8 Non-human Uses of Bacteriophages

Strain specificity has an already established use in the laboratory in typing systems used for identification of bacterial strains and newer diagnostic tests such as KeyPath (MicroPhage, Inc., Longmont, Colorado) to rapidly identify MRSA from blood cultures [65]. The Eliava Institute has been using phages to track enteric pathogens in the environment, and for rapid detection of anthrax and brucellosis [66].

Anti-Listeria phage cocktails were among the first phage products to obtain a Generally Recognized as Safe (GRAS) status from the United State Food and Drug Administration (FDA) [59]. ListShield™ and LISTEX™ P100 are marketed as food additives intended to disinfect processed poultry products and meat. Omnilytics, Inc. (US) specializes in supplying customized phage preparations (Omnilytics' Agriphage™) tailored against the prevalent crop pathogens for agricultural use [67]. *Staphylococcus aureus* phage lysate Staphage Lysate® has been shown to be effective in treating and controlling recurrent pyoderma in dogs [68].

8.9 Human Applications of Bacteriophages

Work with phages carried out in the 1930s report successful treatment of skin infections, surgical infections, typhoid fever, Salmonella and Shigella spp.-related colitis, septicaemia, and urinary tract infections [21, 69]. One of the largest studies, involving 30,769 children, on the effectiveness of phages against bacterial dysentery was conducted in Tbilisi, Georgia, in 1963–1964. Children living on one side of the streets were given anti-Shigella phages orally and those on the other side served as the controls. The incidence of dysentery was 2.6-fold higher in the control group [70].

Numerous reports of successful topical applications of phages, particularly from Eastern European countries, are available [54]. Oral administrations may be useful in fighting enteric infections due to *C. difficile* [40]. Nestlé Research Centre and other subsidiaries of Nestlé S.A. have conducted RCTs on patients, including children, suffering from diarrhoea [44, 45].

The first fully regulated, placebo-controlled, double-blinded, randomized Phase I/II trial on phage therapy was conducted in the UK in 2009

on patients suffering from chronic *P. aeruginosa-otitis*. A single local application of a cocktail of phages (Biophage-PA) resulted in decreased colony counts on culture, improved symptoms and clinical indicators, without adverse reactions [23].

Much of the published research on human application of phages is from case studies subject to experimenter's bias [37]. This can be traced back to d'Herelle's first known use of phages at the Hospital Des Enfants-Malades where phages were administered to all the sick patients without a placebo group [5]. In order to conclusively demonstrate the consistent efficacy of phages, more double-blind, randomized controlled trials, complying with regulatory and ethical guidelines, are required for greater acceptance of phage therapy [71].

8.9.1 Bacteriophage Therapy for Skin Infections

Topical application of bacteriophages is the most studied route of administration. Phages have been successfully used against ulcers, pyogenic infections, burns, and wounds [22, 37, 63, 72].

PhageBioDerm®, a commercially successful biodegradable wound dressing consisting of a stabilized hydrogel system impregnated with ciprofloxacin, benzocaine, chymotrypsin, bicarbonate, and 6 lytic phages against *S. aureus*, *Streptococcus spp*., *P. aeruginosa*, *E. coli*, and Proteus spp., was approved for human use in Georgia in 2000 [40, 54]. Studies have reported successful treatment of ulcers that failed to respond to conventional therapy [72]. Phase I trials on chronic venous leg ulcers and burn wounds have not reported any adverse events associated with bacteriophage use [22, 37].

Phages against *Propionibacterium acnes* involved in the pathogenesis of acne have displayed a broad ability to kill clinical isolates of *P. acnes* [73]. These phages, incorporated into an aqueous cream, retained their antibacterial activity up to 90 days when stored appropriately [74]. A number of trials evaluating phage therapy for burn wound infections, diabetic foot, and acne have been registered in the USA over the past few years [75–77].

8.10 Future Possibilities for Bacteriophage Therapy

Phage therapy can be an important component of personalized medicine, tailored against bacteria isolated from the site of infection [46]. This approach has been shown to be successful at some centres, but would require facilities for phage susceptibility testing [37]. Bacteriophages have been used to transfer gene cassettes that confer susceptibility to antibiotics, thereby reversing drug resistance in bacteria [21].

Phages bearing chloramphenicol on the surface have been shown to specifically target *S. aureus* in vitro [40]. Preparations of bacteriophage lysins could be effectively used in infections caused by Gram-positive bacteria: lysins against *Bacillus anthracis*, *Enterococcus spp.*, and *Streptococcus pneumoniae* have been identified [40]. Liquid-based phage skin disinfectants could be formulated to target difficult-to-treat nosocomial pathogens such as MRSA, *Pseudomonas aeruginosa* or *Acinetobacter baumannii*, without affecting the normal flora [78].

8.11 Conclusions

Bacteriophages have been shown to be potent antibacterial agents targeting most of the known human bacterial pathogens. Animal and human studies have so far not reported serious adverse effects. Local applications of phages have been effective in treating ulcers, wound and burn infections. Commercially successful wound dressings such as PhageBioDerm® have been in use for almost two decades. Similar topical formulations against local infections could be among the first to gain widespread use.

While using bacteriophages therapeutically appears promising, care must be taken to ensure that resistance does not develop. One of the ways that this may be done is to ensure that adequate concentrations of the phages are maintained at the site of infection during therapy. Therapeutic use must be preceded by rigorous clinical trials. Regulations, definitions and standards need to be established by internationally recognized organizations.

Though it is unlikely that phages will replace conventional antibiotics anytime in the near future, robust studies providing reliable and reproducible results will enable bacteriophage therapy to complement antibiotics. Pharmaceutical companies play a critical role in bringing phage therapy to patients suffering from infectious diseases.

While we move towards developing and adopting new weapons to fight infections, it is imperative that we avoid the mistakes that led to the development and spread of antimicrobial resistance.

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9 Bacteriophages as Biocontrol Agents of Biofilm Infections Associated with Abiotic Prosthetic Devices

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9.1 Introduction

Abiotic prosthetic devices or cellular, tissue, or organ substitutes which help restore biological functions in the face of total organ failure form an essential part of regenerative medicine [1]. A combination of advances in the fields of bionic engineering, material science, tissue engineering, nanoengineering, and electrical engineering has led to phenomenal developments in modern healthcare treatments for various ailments. Commonly used prosthetic devices include central venous and urinary tract catheters, artificial muscles in prosthetic limbs, orthopedic prostheses and joints, heart valves, endotracheal tubes, intrauterine devices, extended wear lenses, meshes, retinal and cochlear implants, etc. [2, 3]. A potential threat in the successful implantation of the abiotic prosthetic devices is the possibility of microbial infections. Infections at the site of device insertion, septic thrombophlebitis, septicemia, endocarditis, mesh erosion and tissue decay, perforation and encapsulation of intrauterine devices, metastatic abscesses and their translation into invasive infections are a cause for significant morbidity and complications that are difficult to diagnose and treat [2]. Comparative microscopic, microbiological, and biochemical studies show microbial growths in the form of biofilms associated with infections on abiotic prosthetic devices as they provide a suitable substrate for microbial adsorption and colony maturation [4, 5]. Microbial colonies adhering onto implants in the body secrete an outer covering made of exopolymeric substances (EPS), which protects its residents from the external stressors such as the immune response as well as antimicrobial drugs [6]. According to a study by the National Institute of Health, 65% of infectious disease is associated with biofilms and 80% of such are chronic infections. Such biofilm-mediated infections are difficult to diagnose and highly persistent in the face of antimicrobial treatments [7].

Biofilm-mediated persistent infections and their mechanisms of acquiring antibiotic and host defense resistance have been extensively researched [7–9]. Several biofilm control strategies have been devised in order to prevent the development of biofilms on the substratum provided by such devices [2, 10, 11]. A promising and unexplored strategy involves the use of biological control agents such as bacteriophages, which specifically target the pathogen while

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having no ill effects on the human eukaryotic cells [12–14]. Bacteriophages are intracellular parasitic lytic agents of bacterial hosts, typically nanometer in size, composed of a genome made of DNA or RNA ensconced within a proteinaceous coat (capsid) [15]. Use of bacteriophages (phages) in targeting in vivo infections has an ancient history which fell into disuse in major parts of the world during the era of antibiotics [16, 17]. However, with the alarming increase in antimicrobial resistance particularly amongst biofilm-associated infections, phage therapy is again being explored in earnest [18–20]. This article focuses on recent understanding of the role of bacteriophages as control measures for biofilm-related infections associated with abiotic prosthetic devices.

9.2 Biofilm Formation on Abiotic Prosthetic Devices

Microbial growths in the form of biofilms on abiotic prosthetic devices or implants have serious healthcare and economic consequences [2, 3]. The patients' normal flora or microorganisms from the surrounding environment are likely to be the sources of infection occurring either during peri- or postoperative stage. Many infections occur during hospitalization and are referred to as nosocomial or hospital-associated infections (HAI) [2]. Typically, insertion of abiotic device is likely to be present either for short duration, i.e., catheter tubes, or for years as in case of prosthetic limbs, heart valves, etc. In either cases, the risk of biofilm-related infections and resulting complications are always a potential threat. Although biofilm formation is highly heterogeneous based on the type and strain of infecting species, it can be broadly divided into the four stages (Fig. 9.1).

9.2.1 Stage 1. Adsorption

Microorganism adhesion onto the surface is affected by several factors including surface charge, energy, hydrophobicity, and surface topography [21]. Surrounding host proteins such as fibrinogen, fibronectin, vitronectin, immunoglobulin, and albumin adsorb onto the surfaces of implants developing it into a surface that accelerates bacterial adhesion [22]. The role of microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), polysaccharide intercellular adhesin (PIA), accumulation-associated protein (Aap), extracellular matrix protein (Emp), protein A, and *Staphylococcus aureus* surface protein G (SasG) help in biofilm colonization [21, 23]. Physical forces associated with bacterial adhesion include Van der Waals forces, steric interactions, and electrostatic (double layer) interaction, collectively known as the DVLO (Derjaguin, Verwey, Landau, and Overbeek) forces [21]. In these early stages, the biofilm can be easily eradicated before reversible adsorption converts to an irreversible process. Biofilm biocontrol is most effective when initial adhesion of microbes can be prevented.

9.2.2 Stage 2. Colony Formation

Following irreversible adhesion to suitable substrate, the genetic switch between free living and biofilm mode is induced by secondary messenger molecules cyclic guanosine mono phosphate (cGMP) [24]. cGMP downregulates motility and upregulates the genes responsible for the production of multicomponents which comprise the architecture and biomass of the biofilm exopolymeric matrix (EPM) [24]. The composition of the EPM although largely dependent on the nutrients available in the surrounding milieu is also highly species specific and maybe comprised largely of water with extracellular DNA, proteins, lipids, as well as heteropolymers of soluble polysaccharides [25–27]. Confocal and atomic force microscopy has defined layers of spatial architectures within biofilms with channels and pores for an effective circulatory system within its confines [28, 29]. As the biofilm grows, heterogeneity wthin the structure regarding genotypes and metabolic phenotypes emerge, resulting in the development of chemical gradients and environmental

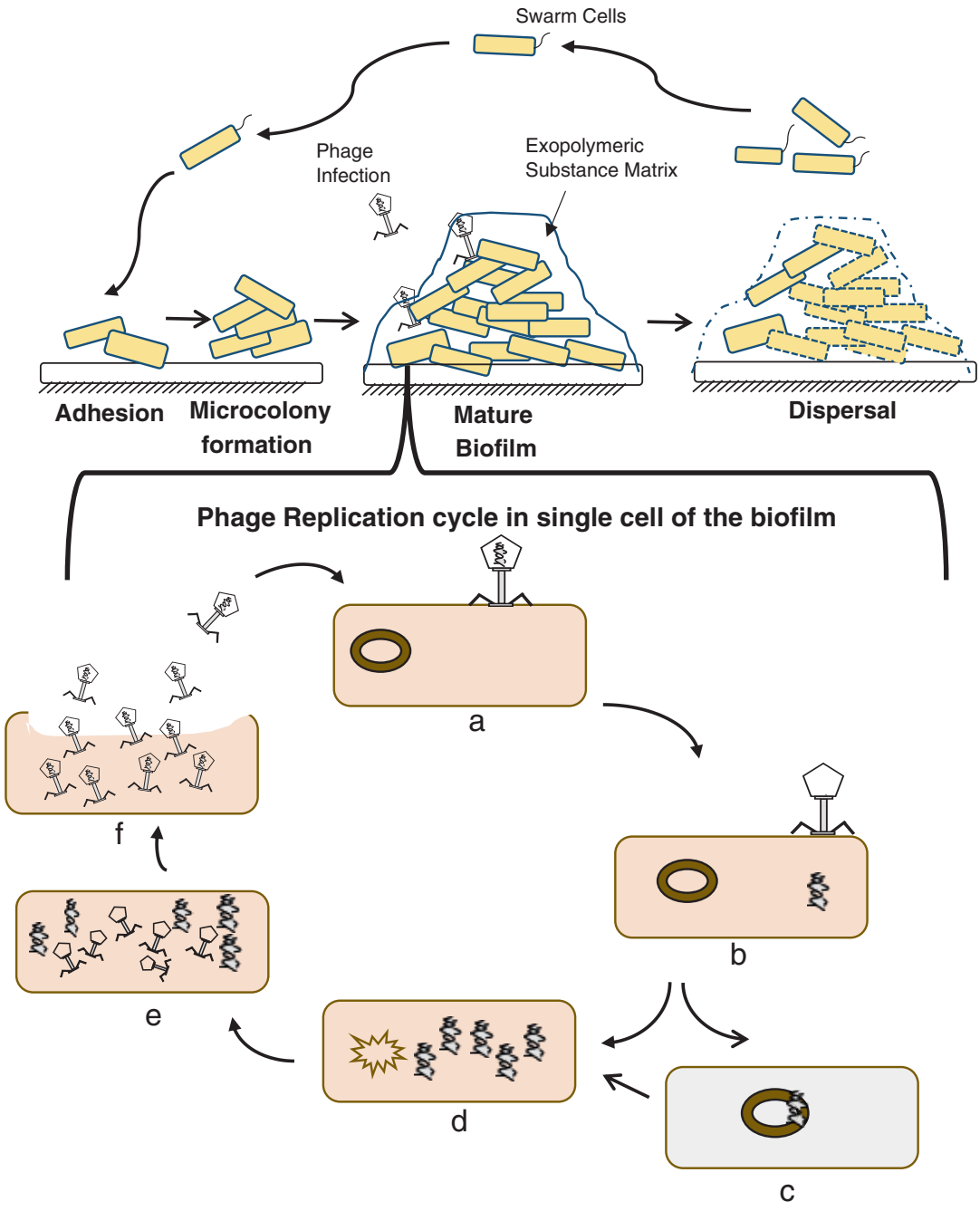

Fig. 9.1 Stages in biofilm formation: adhesion, microcolony formation, maturation, and dispersal leading to swarm cells which may form a fresh biofilm by adhesion to suitable substrate. Following phage application to biofilm, phage replication cycle in a single cell of the biofilm will consist of following steps: (**a**) adsorption of phage on specific host receptor, (**b**) entry of genomic material. Phage life cycle may enter one of two alternative states: lysogency (**c**) or lytic cycle (**d**). (**c**) Lysogeny wherein phage DNA integrates into host chromosome and be transferred to daughter cells until lytic cycle is reactivated. (**d**) Lytic cycle: Phage DNA replicates to create several virion DNA copies. (**e**) Several copies of structural capsid and proteins of phage are formed. (**f**) Assembly of genomic DNA within the capsid structure occurs followed by cell lysis and release of progeny phage to continue the life cycle

micropockets [27]. This heterogenity further contributes to the antibiotic recalcitrant nature of the biofilm.

9.2.3 Stage 3. Maturation [30]

Acquiring certain cell density within the confines of the exopolymeric substances turns on the production of autoinducer signaling molecules by the cells which control the formation of quorum sensing molecules (QSM) [11]. Quorum sensing (QS) is a cell population-dependent gene regulatory mechanism for coordinated community behavior which is characteristic of biofilms [31]. QS signal molecules have a low molecular weight and belong to a wide range of chemical classes including acyl homoserine lactones (AHLs), furanosyl borate diesters (AI2), cis-unsaturated fatty acids (DSF family signals), and peptides. QSM and their regulation have been excellently reviewed and also a major target for anti-biofilm strategy development [32]. LuxI/LuxR and RhII/R quorum sensing systems are typically seen in gram-negative bacteria such as *Ps. aeruginosa* and Enterobacteriaceae family using specific or a combinations of acyl homoserine lactones (AHL) as QSM [30]. The secreted AHL molecules (synthesized by autoinducer synthetase LuxI) upon attaining a certain concentration bind to LuxR-like protein, which in turn activate transcription of several downstream genes which determine the coordinated biofilm behavior. In gram-positive bacteria, signaling peptides are recognized by a two-component signal transduction system which consists of a histidine kinase-based ATP-binding cassette (ABC) membrane protein and a cytoplasmic response regulator protein represented by the *agrBDCA* quorum sensing system in *S. aureus* [31]. In *Streptococcus* species a conserved quorum sensing system represented by *ComCDE* quorum sensing system regulates bacteriocin production as well as several virulence factors including biofilm formation [33]. A LuxS/AI2 autoinducer interspecies quorum sensing molecule composed of interconvertible furanosyl borate diester has been described for many gram-negative and gram-positive bacteria [34]. In a mature biofilm, QS circuits has been reported to affect antibiotic resistance by upregulating antibiotic degrading enzymes, multiple drug efflux pumps, horizontal gene transfer of antibiotic resistant carrying extrachromosomal elements as well as retarding antibiotic permeability by production of exopolymeric matrix [11, 32]. Also, horizontal gene transfer within the close architecture of the biofilms permits the transfer of antibiotic resistance genes via extrachromosomal elements as well as extracellular DNA [8].

9.2.4 Stage 4. Biofilm Dispersal

During the stationary phase of the biofilm wherein cell density is so high as to cause nutrient deficiency, QSM initiate the dispersal or disassembly of the biofilm [35]. Initially, as synthesis of EPM is inhibited, QSM induce production of biofilm EPM degrading enzymes as well breakdown of non-covalent interactions within the biofilm begins. An increase in number of swarming motile cells which break away from the parent biofilm for fresh colonization of niches is observed [36]. In *P. aeruginosa*, LasI/LasR positively regulates the expression of the periplasmic tyrosine phosphatase TpbA [35]. TpbA dephosphorylates the membrane-anchored GGDEF protein TpbB deactivating its DGC activity and thus reducing c-di-GMP levels in the cell. As a result, the c-di-GMP receptor PelD is no longer bound to c-di-GMP and PEL polysaccharide production is decreased [37]. QSM such as AHL and PQS signals also promotes the synthesis of biosurfactant rhamnolipids whose overproduction results in biofilm detachment [38]. In cases of immunocompromised patients, such dispersed biofilms may result in spread of systemic infections [2].

The main concerns associated with biofilm formation on abiotic prosthetic devices are heightened resistance to antibiotic regimens and the development of persister cells within the biofilm niches which are difficult to eradicate [6–8]. The various mechanisms for antibiotic resistance within biofilms are summarized in Table 9.1.

Table 9.1 Mechanisms of antimicrobial resistance in biofilm associated with abiotic prosthetic devices

Property	Mechanisms	References
Biofilm exopolymeric substances (EPS) and matrix	Retard penetrations of antimicrobial compounds Adsorb antimicrobial compounds Harbor antibiotic degrading enzymes	[6, 25–27]
Heterogeneity in growth rate and metabolism	Biofilm composed of aerobic and microaerophilic pockets, nutrient deficient/rich areas create conditions causing cells to become refractory to effect of antibiotic action	[27]
Quorum sensing	Regulate virulence factors including antibiotic resistance genes, motility and virulence factors	[31, 32, 34]
Multiple drug efflux pumps	Upregulated within the biofilm population under stress conditions	[9]
Genetic transfer	Biofilms matrix also contains eDNA and permits genetic transfer of antibiotic resistance gene by conjugation, transformation and transduction	[6]
Persister cells	Stationary phase cells develop into dormant persister cells which are highly antibiotic resistant	[7, 8]

9.3 Biofilm Infections Associated with the Use of Abiotic Prosthetic Devices

In the multidisciplinary regenerative medicine field, replacement of non-functional organs or systems with artificial implants has revolutionized modern medicine [1]. Indwelling abiotic prosthetic or implant devices have also become a niche area for the development of highly antibiotic-resistant device-related biofilm infections. Device-related infections (DRI) are defined by Center for Disease Control (CDC) as infection in a patient with a device (intravascular catheter, endotracheal tube or indwelling urinary catheter) that was in use for at least 48 h before the onset of infection [39]. The most frequently isolated bacterial biofilm formers associated with DRI include gram-negative *Klebsiella pneumonia, Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli,* gram-positive *Staphylococcus aureus*, and emerging pathogens coagulase-negative *Staphylococci* (CoNS). *Candida* sp. amongst fungi are most commonly isolated with implant devices. Most of these are opportunistic pathogens derived from normal microflora or they may be nosocomial in origin [2, 39, 40].

The following is a brief account of the common DRI associated with biofilms. The various biomaterials used with medical devices and the associated biofilm-related infections are summarized in Table 9.2.

9.3.1 Catheters

Catheter is a thin tube made from medicine grade material that can be inserted in the body or used for drainage, supply of fluids or gases, surgical operations applicable for cardiovascular, urological, gastrointestinal, cardiovascular, and ophthalmic uses [41]. The most common biofilm-related infections are reported for the use of indwelling vascular and urinary catheters. Catheter-associated urinary tract infections (CAUTI) accounts for 80% of urinary tract infections leading to patient morbidity which may develop into further complications such as cystitis, pyelonephritis, gram-negative bacteremia, prostatitis, epididymitis, endocarditis, vertebral osteomyelitis, septic arthritis, endophthalmitis, and meningitis [42, 43, 59]. Acidic pH of the urine also results in the formation of crystalline biofilms within the catheter which is the cause of bladder and urethral trauma [2]. In situ central venous catheters (CVC) are sites for biofilm formation both on the outer sides and luminal region [2]. Catheter-related bloodstream infections (CRBSI) or central line-

Table 9.2 Biofilm related infections on commonly used abiotic prosthetic devices

Abiotic prosthetic devise	Biomaterials used	Diseases	Biofilms	References
Intravascular catheters	Polyvinyl chlorine, polyurethane, latex, silicone	CVC septicemia	*Staphylococcus aureus, Candida, Enterobacteriaceae*	[41, 42]
Indwelling urinary tract catheters	Silicone rubber, nylon, polyurethane, polyethylene terephthalate (PET)	Bacteriuria, CAUTI	*Pseudomonas aeruginosa, E. coli, Proteus, Klebsiella, Enterobacteriaceae, Enterococcus, Enterob acter faecalis*	[42, 43]
Intracardiac prostheses, total artificial heart, permanent pacemakers (PPMs), implantable cardioverter defibrillators (ICDs), cardiac resynchronization devices (CRTDs)	Titanium, graphite, pyrolytic carbon, and polyester	Endocarditis	*Enterococci, S. aureus* CoNS, *Klebsiella* sp., *E. coli, Streptococci, Hemophilus* sp., *Actinobacillus actinomycetemcomitans, Cardiobacterium hominis, Coryneb acterium* sp., *Chryseobacterium* sp., *Bacillus* sp., *Mycobacteria*	[44–47]
Endotracheal tubes (ETT)	Polyvinylchloride, polyurethane	Nosocomial pneumonia, ventilator-associated pneumonia	*Streptococci, Ps. aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Candida albicans*	[2, 48, 49]
Corneal/ retinal implants	Polymethylmethacrylate (PMMA), silicone, hydrophobic and hydrophilic acrylate and collamer photovoltaic conjugated polymers	Endophthalmitis, keratitis, periorbital and sclera buckle infections	*S. aureus, CoNS, Propionibacterium acnes, Pseudomonas aeruginosa, non-tubercle mycobacteria, Serratia,* Fungi	[50, 51]
Orthopedic prostheses	Titanium (and its alloys), stainless steel, cobalt-chromium, various polymeric biomaterials (e.g., ceramics, hydroxyapatite, and polyethylene, PMMA, cement)	Prostheses infections	*Pseudomonas aeruginosa,* MRSA, *Propionibacterium acnes, Haemophilus influenzae, Enterococci, Streptococcus viridans, Escherichia coli, Citrobacter, Acinetobacter, Serratia marcescens, Klebsiella pneumoniae, Coryneb acterium*	[45, 52]
Dental implants, periodontal reconstruction	Titanium, zirconium, ceramics	Peri-implantitis	*Prevotella intermedia, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella nigrescens, Treponema denticola, Bacteriodes forsythus*	[3, 53, 54, 55]
Intrauterine contraceptive devices, penile prosthetics	Copper, stainless steel, plastics—PVA progesterone, silver	Endometritis, pelvic inflammatory disease, peritonitis	*Staphylococci, Group B Streptococci, Micrococcus, Corynebacteria, Candida*	[56–58]

associated bloodstream infections (CLABSI) are chronic complications associated with biofilms of CoNS, *S. aureus*, and members of Enterobacteriaceae family which require catheter removal which is in itself a high-risk procedure [41, 60].

9.3.2 Endotracheal Tubes (ETT)

Biofilm formation on the inner surface of ETT in mechanically ventilated patients has a very high rate of incidence and it has been related to the pathogenesis of ventilator-associated pneumonia (VAP) and nosocomial pneumonia [48, 49, 61]. Advances imaging techniques such acoustic reflection technique, optical microscopy, and atomic force microscopy have been used for imaging the ETT biofilm in clinical study along with microbiological colonization data [49]. Majority of biofilm were correlated with VAP pathogens with 73% of the cases being mainly *Pseudomonas* species, *Staphylococcus aureus*, *Candida albicans,* Enterobacteriaceae. *Streptococcus viridans*, *Acinetobacter baumannii*, CoNS, and diphtheroid bacilli were also found to colonize ETT.

9.3.3 Orthopedic Implants

Orthopedic implants are an integral part of the treatments of osteoarthritis as well as bone fracture management. Periprosthetic joint infections (PJI) are a cause of high morbidity and economic loss to operated patients [52]. Trampuz and Zimmerli (2008) classified PJI into three categories based on infection onset: (i) early infection in less than 3 months post implantation caused by highly virulent organisms, e.g., *S. aureus, E coli*; (ii) delayed infection occurring 3–24 months post implantation caused by CoNS or *Propionibacterium acnes*; and (iii) late infection post 24 months caused by *S. aureus, Streptococci,* and gram-negative rods usually as a consequence of hematogenous spreading from skin or soft tissue infections [44]. Biofilms can be found attached to the hardware, cement, bone, and fibrous tissue and even in joint fluids [45, 46]. Diagnosis of PJI is cumbersome and difficult wherein direct culture of organism is one criteria for which molecular diagnostic methods such as polymerase chain reaction amplification of 16 s rDNA sequence of biopsy or joint fluid in addition to fluorescent in situ hybridization (FISH) techniques have been employed [52].

9.3.4 Intracardiac Implants

Intracardiac prostheses, total artificial heart, permanent pacemakers (PPMs), implantable cardioverter defibrillators (ICDs), and cardiac resynchronization devices (CRTDs) are some of the devices associated with cardiovascular implantable electronic device infections (CIED) [40, 47, 62]. Prosthetic valve endocarditis (PVE) is a biofilm infection on the heart valve and associated heart tissue wherein *S. aureus,* CoNS *S. epidermis, Candida,* and *Enterococcus* are implicated in 80% of the cases [63]. PVE occurs in 71% cases within the first year of implantation and complications include heart failure (32.8%), stroke (18.2%), intracardiac abscesses (29.7%), cardiac surgery (48.9%), and hospital death (22.8%) [63].The pacemaker pocket as well as leads of the implantation in the endocardium are susceptible to *S. epidermis* and *Candida* biofilms. Treatment regimen requires rigorous antibiotic therapy and in most cases implant replacement [64]. *Corynebacterium* sp., *Propionibacterium acnes*, gram-negative bacilli including *Pseudomonas aeruginosa*, and nontubercle *Mycobacteria* species account for a minority of CIED infections [62].

9.3.5 Intrauterine/ Penile Prosthetic Devices

An important risk factor for recurrent vulvovaginal candidiasis was found to be biofilm-related infections associated with intrauterine devices (IUD) [56]. In a study wherein 56 IUD were studied, 26 were Candida positive which were represented with highest biofilm formation in *Candida*

krusei and *C. glabrata* strains, followed by *C. albicans* and *C. tropicalis*. The minimum inhibitory concentrations for fluconazole and amphotericin B were 64–1000 times higher for biofilm-forming isolates over the free-living counterparts [57]. Patients treated for erectile dysfunction, prostate cancer, and Peyronie's disease, amongst others, are being treated with inflatable penile prosthesis implants. Biofilm-related infections were found on penile prosthesis devices in 80% of the cases where clinical symptoms associated with biofilm infections included pus and induration over the device with presentation of fever, erythema, and chronic pain [58].

9.3.6 Dental Implants

Oral rehabilitation systems regularly replace old teeth with dental implants (artificial teeth) in combination with artificial crowns. Dental implant-supported fixed prosthesis are in contact with oral fluids and abutments on one part while the other is in contact with tissue. Maximum biofilm formation has been reported around the implant-abutment connection or below the original bone margin level [3]. Implant-related biofilm infections may lead to peri-implantitis that results in the progressive loss of bone tissue [30]. The bacteria that cause per-implantitis and periodontitis are mainly anaerobic gram-negative bacteria such as *Prevotella intermedia, Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Prevotella nigrescens, Treponema denticola, and Bacteroides forsythus* [30].

9.3.7 Other Device-Related Infections

Cochlear implant, intraocular lens, breast implants, and implantable neurological simulators are some of the other biofilm-associated device-related infections [2, 6, 50, 51]. Microbial biofilms formed on breast implants might contribute to a chronic inflammatory response and thus formation of capsular fibrosis and subsequent capsular contracture [65]. Pathophysiology of biofilm-related CC suggests the involvement of *Staphylococcus epidermidis* is a part of the microflora of the skin and the endogenous flora of the breast. Other bacteria isolated from biofilms on implants include *Propionibacterium acne, Staphylococcus aureus*, members of *Staphylococci, Streptococci, Bacillus* species, *Escherichia coli*, *Mycobacterium* species, *Corynebacterium,* and *Lactobacilli* [2].

9.4 Bacteriophage as Biocontrol Agents (Phage Therapy)

One of the most abundant predatory acellular biological entities on our planet are viruses which structurally consists of genetic material (either DNA or RNA) encapsulated in a proteinaceous coat. Viruses that infect bacteria are known as bacteriophages [15]. These bacteriophages (phages) are nano-sized intracellular obligate predators with a high degree of specificity for bacterial cells. Following specific phage-host receptor interaction, the genetic material of the phage is injected into the host. The successive spatial and temporal events in the phage life cycle may follow any of the following turns: Lytic infections involve hijacking of the host replication machinery for the generation of several copies of progeny virion, its assembly and eventual host cell lysis for progeny release in the surrounding milieu. Alternatively, in lysogeny, the phage genome may integrate into the host chromosomal material or assume episomal status within the cytosol for several host multiplication rounds. Upon encountering fortuitous times, the phage may reactivate its lytic genes and ensue the lytic course of events. Hence, lytic phage infections can successfully eradicate an entire bacterial population without affecting surrounding human cells and be contained when the vulnerable population has been cleared (Fig. 9.1).

Use of appropriate phages in combating infectious pathogens has been aptly termed as phage therapy and has been in use since the 1920s. Its historical significance has been extensively covered in recent excellent reviews on the subject

[19, 66]. The advent of antibiotic era cast a shadow on the use of phage therapy post World War, although it has been practiced successfully since in the Eliava Institute in Tbsilli, Georgia, and the Hirsfeld Institute, Wroclaw, Poland. The interest in phage research and therapy however continued in research institute all over the world as evidenced by their large numbers in the literature databases. The translation of phage therapy in research laboratories to their use in the biotechnology industry and its commercial applications has taken on a furious pace with rapidly emerging antimicrobial drug resistance amongst bacteria.

Bacteriophages are naturally occurring bacterial population control predators which thrive when provided with hosts confined within the biofilm EPS and hence serve as effective biofilm control agents [14, 67]. The advantages of bacteriophages as biofilm-controlling strategies are manifold as described below:

1. *Target specificity*: Biofilms offer large numbers and concentrated host for rapid multiplication of the phages. Unlike antimicrobial drugs, phages can differentiate between pathogens and beneficial microbiomes. Since bacteriophages are highly specific in their target identification and lysis, the phages can be directed for pathogenic biofilm and beneficial normal microflora can be spared during treatment regimen.
2. *Autodosing*: Phage thrive on the presence of host. Once phages have been delivered to target locations, they perpetuate as long as hosts are present and eventually are eliminated from the system and there are no side effects of extra dosage. This system of autodosing makes phage therapy highly attractive. However, in practice, presence of immune response and other environmental factors may require repeated application of phage treatment for efficacy.
3. *Non-toxicity*: Typically lytic tailed phages belonging to the Myoviridae, Siphoviridae, and Podoviridae families are used for phage therapy. Phages are nucleoprotein and there is no evidence of any harmful effects of phage on the immune response. However, it is imperative that highly purified phage preparation be used for human applications.
4. *Phage genetic engineering*: Phage genome show great plasticity and it has been possible to genetically re-engineer phage as homing devices to infectious sites [68]. Phage display technology helps express peptides, antibodies, enzymes, quorum sensing antagonists, and antimicrobial compounds within the phage capsid. Phage-mediated delivery of CRISPR/Cas-encoded RNA-guided nucleases (RGNs) is a recent strategy in causing digestion of target bacterial DNA in a sequence-specific manner. Staphylococcal ϕMN1phage particles carrying CRISPR/Cas in engineered phagemid with spacers targeting certain *S. aureus* virulence genes killed virulent, but not avirulent, *S. aureus*. A reduction was observed in virulent *S. aureus* strain cells on a skin infection mouse model [69]. Use of multiple CRISPR spacers encoded on phage genomes were found to be efficient in degrading multiple DNA targets in the infected host cell [13].
5. *Stability and formulation diversity*: Phage preparations are stable and can be formulated into various creams, ointments, and colloid suspensions and impregnated into surfaces without losing their viability or biofilm control potential [70].

9.5 Strategies Adopted for Anti-Biofilm Phage Treatments

9.5.1 Phage-Modified Polymeric Substrates

Phage sequestered onto the adsorbing biomaterials or coating surfaces with hydrogel-containing phages have been developed to prevent microbial adhesion [71] (Fig. 9.2a). Covalent anchorage of phages onto adsorbing surfaces ensures that the phage is capable of injecting its genetic material in the adsorbing bacteria and result in the generation of several copies of progeny which are free living. *E. coli*- and *S. aureus*-specific T1 and ϕ phages were simultaneously cova-

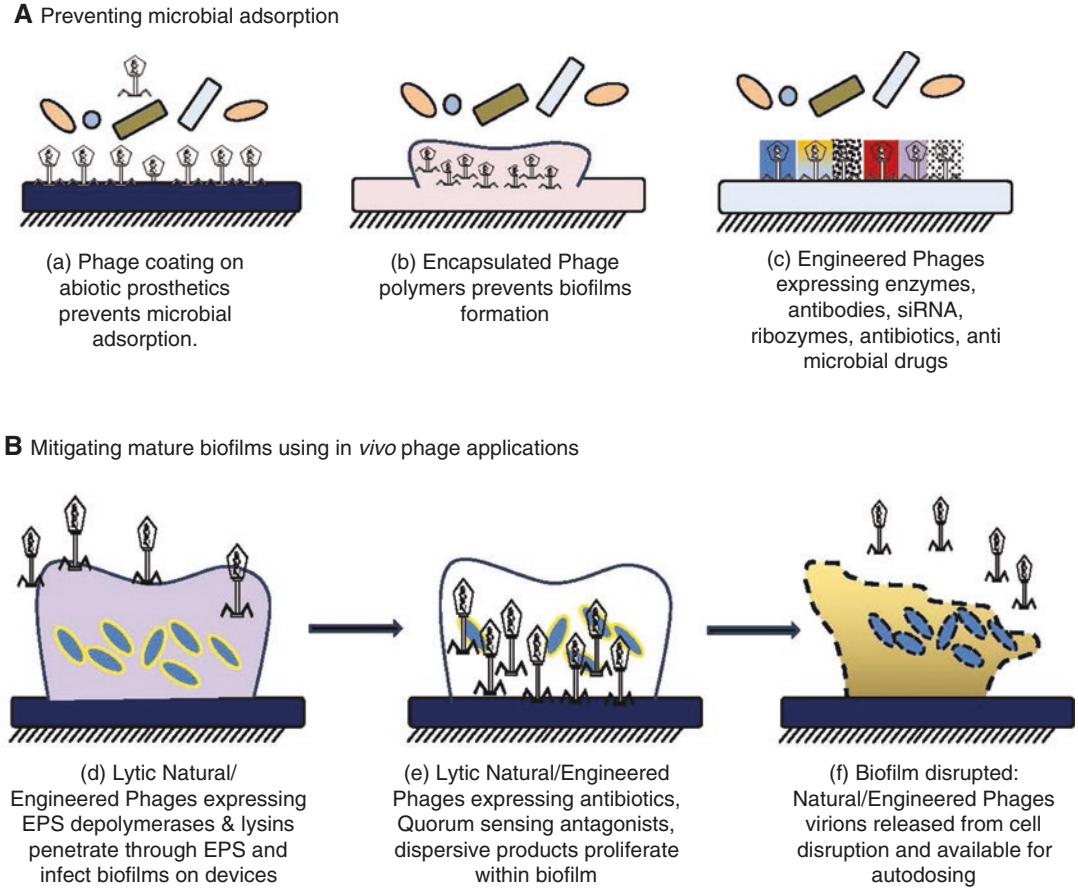

Fig. 9.2 Bacteriophage applications to prevent biofilm infections on abiotic prosthetic devices. (**A**) Prophylactic prevention by inhibiting microbial adsorption and adhesion by phage coating (**a**), encapsulated phage polymers (**b**), or coating with engineered phages expressing antibiofilm compounds (**c**). Phage coating can also function as biosensors for diagnostic applications. (**B**) Therapeutic approach by phage application on mature biofilms. Penetration through mature biofilm exopolymeric substances (EPS) by production of depolymerases and lysins (**d**). Upon phage penetration, lysis of biofilm residents by natural or genetically engineered phages expressing antibiotics, quorum sensing antagonists or biofilm-dispersive products like nitric oxide and enzymes (**e**). Once the biofilm is disrupted, virions would be released and available for mitigation of other biofilms on abiotic devices via autodosing regime (**f**)

lently adsorbed onto polyethylene and polytetrafluoroethylene surfaces which could effectively eliminate biofilm formation [72]. A US patent (US20160010077 A1) has also been filed by the University of Southern Mississippi for a method for the covalent attachment of bacteriophages on to polymeric surfaces (http://www.google.sr/patents/US20160010077). Triggered release of phages from agarose/hyaluronase hydrogel matrixes for the control of skin biofilm infection model of *S. aureus* was also found to be an effective strategy [73].

9.5.2 Recombinant Filamentous Phages as Nanocoatings

Filamentous phages such as M13 phage, RS2 RNA phage, and T4 phage are being used as nanoengineering biosystems which are being used as nanoscaffolds to express antimicrobial compounds and as biosensors [20, 74, 75]. Filamentous phages coat proteins coated with aptamers, ribozymes, siRNA and vaccines form excellent nanocarriers [74].

9.5.3 Phage Therapy, Phage Cocktails, and Cotherapy

Phage therapy for biofilms is usually carried out by direct application over the biofilm formed on the abiotic prosthetic device surface. Factors affecting biofilm clearance success in direct applications include phage dosage, route of administration as well as the stage of biofilm maturation and its resident clinical species [16, 76, 77]. Phage cocktails (combination of phage with varying host specificities) have also proved very efficacious in controlling polymicrobial infections on abiotic prosthetic devices [78]. Medical applications of phage therapy have undergone clinical trials as in the case of phage burn (funded by European commission), a phage cocktail targeting *Ps. aeruginosa* and *E. coli* infections associated with burn patients [20].

Use of phages alongside sub-minimum inhibitory concentrations of antibiotics in solution or in an immobilized form have been shown to be efficacious in controlling mature biofilms [79, 80]. Meropenem-phage and amikacin-phage combination showed synergistic activity in reducing planktonic and biofilm formed *Ps. aeruginosa* biofilms [81]. Phage antibiotic synergistic treatment for *Ps. aeruginosa* PA14 biofilm was tested with phage and 5 different bactericidal antibiotics [82].

9.5.4 Phage Enzybiotics

Phage-derived products such as bacteriolytic enzymes: lysins, depolymerases, as well biofilm exopolymeric matrix degrading enzymes are being exploited as anti-biofilm agents while circumventing potential threats associated with the use of live phages [83, 84]. Engineered bacteriophage enzymes have been employed to disperse biofilms by breaking down components of the extracellular polymeric matrix [85]. These phage-derived endolysins, including the novel Artilysins, show activity against persister cells of gram-positive, gram-negative, as well Mycobacterial origin [86]. The T7 phage was genetically engineered to express the dsp B gene encoding biofilm-dispersing enzymes from *Acinobacillus actinomycetemcomitans* which drastically reduced *E. coli* biofilm counts even as such enzymes have low substrate specificities [87]. Use of T7-engineered phages expressing quorum sensing quenching enzymes AiiA lactonase effectively inhibited mixed biofilm of *Ps. aeruginosa* and *E.coli* [88]. *S. aureus* biofilm control with the use of a chimeric protein CHAPSH3b derived from peptidoglycan hydrolase of phage vB_SauS-philPLA88 and lysostaphin was reported [89].T4 lysozyme is fused with cellulose-binding module for facilitating phage on wound dressings and retained antimicrobial activity against *E. coli* and *Micrococcus lysodeikticus* bacteria [90].

9.5.5 Phage Directly Affecting Antibiotic Resistance within the Biofilm

Antibiotic resistance is one of the major problems of biofilm-associated prosthetic device infections. Isolation of phages that exert selective pressure on bacteria to confer them sensitive to the current regimen of antibiotics is a new strategy in phage biocontrol. A lytic bacteriophage OMKO1 (family Myoviridae) of *Pseudomonas aeruginosa* that utilizes the outer membrane porin M (OprM) of the multidrug efflux systems MexAB and MexXY as a receptor-binding site has been isolated [91]. Phage-OMKO1-resistant strain (oprM knockout) showed increased sensitivity to ceftazidime, ciprofloxacin, tetracycline, and erythromycin antibiotics. A novel method of introducing antibiotic sensitizing gene cassette through genes rpsL and gyrA to two antibiotics, streptomycin and nalidixic acid, respectively, through temperate phage therapy to reverse engineer antibiotic resistance in *E. coli* pathogens is reported [92]. Gene transfer of antibiotic resistance genes through conjugative plasmids has higher frequency rates within the closely confined populations within the biofilm. A lytic plasmid-dependant phage PRD1 and antibiotic-resistant plasmid RP4 co-evolution was

studied in *E. coli* and *Salmonella enteric*. Infections with PRD1 drastically reduced the frequency of antibiotic resistance cells [93]. Bacteriophages could play a significant role in restricting the spread of plasmid-encoded antibiotic resistance [94].

9.5.6 Overcoming Bacterial Phage Defense Systems and CRISPR-Dependent Biofilm Inhibition

Development of resistance to phage infections by the host may result in ineffective biofilm control. Bacterial cells may acquire resistance either by altering phage entry receptors or through viral nucleic acid degradation post entry via the CRISPR (Clustered regularly interspaced short palindromic repeats) and CRISPR-associated Cas9 cascade proteins [69, 95]. CRISPR are prokaryotic adaptive immune systems which involve an array of repetitive sequences with spacers acquired from potential foreign DNA sources such as viruses, plasmids, or transposons. Upon reinfection with foreign DNA source, CRISPR RNA (crRNA) activates Cas proteins to degrade the complimentary foreign DNA.

Bacteriophages in response encode anti CRISPR proteases that inhibit the CRIPR defense system [96]. Genetically engineered phages that encode anti CRISPR proteases have been designed. *Pseudomonas* DMS3 temperate phage through elegant experiments involving the CRIPR Cas system was shown to modulate biofilm formation and swarming motility behavior [97]. In *Streptococcus thermophilus* phages mutations within the protospacer regions provide protection against CRISPR cas system. In *Vibrio cholera* phage-encoded CRISPR/Cas system is used to counteract a phage inhibitory chromosomal island of the bacterial host. A recent study describes a novel Bacteriophage extrusion (BREX) system which involves a six cassette gene system in *Bacillus subtilis* wherein host DNA is methylated at fifth position of a non-palindromic 5-TAGGAC-3 hexamer sequence and phage inhibited by blocking phage DNA replication of both lytic and temperate phages [98]. Phage-transferable CRISPR-Cas systems are capable of specifically killing pathogens or resensitizing them to antibiotics [69, 99].

9.5.7 Phages and Quorum Sensing

In a recent work, it was shown that phage phiCDHM1 infecting *Clostridium difficile* harbors QS gene homologs (*agr3*) which can influence pathogen behavior [100]. The decision between lytic and lysogenic behavior of phage infection is also shown to be guided by arbitrium system which consists of the production of oligomeric signaling peptides [101]. Understanding phage communication signals can help better manipulate biofilm dispersal strategies using phage therapy. Engineered T7 phages expressing quorum quenching molecules lactonases have successfully inhibited *Ps. aeruginosa* and *E. coli* biofilm [88].

9.5.8 Phages as Theranostics

Theranostics combines specific targeted therapy wherein diagnosis and therapy are combined in a single agent [102]. Classical phage typing involving the use of specific phages for bacterial strain identification has now been extended to the use of phages as biosensors or diagnostic markers [20]. Fluorescent-labeled Mycobacteriophage DS6A can differentiate between members of MTB complex [103]. NanoLuc reporter phage has been developed for the detection of *E. coli* OH:157 foodborne pathogen [104]. Similar technological advances can be applied for the diagnosis as well as therapy of DRI-associated biofilms.

9.5.9 Phages as Vaccine Delivery Agents

Prophylactic measures using phage nanosystems used as vaccine-carrying agents prior to a planned implantation for the prevention of biofilm formation by common skin microflora or nosocomial infections are being explored [75]. A

combination of phage display vaccines and phage DNA vaccines are being developed wherein antigens as fusion products are expressed on the major surface proteins of phages such as M13 and T4 phages while it carries gene for vaccine candidate in its genome under a strong expression promoter [105].

9.6 Control of Biofilm-Associated DRI with Phage Applications

Applications of the above phage control strategies are being successfully applied for the mitigation of biofilm-related infections on medical implants in in vitro studies, animal models, and human therapies. The control of catheter-induced urinary tract infections (CAUTI) caused by in vitro-induced *Proteus mirabilis* biofilms with two novel virulent phages, the podovirus vB_PmiP_5460 and the myovirus vB_PmiM_5461 was reported [106]. Further, phage-coated catheters using a dynamic biofilm model simulating CAUTIs showed a significant reduction of *P. mirabilis* biofilm formation up to 168 h of catheterization [106]. The potential of a 3 phage cocktail in treatment of established infection as well as early colonization of in vitro model of catheterized urinary tract infection showed significant decrease in crystalline biofilm formation [107]. Another study investigated the effect of pretreating hydrogel-coated silicone catheters with mixtures of mixed species (*Pseudomonas aeruginosa* and *Proteus mirabilis*) bacteriophages on the development of single- and two-species biofilms in a multiday continuous-flow in vitro model using artificial urine media. Phage pretreatment reduced *P. aeruginosa* biofilm counts by 4 log10 CFU/cm2 ($P \leq 0.01$) and *P. mirabilis* biofilm counts by >2 log10 CFU/cm^2 ($P \leq 0.01$) over 48 h [108].

Catheter-related bloodstream infections (CRBSI) are indicative in patients requiring long-term treatment of parenteral nutrition, chemotherapy, or hemodialysis [60]. Antibiotic lock therapy (ALT) is a catheter sterilization method using high concentrations of antibiotics into the catheter lumen for extended periods of time. In a rabbit model, treatment of 24-h *S. aureus* biofilm-infected central venous catheters with a *S. aureus*-specific bacteriophage K antimicrobial-lock technique significantly reduced *S. aureus* bacterial colonization and biofilm presence [109]. Mean colony-forming units (CFU/ cm^2) of biofilm measured in the distal catheter segment were significantly decreased in experimental animals (7.6×10^3 CFU/cm^2) as compared with controls (1.2×10^5 CFU/cm^2). Scanning electron microscopy demonstrated that biofilms were present on the surface of five of five control catheters but only one of five treated catheters ($P = 0.048$).

In catheter-induced aortic vegetation and experimental endocarditis due to *Ps. aeruginosa* biofilm studied in Wistar rats, synergistic action between intravenous supply of phage cocktail and antibiotic ciprofloxacin reduced bacterial load in comparison to control [90]. Synergistic activity of phage and antibiotic combination has proved to be highly effective in controlling antibiotic-resistant biofilms. *In vitro* fibrin clots as well as aorta-induced experimental endocarditis treated with phage/ciprofloxacin combinations were highly synergistic, killing >6 log CFUs/g of vegetations in 6 h and successfully treating 64% ($n = 7/11$) of rats in comparison to single-dose phage therapy or ciprofloxacin mono treatments that killed 2.5 log CFUs/g of vegetations in 6 h ($P < 0.001$ vs. untreated controls) .

Prosthetic joint infections (PJI) are a devastating postsurgical complication [46]. *S. aureus* biofilms account for 20–40% arthoplasty infections following knee or hip joint replacements leading to prolonged antibiotic treatments, multiple surgeries, and replacement of prosthetics or eventual amputations. The use of engineered bacteriophages targeting *S. aureus* and other microbial infections has been successfully demonstrated in the following studies. In an implant-related infection model in rats, MRSA and *Pseudomonas aeruginosa*-specific bacteriophages were tested with antibiotic regimen of teicoplanin for MRSA and imipenem, cilastatin, and amikacin for *Ps. aeruginosa,* respectively [110]. *S. aureus*-specific phage along with linezolid (incorporated in hydroxymethyl propyl cellulose biopolymer)

allowed gradual release of the two agents at the implant site in a mouse model of prosthetic joint infection with *S. aureus* ATCC 43300(MRSA) resulting in reduction in bacterial adherence as well inflammation [111].

Bacteriophage treatments in face of chronic symptomatic antibiotic-resistant infections have also been implicated in having anti-inflammatory properties [112]. In a clinical study, thirty-seven patients, some with periprosthetic infections with chronic antibiotic-resistant bacterial infections, were treated with oral bacteriophage therapy and their inflammation markers such as C reactive protein and mean WBC were found to diminish [112].

9.7 Outlooks and Challenges

Biofilm infections of prosthetic devices not only cause inflammatory responses but also lead to complications due to loosening of implanted devices, wound dehiscence, or disruption of prosthetic valves and embolism. Bacteriophage therapy is a promising alternative to counter effects of recalcitrant biofilms and several phage strategies have been described for the control of infectious microorganisms. Even so, specific study targeting their behavior with biofilms is still being explored. Some of the greatest challenges that phages face within the biofilm are penetration through the exopolymeric substances (EPS) which is being hopefully addressed with several genetically engineered phages expressing lysins and EPS degrading enzymes. Even within biofilms, the presence of multiple species is common and hence the use of broad range phages is required for effective biofilm removal [113]. A host range expansion protocol wherein coculturing of several *Ps. aeruginosa* cultures with four phage mix was used to develop a phage cocktail with the requisite host range [114]. Two sequential multihost strategies have been evolved for the isolation of polyvalent bacteriophages PX1 of the Podoviridae family and PEf1 of the Siphoviridae family using *Pseudomonas putida* F1 or *Escherichia coli* K-12 and subsequently used to infect model problematic bacteria [115].

Phage dosing for biofilms clearance on prosthetic devices is very critical. Initial high dose of phage application when bacterial density is high results in an immediate arrest of biofilm growth. However, if there is low bacterial density, initial phage concentrations may decay as a consequence of lack of adequate host supply [116]. Slow release of appropriate dosages of phages using phage encapsulation technology can be used to maintain *in situ* phage amplification in response to microbe within the biofilm [117, 118].

Immune response to phage administered is still of concern in human phage therapy and studies documenting humoral responses to phages have been recorded [119]. Immune response to *Pseudomonas* phages F8 and T4 in mice showed an upregulation of innate (phagocytes) and specific immune response (antibodies) to the circulating phages similar to that observed for eukaryotic viruses [120]. Mathematical modeling of the experimental data also showed that preimmunization or natural pre-exposure to a phage may hamper its effectiveness as a therapeutic agent.

Use of biofilm-dispersing agents in co-therapy approaches or the use of genetically engineered phages expressing dispersive enzymes, nitric oxides, and quorum sensing antagonists is effective in targeting biofilms [68, 87]. Following a biofilm-dispersive regimen, the use of antimicrobial agents at much lower inhibitory concentrations appears to be a comprehensive antibiofilm strategy.

Detection of biofilms remains one of the greatest challenges of biofilm infections [5]. New molecular methods should be introduced in the practice along with microscopy which can substantially reduce time taken in conventional culture methods [4]. These innovative methods are expected to provide a more sensitive bacterial enumeration and detection that would contribute to better treatment regimens. Phage theranostics combine diagnosis and therapeutic applications for effective and timely control of biofilm-associated device-related infections [102].

Apart from preventing infections on prosthetic devices, bacteriophages are also finding

use in regenerative medicine as nanoscaffolds for tissue regeneration [20]. Genetically engineered M13 phages have long rod-shaped nanostructures which self-assemble as scaffolds used for tissue regeneration [71]. Additionally, the M13 major coat protein can be genetically engineered to express cellular differentiating markers helping in osteogenesis and neovascularization. Phage-based regenerative medicine shows great promise with developing technologies such as 3D printing and precision-based nanomedicines [20].

Since there is currently no legislation regarding the use of bacteriophage therapy, the development of bacteriophages as novel drugs comes under the purview of the Food and Drug Administration (FDA) in the United States. Similarly the efficacy of phage therapy is yet not approved by the European regulatory standards [121]. Developing a cost-effective phage therapy module requires deliberation from both the legislative/regulatory bodies and the pharmaceutical industry. In Belarus, Russia, and Ukraine, a number of companies including Microgen are already marketing phage cocktails for a number of infections which are available as registered medicines [122]. Intralytics Inc. in the USA has developed a patented and FDA-approved phage cocktail against *E. coli* and *Listeria monocytogenes* for the food industry marketed as ListShield™ (http://intralytix.com/). It is hoped that coordinated efforts from the medical community, pharmaceutical companies, and legislative bodies will make phage therapy an economical and highly effective treatment option for the control of DRI.

9.8 Conclusions

Awareness regarding the potential of phage usage has increased manifold. Commercial patents afforded for biofilm control of infections in food industry as well as the success of clinical trials worldwide are providing a major credence to phage therapy. It is hoped that combination treatments will gain popularity in mainstream medicine with phage treatment units in hospitals worldwide.

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10 Extracellular Vesicles Derived from Mesenchymal Stem/Stromal Cells: Current Approaches to Enhance Their Release and Therapeutic Potential

Richard Schäfer, Benjamin Koch, and Patrick C. Baer

10.1 Introduction

Cell-based therapies have been widely used in experimental and clinical studies as a new therapeutic approach for several diseases. In particular, transplantation of mesenchymal stem/stromal cells (MSCs) is a very promising therapy option to support organ and tissue regeneration. During embryonic development MSCs originate from the somatic lateral plate mesoderm [1]. Later, MSCs can be isolated from umbilical cord/cord blood, amniotic fluid and the placenta, but also from nearly all tissues and organs of the adult organism [2]. In vivo MSCs can be traced close to the vasculature, but they can also be detected in other distinct localizations such as the endosteum or the medullary cavity of the bone [3, 4]. Observations that MSCs can be differentiated in vitro into mesodermal lineages such as osteocytes, chondrocytes, and adipocytes [5] suggested a "stem cell" character of MSCs. However, this has been heavily debated due to lack of evidence, or at least inconsistent reproducibility, of functional MSCs' transdifferentiation into non-mesodermal cell types [6]. Yet, the existence of MSCs subpopulations featuring different degrees of stemness or plasticity in vivo and in vitro cannot be completely ruled out [4, 6, 7].

Currently, the most widely used MSC isolation technique is outgrowth and subculturing of adherent fibroblastoid cells, and MSCs ex vivo expansion is feasible for up to 50 cumulative population doublings [8], hereby providing substantial cell numbers for manufacture of MSC therapies. MSCs research has been growing steadily for decades, and MSCs productions for clinical applications are on the rise [9]. But what, besides their relatively simple isolation procedure and their ex vivo upscaling potential, makes MSCs attractive as cell therapeutics for regenerative medicine? MSCs have been successfully evaluated for decades in a great variety of preclinical disease models such as cardiac and cerebral ischemia, lung injury, bone defects, as well as autoimmune diseases [10–14]. Meanwhile, MSCs have been used in the clinic and current clinical indications for MSCs (mainly derived from bone marrow and adipose tissue) in regenerative medicine, such as organ ischemia or

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skeletal degenerations, are measuring up to immunomodulation therapy of graft-versus-host disease (GvHD) [9].

Despite promises and hopes for successful treatment of severe conditions by MSCs, there are substantial challenges to overcome before MSC therapies can be sustainably implemented in clinical medicine. Particularly, MSCs' heterogeneity [2] and lack of deep understanding their mechanisms of action are hampering rapid progress. What we know so far is that MSCs produce various growth factors and cytokines suggesting that both their regenerative and immunomodulatory functions are mediated by such secreted and/or released proteins interacting with local effector cells [9, 15]. Specifically, extracellular vesicles (EVs) are regarded as a relevant means for factor trafficking between MSCs and other cell types [16], and, as proof-of-principle, MSC-derived EVs have been already successfully applied in the clinic [17].

In the following, the concept of MSC-EVs, as well as approaches to enhance their release and to improve their therapeutic potential, will be discussed.

10.2 Extracellular Vesicles

All eukaryotic cells and even prokaryotes release nano-sized, membranous vesicles, termed EVs [18]. Initially believed to take part only in waste management [19], it has meanwhile become clear that EVs are involved in many biological processes and diseases, and that they may have great potential as biomarkers and possibly also for therapy development in regenerative medicine [20, 21]. According to their origin and size, the following particle types are subsumed under the EV concept: exosomes (about 30–150 nm, released by exocytosis from multivesicular bodies), microvesicles (ca. 100–1000 nm, shed from the plasma membrane), and apoptotic bodies (about 400–5000 nm, released by blebbing of apoptotic cells) (Table 10.1) [22]. According to our current understanding, exosomes are primarily described as mediators of short- and long-range communication, while they, together with microvesicles and apoptotic bodies, take also part in waste disposal and recycling [23–26]. EVs' cargo can consist of proteins, cytokines, lipids, RNA [e.g., mRNA, ncRNA], and DNA (e.g., mitochondrial DNA) (Fig. 10.1) [22, 27, 28]. EV-mediated changes in cellular activity in both healthy and diseased conditions can be affected by exosomes carrying MHC complexes [29], anti-inflammatory noncoding RNA [30], factors promoting angiogenesis (e.g., PDGF, EGF, VEGF, NF-κB pathway proteins) [31], or wound healing [32]. Key in isolation of EVs and EV subpopulations are reproducible, affordable, and efficient technologies. Current methods can only enrich but not selectively purify EV subpopulations, and protein-RNA-complex contaminations are still an issue [33–35]. Therefore, it is of no surprise that EV studies suffer from inconsistencies of reproducibility [36]. However, the number of studies dealing with EVs has continuously increased in the past decade and therefore many efforts to standardize isolation and characterization are made. Current techniques for EVs isolation are

Table 10.1 Extracellular vesicles: characterization by size, markers, and contents [21, 22]

Type	Origin	Size	Markers	Contents
Exosomes	Multivesicular bodies (endosomal pathway), internal budding, exocytosis	30–150 nm	Tetraspanins, ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8	Proteins, lipids, coding and noncoding RNA, cytosol
Microvesicles	Plasma membrane budding	100–1000 nm	Integrins, selectins, CD40 ligand	Like exosomes
Apoptotic bodies	Cell fragmentation/blebbing	400–5000 nm	Phosphatidylserines	Proteins, lipids, DNA, rRNA, organelles and cytosol

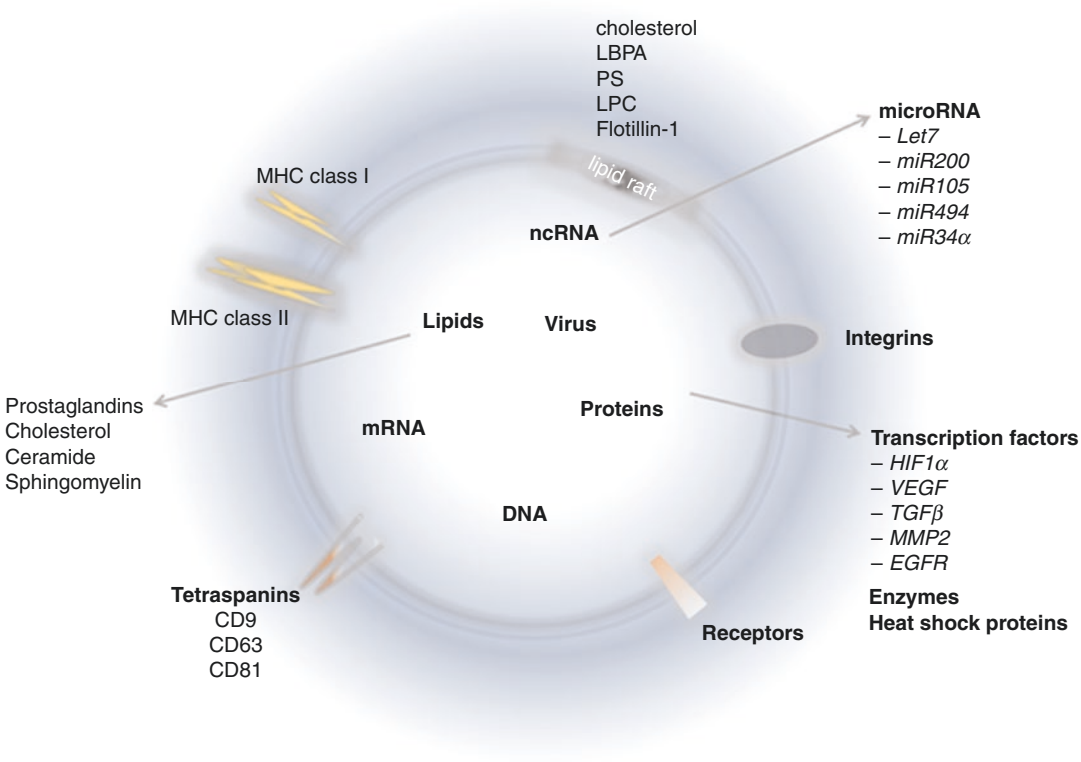

Fig. 10.1 Overview of characteristic extracellular vesicle (EV) contents. *LBPA* lysobisphosphatidic acid, *PS* phosphatidylserine, *LPC* lysophosphatidylcholine, *ncRNA* noncoding RNA

precipitation, differential ultracentrifugation, density gradient enrichment, size-exclusion chromatography, and immune-affinity capture technology. Differential ultracentrifugation is widely used but without further purification steps (such as sucrose density gradient ultracentrifugation) contaminating proteins are often present in the EV preparations [20]. Also, ultracentrifugation produces aggregates of EVs and non-vesicular macromolecules [37] and introduces many, likely uncontrollable parameters, like *g*-force, rotor-type, and angle [36]. In comparison, immune-affinity purification holds promise for more pure isolates [22].Currently, there are more and more supporters for size-exclusion chromatography [34, 38], especially in combination with preceding concentration steps [39], as it delivers better preserved bioactive vesicles compared to other isolation techniques [40].

10.3 Preconditioning Regimens to Enhance MSCs Regenerative Potential

Cell-based therapies have been widely used in experimental and clinical studies as a new therapeutic approach for several diseases. The protective effects of MSCs, their conditioned medium (CM), or EVs derived from MSCs have been shown to promote regeneration after various organ and tissue injuries. The mechanisms by which MSCs enhance regeneration and ease inflammation and injury are not completely understood, but multiple pathways might mediate the release of soluble mediators, EVs, organelle transfer, and cell-to-cell contacts [41]. Comprehensive profiling of the factors secreted by MSCs revealed that their secretome consists of various cytokines, chemokines, growth factors, extracellular matrix proteins, and molecules

of vascularization and hematopoiesis pathways. Factors that limit the regenerative capacity and the therapeutic efficacy of transplanted MSCs are their poor migration and survival in the target tissue. Transplantation of MSCs or application of their CM including EVs or even purified EVs requires MSCs with maximum regenerative capacity. Therefore, the rationale should be primarily to develop new strategies for improvement of the regenerative efficiency of MSCs and the vesicles released by MSCs (Table 10.2). In vitro pretreatment ("preconditioning") strategies have been shown to enhance survival, engraftment, and paracrine properties of MSCs and, therefore, optimize their reparative and regenerative capacity [9].

Recent data indicate that the regenerative potential of MSCs could be boosted by pretreatment with environmental or pharmacological stimuli, enhancing their therapeutic efficacy. The factors and vesicles released by preconditioned MSCs are manifold and exert immunomodulatory, anti-apoptotic, pro-angiogenic, and trophic effects [42]. Currently used MSCs preconditioning regimens include their culture in a hypoxic or anoxic atmosphere, incubation with trophic factors (growth factors, cytokines, or hormones), application of lipopolysaccharides or pharmacological agents, as well as overexpression of specific factors by genetic modification of the cells [43–46]. Nevertheless, genetic modifications such as overexpression of genes involved in migration, apoptosis, or survival can be complex to translate into clinical-grade protocols. Therefore, alternative preconditioning regimens without active manipulation in the genome might be considered.

Hypoxic preconditioning has been shown to enhance cell survival, proliferation, and also the angiogenic potential of MSCs [47–50]. Also, hypoxic preconditioning protects MSCs by activation of anti-apoptotic signaling mechanisms and enhances their angiogenic potential by induction of the expression of proangiogenic genes in vitro [51]. Furthermore, preincubation under hypoxia leads to metabolic changes resulting in higher in vivo cell survival after transplantation [48], and also induces the expression of genes that are involved in migration and homing (e.g., CXCR4 and SDF-1) [52]. The downstream signaling pathway during hypoxic pretreatment is the induction and translocation of HIF1α to the cell nucleus with the activation of gene expression (e.g., VEGF), and also the generation of reactive oxygen species (ROS) [50]. The findings of Lee and coworkers showed that hypoxic preconditioning of MSCs promotes proliferation and angiogenic cytokine secretion via the HIF1α-GRP78-Akt signal pathway, and improves the survival of the cells in an in vivo model of hind limb ischemia [53]. MSCs treatment by anoxia also enhances their survival and promotes their regenerative capacity [54]. As underlying mechanism of these beneficial effects

Table 10.2 Characteristics of extracellular vesicles derived from MSCs (Modified from [95])

Type [Human]	Protein content	RNA content
Adipose-derived MSCs	CD105, CD90	miR29c, miR150
Wharton-Jelly MSCs	CD9, CD44, CD63, CD73	miR15a,-15b,-16
Bone marrow MSCs	CD44, CD29, α4- α5-integrins, CD73, TIA, TIAR, HuR, STAU1, STAU2, AGO2	POLR2E, SENP2/SUMO1, RBL1, CXCR7, LTA4H, CLOCK, IRF6, CRLF1, IL1RN, miR-24, -103-1, -140, -143-5p, -340, -223, -451, -564
Embryonic MSCs	OCT4, WNT3 [isoform A and B]	OCT4, NANOG, GATA4, SOX2, KLF4, LIN28, miR-292, -294, -295
MSCs from induced pluripotent stem cells	CD9, CD24, CD63, CD81, integrins, glycoproteins	OCT4, NANOG, SOX2, miR-302/367 cluster miRNAs
Liver-derived MSCs	CD29, α4-integrin, CD44	MATK, MRE11A, CHECK2, MYH11, VASP, CDK2, STAU2, miR-451, -223, -24, -125b, -31, -122

anoxia induced increased phosphorylation of cell survival factors such as Akt and endothelial nitric oxide synthase [55].

ROS and reactive nitrogen species are biologically active oxidants and are regarded as important physiological signaling molecules. Various reports indicate the role of ROS as second messengers in the O_2 sensing [56, 57]. Preconditioning by ROS has been shown to enhance the proangiogenic properties of MSCs [57]. ROS generation increased MSCs secretion of the proangiogenic and anti-apoptotic factors VEGF and HGF, but did not affect MSCs ability to differentiate into cells with endothelial phenotype in vitro [57]. Applying a pharmacological preconditioning strategy with the mitochondrial inhibitors to modulate ROS generation in MSCs, Carriere et al. [57] described a strongly improved revascularization and increased number of CD31positive cells in the ischemic area of their in vivo model.

In vitro pretreatment with pharmacological or chemical agents is an alternative preconditioning concept to boost MSCs regenerative potential. For example, preincubation with sildenafil (or a silencing vector to phosphodiesterase-5) significantly improved viability and decreased necrosis and apoptosis of MSCs. It increased the release of growth factors in MSCs, and enhanced their regenerative potential in an in vivo model of myocardial infarction [58]. Incubation of MSCs with deferoxamine, an iron chelating drug, has been shown to stabilize HIF-1α under normoxic conditions as well as the activity of two metalloproteases [59]. The stabilization of HIF-1α resulted in its increased translocation to the nucleus and in increased transcription of genes involved in cell migration [60]. In addition, deferoxamine preconditioning prior to transplantation increased homing of MSCs through modulating the expression of chemokine receptors as well as metalloproteases [59]. Other pharmacological approaches include the pretreatment with atorvastatin [61], diazoxide [62], or curcumin [63]. For example, curcumin has been reported to cause potent antioxidant and anti-inflammatory properties, and free radical-scavenging activity [63]. Consequentially, pretreatment of MSCs with curcumin improved tolerance to oxidative stress injury and resulted in enhancement of their therapeutic potential in myocardial repair after myocardial infarction [63].

Another promising approach to enhance MSCs therapeutic potential is the preincubation with growth factors or other small molecules via the culture medium (reviewed in [9]). In this regard, the growth factors EGF, GDNF, and IGF-1, the pro-inflammatory cytokine TNFα, the chemokine SDF-1 (CXCL12), or hormones such as angiotensin-II have been shown to enhance regenerative capacity or the paracrine functions of MSCs [64–72]. EGF promoted in vitro expansion of MSCs without altering their multipotency [71–73] and enhanced MSCs motility and migration [72–74], and also the release of factors like VEGF, HGF, HB-EGF, and interleukin (IL)-6 and -11 [71, 75]. Others have shown that pretreatment with TGF-β increased VEGF production of MSCs in vitro [66]. TNF-α pretreated MSCs increased the release of cytokines, chemokines, and proteases compared to untreated MSCs. In this study, the enhanced secretion of 118 proteins into the culture medium upon TNF-α incubation was identified [76], specifically, many of them known to be critically involved in inflammatory processes (e.g., IL-6, IL-8, and MCP-1). Inflammation is a key response to organ and tissue injury, with cytokines and chemokines also being associated with regeneration processes. Enhanced expression of IL-6, IL-8, or MCP-1 goes along with enhanced migration of monocytes to the site of injury, hereby promoting aproinflammatory response.

Taken together, enhancement of the regenerative capacities of MSCs by preceding in vitro preconditioning regimens is a promising strategy for regenerative therapies, which may also decrease the amount of cells for transplantation and, therefore, possibly reduces the risk of side effects. Due to the proposed main mechanistic concept by paracrine activation, the application of CM (including regenerative factors and EVs) or EVs might be an alternative or complement of the cell therapy.

10.4 Current Approaches to Enhance the Release and Potential of MSC-EVs

Although there are a substantial number of studies showing the highly promising effects of preconditioning strategies on the therapeutic potential of MSCs or their CM, only few studies have been published to date focussing on the specific involvement of isolated EVs in this context. Increasing amounts of experimental data have revealed that MSC-derived EVs can stimulate angiogenesis, modulate the immune status, and exert paracrine effects that improve organ or tissue regeneration following injury. EVs were shown to carry variety of biomolecules such as growth factors, receptors, enzymes, transcription factors, signaling and immunomodulatory molecules, DNA, RNA transcripts, and noncoding RNA including retrotransposons, vault RNA, long noncoding RNAs, and microRNAs (Fig. 10.1) [77, 78], and are major communication mediators between cells [79–81]. EVs are taken up by cells and can alter gene expression or activate intracellular signal cascades. Hypoxic or anoxic microenvironment or oxidative stress can increase the amount and concentration of EVs in culture [82–84]. In this context, in vitro preconditioning regimens of MSCs prior to EV isolation and their transplantation in an in vivo injury model seem to be promising approaches to enhance the regenerative potential of EVs.

It has been shown that hypoxic preconditioning not only stimulates the secretion of growth factors, cytokines, and other proteins, but also the release of exosomes and microvesicles from MSCs. EVs from hypoxia-preconditioned cells had better therapeutic effects in organ injury through specific cargoes compared to EVs from non-preconditioned cells [85]. A recent study by Cui and coworkers examined whether exosomes derived from hypoxia-preconditioned MSCs (hypEx) and non-preconditioned MSCs (npEx) could prevent memory deficits in Alzheimer disease (AD) [86]. The results showed that neurologic conditions were significantly improved, plaque deposition and Aβ levels were lower, and expression of many effector proteins was different in the hypEx group compared to the npEx group. Furthermore, hypEx increased the level of miR-21 in the brain of AD mice [86], which may induce a positive effect during pathophysiological processes in the brain [87]. Others investigated whether hypEx were superior for myocardial repair, compared to exosomes from normoxia-treated MSCs [88]. The study showed that infusion of hypEx resulted in significantly higher survival, smaller scar size, and better cardiac functions recovery. In addition, significantly higher levels of miRNA-210 were detected in hypEx. Hypoxia treatment of MSCs increased the expression of neutral sphingomyelinase 2 (nSMase2) which is crucial for exosome secretion. Blocking the activity of nSMase2 resulted in reduced miR-210 secretion and abrogated the beneficial effects of hypEx. The authors therefore concluded that hypoxia augments miR-210 and nSMase2 activities, which is responsible at least in part for the enhanced cardioprotective potential of hypEx [88]. Feng et al. [89] showed that exosomes enriched with miR-22 were secreted by MSCs following ischemic preconditioning (repeated cycles of anoxia with intermittent reoxygenation). These miR-22 enriched exosomes reduced apoptosis of cardiomyocytes in vitro, and reduced cardiac fibrosis in an in vivo model.

Another recent study investigated the influence of an in vitro preconditioning stimulus, i.e., hypoxia or isoflurane, on EV concentration and composition of cardiomyocytes, fibroblasts, and a myoblast cell line [90]. Whereas the authors found no significant influence of the preconditioning regimen on secretion of EVs and their morphology, the protein and miRNA (e.g., miR-761) load was affected by the in vitro pretreatment. Also, EV markers (e.g., CD63, heat shock protein 70) were significantly upregulated. In another study, pretreatment of cardiomyocytes with hypoxia resulted in the upregulation and enrichment of miR-30a in their exosomes [91].

The main downstream signaling pathway during hypoxic pretreatment is the induction and translocation of HIF1α to the cell nucleus. In this context, it has been shown that overexpression of HIF-1α in MSCs enhanced their exosome secretion

and improved therapeutic potential by inducing angiogenesis in transplanted tissues [92].

Lu et al. [93] showed that the trophic functions of adipose-derived MSCs for their use in bone tissue regeneration were further potentiated when cells were preconditioned with tumor necrosis factor-alpha (TNF-α). This effect was mainly mediated by their EVs, as the removal of EVs from the medium largely diminished their effects on proliferation and osteogenic differentiation of primary osteoblasts. The study further showed that the cellular content of Wnt-3a was elevated in MSC-EVs after preconditioning with TNF- α, and inhibition of Wnt signaling decreased the effect of MSC-EVs on osteoblasts [93]. Interestingly, preconditioning with interferon-γ, another proinflammatory cytokine, abrogated the protective effects of MSC-EVs in an animal model of ischemic acute kidney injury [94]. Specifically, EVs from untreated control MSCs ameliorated kidney dysfunction and acute tubular necrosis, whereas EVs from preconditioned MSCs did not influence the development of kidney injury [94]. Also, this study demonstrated that interferon-γ pretreatment leads to the production of EVs, which originate from distinct internal vesicle routes. EVs from untreated and preconditioned cells contained different and unique proteins, and had different therapeutic potential.

Although not finally proven, it appears that the regenerative potential of EVs is promising and may be even greater by preconditioning. Nevertheless, recent preconditioning regimens mainly focus on hypoxic or anoxic microenvironment. The results from these studies that show the potential of pretreatment regimens on MSC preparations demonstrate the urgent need to further investigate the mechanistic influence and involvement of MSC-derived EVs in organ and tissue regeneration, and the enhancement of these processes by preconditioning regimens. Elucidating the multiple functions of EVs will eventually contribute to further understanding the complex self-regeneration mechanisms of the organism and the therapeutic capacities of MSCs, hereby optimizing their clinical application to support organ or tissue regeneration in the future.

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11 Anti-Biofilm Activity of Viruses, Bacteria, Fungi, and Lichens: Mechanisms and Impact on Clinical Practice

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11.1 Introduction

Biofilms are pluricellular structures displaying sophisticated regulatory mechanisms that allow the survival of bacteria or fungi in hostile environments such as those found in human hosts during clinical infection. When adopting a sessile lifestyle, bacteria gain the adaptive ability to tolerate a wide range of antimicrobials, becoming increasingly resilient. In such cases, antimicrobial treatment may fail not necessarily due to resistance but rather through tolerance and target evasion [1, 2]. Biofilms have different characteristics in Gram-positive [3] and Gram-negative germs [4, 5] and, consequently, different mechanism may be required to fight biofilm-driven infections.

In the clinic, there is an acute need to find new options for the treatment of biofilm-driven infections, and research on biofilm-active agents is well underway. Theoretically, if the three-dimensional biofilm structure is specifically targeted, the remaining planktonic cells can be easily reached by common antimicrobials, and the infectious process can thus be stopped. However, despite the abundant research on this topic, the transition from bench to bedside is not always as straightforward. Through this chapter, we aim to characterize the existing body of knowledge on the topic of natural anti-biofilm agents, by reviewing the specific literature, in order to identify the main types of agents, their mechanisms and their potential clinical role and impact on medical practice.

11.2 Viruses

A well-described category of natural anti-biofilm agents is that of bacteriophages, which are viruses infecting bacterial cells and either destroying these bacterial cells or circumventing their ability to form biofilms.

As bacteriophages display target specificity, different bacteriophages target different bacteria. Most of the literature on this topic specifically discusses bacterial lysis, but a lot of recent work has also focused on their specific ability to inhibit biofilm formation or to contribute to the disruption of mature biofilm. As bacteriophages play a wide range of roles, they can also be involved in biofilm-building activities, by contributing to polymer assembly, and increasing the amount of

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extracellular DNA as is the case with *Pseudomonas aeruginosa's* internal phage Pf [6]. This subchapter will however focus on the anti-biofilm properties of bacteriophages.

One of the best-characterized actions of bacteriophages is bacterial lysis, which leads to a decrease in bacterial load, similar to the mechanisms of other antimicrobial agents. However, particularly in infections with Gram-negative germs, lysis can lead to release of endotoxin [7], specifically its lipid A component [8], potentially associating exaggerated proinflammatory cytokine responses; *Escherichia coli*, for example, can display on its surface up to 10^6 lipid A residues [9] and induce a strong host response. Therefore, the use of lysis-deficient bacteriophages has been proposed as option for decreasing endotoxin release [8], and promising results have been shown in a murine peritonitis model [10]. Nevertheless recent data suggest that in *E. coli* clinical isolates the release of endotoxin with the use of therapeutic virulent phages (LM33_P1 and 536_P1) may be comparable to that associated with amikacin use, and two- to fourfold lower compared to carbapenem (specifically, imipenem) use [11].

Bacteriophage mixtures have been studied in clinical trials and are already marketed in countries such as Georgia, or used as experimental adjunctive local treatment in patients who fail conventional antimicrobial therapy in a few other countries, including Romania [12–15] and Poland [7], where most of the in vitro and clinical experience is available for Gram-positives, but an extending body of work also addresses Gram-negatives [7].

Apart from bacteriophage mixtures, or cocktails, specific bacteriophage-encoded enzymes have been studied for the anti-biofilm activity. Among these, depolymerases have been, until recently, by far the best studied, and are known for their activity against carbohydrates such as those found in capsular polysaccharides and extracellular polymeric substances (EPS). A thorough review by Pires et al. [16] classified depolymerases into three main categories: O-glycosyl hydrolases (divided into six groups, among which three are more frequently encountered: sialidases or neuraminidases, levanases, and peptidases, and three are less common: xylosidases, dextranases, and rhamnosidases), polysaccharide lyases (divided into three groups: hyaluronate lyases, alginate lyases, and pectin/pectate lyases), and other types of enzymes, such as lipases. A large number of the described depolymerases are encoded by bacteriophages from the *Caudovirales* order, and are constituents of the tail structure. For example, multiple types of tail fiber and tailspike proteins have been reported to have depolymerase, or, specifically, endoglycosidase activity [17]. The roles that depolymerases play in biofilm control are twofold. First, they can degrade the EPS and decrease the viscosity of the biofilm matrix, leading to better diffusion of both bacteriophages and other antimicrobials in the bacterial biomass. Second, they can degrade capsular polysaccharides and facilitate bacteriophage adsorption and entry into bacterial cells [17], where bacteriophage-induced bacterial lysis can now occur. Therefore, depolymerases can be further studied for prospective application in clinical practice either as part of bacteriophage therapy or, potentially, as purified enzyme extracts or recombinant depolymerases. Further data is needed to ascertain the degree to which they retain their biological activity under in vivo conditions, but a number of studies do point towards a preserved activity of recombinant enzymes in decreasing virulence in *E. coli* K1 [18, 19]. Specific examples of potential clinical applications include alginate lyase in the reduction of exopolysaccharides produced by *P. aeruginosa* mucoid strains from patients with cystic fibrosis [20, 21], or CHAPK murein peptidase (cysteine, histidine-dependent amido hydrolase/peptidase) derived from anti-staphylococcal bacteriophage K, which inhibits biofilm formation and disrupts mature methicillin-resistant *S. aureus* (MRSA) biofilm [22]. The K bacteriophage-derived modified murein hydrolase domain has also been combined in vitro with a cell wall-binding domain derived from lysostaphin, to generate the chimeric protein P128, which was able to induce a 95.5% reduction in mature 48-h biofilm by *S. aureus* isolates from chronic rhinosinusitis [23].

Lysozymes such as Cpl-1 and Cpl-7 have shown anti-biofilm activity on *Streptococcus pneumoniae*, *S. pseudopneumoniae*, and *S. oralis* 14–16-h biofilms [24].

Bacteriophage-encoded endolysins have long been described as potential antimicrobial agents, through their lytic activity resulting from the hydrolysis of peptidoglycan layers. Recent data also point towards their potential role as anti-biofilm agents, again, either as part of bacteriophage therapy or through their administration as purified extracts, and a notable example is that of endolysin MR-10, which has been shown to decrease bacterial biomass in mature 7-day-old MRSA biofilm, and has been proposed for its potential use in sequential treatment, following initial administration of an antimicrobial (specifically, minocycline) [25]. Another study also demonstrated the activity of nine other endolysins on *S. aureus* mature 24-h biofilm; among these recombinant peptidoglycan hydrolases containing the SH3b domains, four appeared highly active (LysK, lysostaphin, Twort, phiSH2), while others demonstrated a concentration-dependent activity (80α, phi11, P68, 2638A, and WMY) [26]. Further data on endolysins show that LysH5 is also active on mature 24-h *S. aureus* and *S. epidermidis* biofilms, albeit at a lower extent when compared to lysostaphin; furthermore, LysH5 also targets persister cells, and does not lead to biofilm induction when administered at sub-inhibitory concentrations [27]. By comparison, SAP-2 is as efficient as lysostaphin on 2-day mature *S. aureus* biofilm [28]. PlyGRCS also induces a rapid decrease in *S. aureus* mature 24-h biofilm, with a reduction of the biomass to half in as little as 1 h [29].

As described above, peptidoglycan hydrolases have been intensely studied for their potential role in the management of Gram-positive biofilm-related infections. The structural characteristics of Gram-negative germs make them less susceptible to endolysins, as their outer membrane efficiently covers the peptidoglycan layer. Different strategies for facilitating the action of endolysins on Gram-negative bacteria have been assessed; an example is pretreatment with outer membrane permeabilizers, including chelators, such as ethylene diamine tetraacetic acid disodium salt dihydrate (EDTA), or polycationic agents, such as polymyxins, aminoglycosides, or lysine polymers [30], but recombinant proteins such as LysPA26 may also display stand-alone anti-biofilm activity on *P. aeruginosa* [31].

Apart from the already well-described bacteriophage-derived enzymes, other types of bacteriophage proteins, specifically tail tubular proteins such as TTPAgp31 from *Klebsiella pneumoniae* bacteriophage KP32 and TTPAgp44 from *K. pneumoniae* bacteriophage KP34 have been shown to display dual function, with structural and enzymatic activity alike [7]. In a recent study, Brzozowska et al. (2017) [7] have shown that TTPAgp31 degrades multiple types of *K. pneumoniae* polysaccharides, including capsular, cell-free (slime), and lipo-polysaccharides through an α-1,4-glucosidase activity, and displays activity on 20-h mature biofilm, decreasing the biomass by 80% for *K. pneumoniae*, 50% for *S. aureus*, and 60% for *Enterococcus faecalis*, while TTPAgp44 hydrolyzes *E. faecium* capsular polysaccharides through a glucohydrolase-like activity and also displays activity on mature 20-h biofilm, reducing the bacterial biomass by 80% for *E. faecium*, 40% for *P. aeruginosa*, and 40% for *Bacillus subtilis*.

11.3 Bacteria

In natural environments, bacteria often come into contact with each other, and they can display a complex range of interactions, from collaborating within microbial consortia, to competing with each other for scavenging resources, or even directly attacking each other by synthetizing specific molecules, bioactive peptides, or by secondary metabolites.

LytA, an *N*-acetylmuramoyl-L-alanine amidase, is a pneumococcal autolysin which, when purified and administered under in vitro conditions, decreases pneumococcal biofilm biomass by 80%, and also displays synergy with the Cpl-1 bacteriophage-derived lysozyme [24].

Bacillus aneurinolyticus, or *Bacillus brevis*, produce a wide array of secondary metabolites. Among these, tyrocidines TrcA, TrcB, and gramicidin S significantly inhibit biofilm formation by *C. albicans*, while gramicidin S is also able to fully eradicate 24-h mature biofilm, although all studied tyrocidines (TrcA, TrcB, TrcC, TpcC, and PhcA) display some bactericidal effect on mature biofilm, in the range of 28–74% reduction [32]. When looking specifically at TrcA, TrcB, and TrcC, they eradicate 55–74% of mature *C. albicans* biofilms, and they display synergy with caspofungin and amphotericin B [32].

Bacillus safensis, a soil-dwelling germ, can also inhibit biofilm formation and impair yeast-to-hypha transition in *C. albicans*; it also inhibits biofilm formation and capsule formation by *Cryptococcus neoformans*, potentially by impacting the accumulation of glucuronoxylomannan and its organization into a matrix [33].

P. aeruginosa displays an anti-biofilm effect on *Aspergillus fumigatus* through the production of pyoverdine, which acts as a siderophore, decreasing the iron concentrations and inducing iron starvation in *A. fumigatus* [34]. Extracellular products such as polysaccharides from *P. aeruginosa* PAO1 can disperse mature 24-h *S. epidermidis* biofilm [35]. Furthermore, anti-biofilm effects of either planktonic or biofilm-associated polysaccharide extracts or Gram-negative lipopolysaccharides have also been demonstrated for a wider range of bacteria-bacteria interactions, whereby one germ's products or components inhibit the other's biofilm mode of growth [36].

Kolodkin-Gal et al. [37] have shown that biofilm-grown *Bacillus subtilis* produces D-amino acids once the biofilm reaches a mature state (5–8 days growth). Among these D-amino acids, a spontaneously occurring mixture of D-leucine, D-methionine, D-tyrosine, and D-tryptophan is able to disperse mature biofilm and also, when extracted and purified, it also inhibits biofilm formation. The mixture's anti-biofilm activity is explained through the incorporation of these biofilm-disassembling D-amino acids into the cell wall on the third day of growth. Once incorporated, they subsequently impair the anchoring into the cell wall of the amyloid fibers formed by the TasA protein, which is the main component of *B. subtilis* biofilms, along with exopolysaccharides [38]. The study by Kolodkin-Gal et al. [37] also tested the efficacy of this mixture of D-amino acids in inhibiting biofilm by other bacterial species, and they found that both D-tyrosine and the D-amino acid mixture were able to prevent biofilm formation by *S. aureus* and *P. aeruginosa*.

Oral microbiota may play a role in preventing dental caries, by inhibiting biofilm formation by *Streptococcus mutans*. Multiple species of lactobacilli have been studied for their anti-biofilm properties, either through coculturing, administration of cell-free supernatant, or extraction of bacterial products such as bacteriocins. For example, Ahn et al. have shown that *Lactobacillus plantarum* inhibits the production of exopolysaccharide from sucrose by *S. mutans* [39], while Wasfi et al. have shown that four *Lactobacillus* species inhibit biofilm formation, namely *L. salivarius*, *L. reuteri*, *L. plantarum* subspecies *plantarum*, and *L. casei* subspecies *casei*, while only three of these are also active on mature overnight *S. mutans* biofilm: *L. salivarius*, *L. reuteri*, and *L. plantarum* subspecies *plantarum*, through the same mechanism of reducing exopolysaccharide formation [40].

Salivary isolates of *L. paracasei*, *L. rhamnosus*, and *L. fermentum* also inhibit *C. albicans* biofilm formation through their production of exometabolites and organic acids, both when cocultured with the fungi and when administered as mature 24-h growth supernatant [41]. Matsubara et al. have also shown that *L. rhamnosus*, *L casei*, and *L. acidophilus* inhibit biofilm formation and are active on mature *C. albicans* biofilm, while also hindering its yeast-to-hyphae differentiation, which is an important anti-virulence effect [42], and this was also demonstrated by Vilela et al. specifically for *L. acidophilus*, both in vitro and in an experimental candidiasis model of *Galleria mellonella* [43]. The supernatant derived from certain probiotic lactobacilli (*Lactobacillus gasseri* and *Lactobacillus rhamnosus*) can inhibit biofilm formation and disrupt mature 24-h biofilm of *Candida* non-*albicans* biofilms, specifically *C. tropicalis*, *C. krusei*, and

C. parapsilosis, both alone and in a mixed pluri-species biofilm model [44].

Mixed biofilms can be encountered in clinical practice, and Krzyściak et al. [45] have studied the dental caries biofilm, showing that the presence of *S. mutans* increases the number of *C. albicans* colonies and increases the overall biofilm mass. They also showed that coculturing with *Lactobacillus salivarius* significantly decreased biofilm formation by *S. mutans* and *C. albicans* alone, or in mixed biofilms.

L. fermentum was used for purification of a bacteriocin, namely fermencin SD11, which displays antimicrobial activity on oral bacteria such as *S. mutans*, *S. sobrinus*, periopathogenic bacteria such as *A. actinomycetemcomitans*, *Fusobacterium nucleatum*, and *Porphyromonas gingivalis*, as well as *C. albicans*, but has not yet been further studied for a potential anti-biofilm activity [46]. However, other bacteriocins have been shown to display anti-biofilm properties, as is the case with sonorensin, produced by a marine isolate of *Bacillus sonorensis*, MT93, which is bactericidal to both metabolically active and dormant *S. aureus* and *E. coli* strains, and also inhibits biofilm formation by *S. aureus* [47]. Specific strains of *L. fermentum* have also been shown to produce bacteriocins able to inhibit biofilm formation by *P. aeruginosa* PAO-1 [48], and *L. kunkeei* also inhibits biofilm formation by *P. aeruginosa* and attenuates infection in a *Galleria mellonella* model [49]. When extracted or purified for standalone administration, bacteriocins can be considered as postbiotics, as they are bacterial products or by-products of probiotic bacterial metabolism [50].

Okuda et al. [51] have studied two class I bacteriocins (lantibiotics): nisin A produced by *Lactococcus lactis* and nukacin ISK-1 produced by *Staphylococcus warneri* ISK-1, and a class II bacteriocin, lacticin Q, produced by *Lactococcus lactis* QU 5. In their study, only nisin A and lacticin Q were bactericidal on mature 24-h biofilm-embedded *S. aureus*, and their activity could be explained by their pore-forming potential, which is not present for nukacin ISK-1. However, none of the tested bacteriocins were able to completely eradicate mature biofilm in this study.

A bacteriocin produced by *L. plantarum* ST8SH displayed potent anti-biofilm activity on *Listeria monocytogenes* strains, and synergy with vancomycin [52]. Another *L. plantarum* isolate (CIRM653) decreased 24-h mature *K. pneumoniae* biofilm by 77.8%, leading to bacterial dispersal, but also to an increased rate of gastrointestinal colonization by *K. pneumoniae* in a murine model [53].

Another class of bacteriocins, sactibiotics, are small antimicrobial peptides. Such an example is hyicin 4244, produced by *Staphylococcus hyicus* 4244, which showed strong inhibition of biofilm formation, and strong activity on 24-h mature biofilm produced by clinical isolates of *S. aureus* and *S. saprophyticus* [54].

Bifidobacteria have also been studied for their potential anti-biofilm activity, albeit to a lesser extent than lactobacilli. Kim et al. have shown that *Bifidobacterium longum* cell extracts can inhibit biofilm formation by enterohemorrhagic *E. coli* O157:H7 by 36%, and attenuate its virulence in a *Caenorhabditis elegans* model [55],

Among cyanobacteria, *Spirulina platensis* can display antimicrobial [56] and antifungal properties [57], and its methanolic exact has recently been shown to inhibit *P. aeruginosa* biofilms by decreasing the amount of EPS [56]. Furthermore, the aqueous extract of *Spirulina platensis* has been used in the biosynthesis of silver nanoparticles, which were then used to coat Foley catheters in combination with amikacin and nitrofurantoin, and displayed a complete inhibition of colonization or biofilm formation by uropathogenic *E. coli* for 14 days, in a murine model of UTI [58].

Other types of silver nanoparticles have been biosynthesized from *Streptomyces calidiresistens* supernatant, and have been shown to inhibit biofilm formation by *S. aureus*, *E. coli*, and *C. albicans*, albeit the degree of biofilm inhibition was significantly influenced by the type of *Streptomyces calidiresistens* strain used for biosynthesis [59].

Streptomyces hawaiiensis produces acyldepsipeptides (ADEPs) [60]; among these, a semi-synthetic derivative, ADEP4, binds to the ClpP protease and activates proteolysis, leading to the destruction of bacterial cells, both

metabolically active and inactive, and specifically bacterial persisters [61], and its association with rifampin fully eradicated *S. aureus* biofilm in vitro and in a murine thigh infection model [61].

11.4 Fungi and Lichens

In the phylogenetic tree of life, Bacteria and Eukaryota represent different domains, each comprising multiple life forms. Fungi are part of the Eukaryota domain, and can be involved in clinical infections in humans. However, recent research has shown that some of their cell wall components or some of their secondary metabolites may display important roles in limiting infections or biofilms. Here, we will briefly describe anti-biofilm compounds isolated from either clinically relevant fungi or lichen-associated fungi.

Different members of the *Penicillium* genus produce different biofilm-active compounds, including the dipeptide cis-cyclo (Leucyl-Tyrosyl), which inhibits biofilm formation by *S. epidermidis* [62], or norlichexanthone, a non-reduced tricyclic polyketide isolated from *Penicillium algidum*, which inhibits biofilm formation and virulence traits such as neutrophil lysis by MRSA [63]. Other members of this genus produce shearinines, secondary metabolites that can inhibit yeast-to-hyphae transition and biofilm formation, and disrupt 48-h mature biofilm in *C. albicans*, while also displaying synergy with amphotericin B [64]. The hyphal transition and biofilm formation can also be inhibited by other alkaloid and polyketide secondary fungal metabolites, waikialoid A and waikialide A, produced by members of the *Aspergillus* genus; however, these metabolites are not active on mature biofilm [65].

Mannoprotein is a surfactant which has been extracted from *Saccharomyces cerevisiae* cell wall. Mannoprotein does not possess antimicrobial activity on *S. aureus* or *S. epidermidis* but it does inhibit biofilm formation, and disrupt mature staphylococcal biofilms, potentially by influencing cell surface hydrophobicity [66].

Metabolites from *Plectosphaerella cucumerina* such as patulin and emodin can specifically inhibit biofilm formation, disrupt mature 24-h biofilm, and inhibit the production of virulence factors such as protease, elastase, and pyocyanin, by *P. aeruginosa* PAO1 without displaying antibacterial activity [67]. Terreic acid, a secondary metabolite of *Aspergillus terreus*, can inhibit biofilm formation by *E. coli* [68].

Farnesol, a sesquiterpene from *C. albicans* or *C. dubliniensis*, can inhibit biofilm formation by other *Candida* isolates [69], but also by *Pneumocystis jirovecii* [69, 70], *S. epidermidis*, or *S. mutans* [69]. Other fungi-derived terpenes have also been reported to display anti-biofilm activity, including guignardone N and guignardic acid, produced by *Guignardia* spp., which display synergy with fluconazole in the inhibition of *C. albicans* biofilm [69, 71].

Lichens have also been studied for their capacity to produce anti-biofilm compounds, and a recent study by Millot et al. has identified four acetone lichen extracts, from *Cladonia uncialis*, *Evernia prunastri*, *Ramalina fastigiata*, and *Xanthoparmelia conspersa*, that showed promising anti-biofilm activity on *C. albicans*, through a non-lethal effect. The main metabolites identified in the acetone extracts were squamatic acid and usnic acid, evernic acid and usnic acid, evernic acid and usnic acid, and stictic acid and usnic acid, respectively [72].

Potentially one of the best characterized secondary metabolites of licheni-associated fungi, usnic acid has been studied for its anti-biofilm properties. It inhibits biofilms by most group A streptococci [73], *S. aureus* strains isolated from patients with cystic fibrosis [74], *C. albicans* [75], *C. orthopsilosis* [76], but not by *C. krusei* [77], while data for *C. parapsilosis* is contradictory [77]. It has been loaded onto magnetic nanoparticles [78], carboxylated poly(L-lactide) microparticles used for disrupting 24-h mature *S. epidermidis* biofilm [79], and used for coating magnetic polylactic-co-glycolic acid-polyvinyl alcohol (PLGA-PVA) microsphere thin films [80], and used for surface coating of zirconium dioxide bearing and barium sulfate bearing bone cement, to prevent biofilm formation by MRSA

[81]; however, it failed to inhibit biofilm formation by *S. aureus* when used loaded onto polyurethane surfaces [82].

Retigeric acid B, a pentacyclic triterpenoid isolated from the lichen *Lobaria kurokawae*, synergistically attenuates yeast-to-hyphae transition and biofilm formation by *C. albicans*, together with fluconazole [83].

Evernic acid, a secondary metabolite isolated from lichens from the *Evernia* genus, inhibits biofilm formation and quorum sensing of *Pseudomonas aeruginosa* PAO1 [84], and a similar but even stronger effect has been demonstrated by the same author group for zeaxanthin, a tetraterpenoid isolated from the *Cladonia* genus, among other lichens [85].

Pyridoxatin, a product isolated from an endolichenic fungus from the *Acremonium* genus, inhibits biofilm formation and growth of *C. albicans* by inhibiting ergosterol synthesis [86]. Diorcinol D, a diphenyl ether derivative isolated from the lichen endophytic fungus *Aspergillus versicolor*, displays synergy with fluconazole on *C. albicans* in disrupting 24-h mature biofilm, and in reversing azole resistance, potentially by inhibiting efflux pumps and ergosterol biosynthesis [87].

11.5 Conclusions

A multitude of natural compounds have been studied for their potential use as anti-biofilm agents, and promising data show effect on nascent biofilm for most substances, but also on mature biofilm for some of the studied products. However, further research is still needed for most of these compounds, as the available body of knowledge is mostly based on in vitro studies, or in vivo euarthropode, nematode, or murine models.

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Part III

Emerging Technologies

Bionic Reconstruction: The New Frontier

12

Stefan Salminger, Johannes A. Mayer, and Oskar C. Aszmann

12.1 Introduction

Brachial plexus lesions including avulsion injuries of multiple nerve roots most commonly affect young adults as the vast majority of these patients suffer a motor vehicle accident [1, 2]. In these high-velocity accidents whiplash injuries may result in high traction forces on nerve fascicles leading to temporary or even permanent global plexopathies [3, 4]. These disabling injuries often have negative social and emotional consequences and are leading to unemployment in this mostly young population [5, 6].

Aside from standard trauma management, the treatment of such complex nerve injuries should be initiated in a timely manner. In patients suffering an avulsion injury of multiple roots, surgical reconstruction focuses on the restoration of a stable shoulder as well as elbow function [7–9]. Especially after avulsion injuries of the lower roots, return of hand function is hardly ever achieved, resulting in sensory loss, intrinsic wasting, and stiffness. The existing secondary reconstructive procedures for brachial plexus injuries that include muscle and tendon transfers, free functional muscle transplantations (FFMT), arthrodesis, tenodesis, or corrective osteotomy may be able to restore useful hand and arm function in some patients; however, in very severe cases, beyond the scope of biological reconstruction, it may be more appropriate to replace the non-functioning hand with a prosthetic device after elective amputation [10, 11]. This concept of bionic limb replacement has been successfully demonstrated in patients with functionless hands after brachial plexus injuries, massive tissue damage, or congenital deficiencies [10, 12, 13].

12.2 Technique

12.2.1 Initial Review

Upper limb function is first assessed preoperatively for both range of motion and sensation. Prosthetic replacement with an artificial hand requires a stable shoulder, a strong elbow flexion, and at least two myosignals at the forearm to open and close a prosthetic hand. Insufficient motor power to move the biological hand and missing sensation represents the main indications for

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prosthetic limb replacement. Importantly, a structured interview with a psychologist is performed to explore patient concerns and adequate coping mechanisms to deal with the loss of extremity function or the event of an elective amputation.

12.2.2 Identification and Creation of EMG Signals

Nerve conduction and EMG studies should be performed to assess the extent of nerve damage as well as to quantify the EMG activity present in the remaining musculature. If there are two distinct EMG signals, no nerve transfers or muscle transplantations are needed. If EMG signals are missing after the initial nerve injury, but muscles in the forearm are still vital, nerve transfers or standard nerve repair can be performed to establish new EMG signals. However, if the forearm presents as a biologic wasteland, either due to long denervation time or other reasons (e.g., ischemia or fibrosis), then autologous muscle needs to be transplanted to serve as a bioamplifier for prosthetic control. This can be achieved by FFMT using the gracilis muscle from the leg and coapting its motor branch to a target nerve within the forearm. The presence of motor axons in the target nerve can be distinguished by using an intraoperative staining method. Still, the presence of a Tinel-Hoffman sign suggests the existence of regenerated fibers. In patients with known brachial plexopathy, the brachial plexus is explored surgically and its branches electrically stimulated for motor activity. Once the presence of motor fibers is confirmed by intraoperative staining, fascicles of the donor nerve can then be transferred to the target nerve to create a useful EMG signal for prosthetic control. Residual faint muscular activity in other muscle groups can be used as the opposing control signal.

12.2.3 Rehabilitation and EMG-Signal Training

After nerve transfer surgery, a nerve regeneration period of approximately 3–9 months is needed for motor nerves to reach their targets. Once neuromuscular activity is recordable, rehabilitation training can be initiated with visual feedback. EMG activity recorded by surface electrodes can be displayed visually on a computer screen and used to train specific muscle activations.

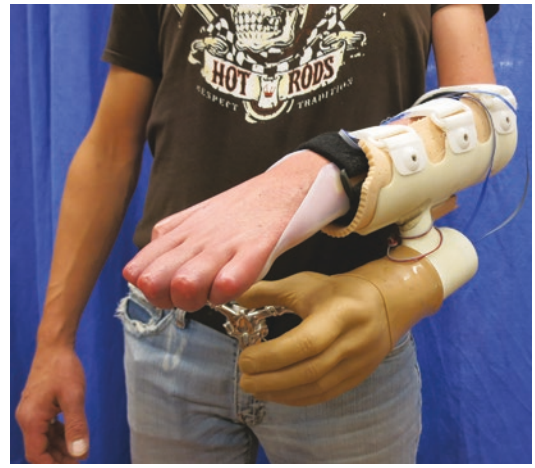

Fig. 12.1 Hybrid hand fitting

Once patients are comfortable with this feedback, these signals can be used to control a virtual hand. This is especially useful while the non-functioning hand is still in place, as it encourages patients by visualizing prospective prosthetic hand use. Once the patient is familiar with the different prosthetic functions in the virtual environment, a "hybrid hand" can be fitted, where a prosthetic hand is mounted to a forearm cast onto the non-functioning hand (Fig. 12.1). This hybrid hand acts as a further rehabilitation tool to encourage confidence in myoelectric control prior to amputation.

12.2.4 Amputation and Prosthetic Fitting

The prosthetic limb will replace the existing human hand, and as such the positioning should be customized to each patient. Based on the technical needs the adequate distance for amputation was determined between 15 and 17 cm distal to the lateral epicondyle. The skin area showing the best sensory capacity is included in the flap design for stump coverage. This results in

Fig. 12.2 Patient after prosthetic fitting

improved comfort and prosthetic feedback for the patient. A compressive garment is used for early edema control. As the forearm of such patients suffering severe brachial plexus injuries is already completely atrophied, prosthetic fitting can take place as early as 6 weeks post amputation (Fig. 12.2).

12.3 Discussion

In some patients an accident may not lead to an amputation, however to a severe nerve injury, which may result in an "inner" amputation, leaving the patient with a non-functional and insensate hand or arm [10]. Although it is possible to achieve shoulder stability and sufficient elbow function in most of the patients, reconstruction of useful hand function still represent a goal difficult to achieve, especially in patients suffering lower root avulsions. Due to accidents dating back decades in some patients, muscle atrophy and complete joint stiffness of the hand, reconstructive procedures are unable to restore useful hand function. In such cases, bionic hand reconstruction was established as a new treatment option to overcome these biological limitations. The proposed concept of bionic hand substitution was also successfully adapted to patients after massive traumatic tissue loss as well as a patient suffering a congenital limb deficiency. This new concept of bionic hand replacement is able to restore useful hand function in patients who were beyond any type of biological reconstruction. In these cases hand transplantation represents no option since the reinnervation of the transplanted hand would fail due to the extensive neurological damage.

Still, the functional capacity of a myoelectric prosthetic device can by no means be compared with that of a biologically sound hand. However, in such severe cases, the useful prospective prosthetic hand function justifies artificial replacement. Still, the prosthetic hand will always act as a helping hand for managing bimanual activities, whereas the prosthesis can take over simple tasks that require brute strength, freeing the sound extremity for demanding dexterous tasks.

Additionally, in patients suffering severe brachial plexus injuries including root avulsions, the chronic pain syndrome, referred to as deafferentation pain, represents an equal burden to the patient as the loss of hand function. In our experience, prosthetic hand replacement leads to a significant pain relief as a consequence of functional reafferentation and replacement of the phantom limb with a functioning artificial hand.

12.4 Conclusions

The presented technique of bionic hand reconstruction overcomes biological limitations of classic reconstructive approaches in patients suffering severe injuries leading to a substantial chronic loss of hand function. Future upcoming technological developments will have great impact on signal processing and interpretation for prosthetic control. Due to the reduced number of myosignals in this patient population, pattern recognition or regression algorithms may not be as beneficial as for conventional amputees. Still,

these advanced systems may be able to extract more information on the existing faint muscle signals and therefore improve prosthetic function. Additionally, prosthetic control will be improved by implantable electrodes providing consistent high-quality myosignals independent from skin texture or transpiration, amount of subcutaneous fat, or prosthetic socket movements [14]. Although skin sensation is limited in most patients suffering brachial plexus injuries, tactile prosthetic feedback may also be realized, thus enhancing prosthetic use and bodily integrity in future prosthetic systems [15].

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13 Carbohydrates in Regenerative Medicine: From Scaffolds to Cell Fate Modulators

Roberto Guizzardi, Mattia Vacchini, and Laura Cipolla

13.1 Introduction

Regenerative medicine can be defined as a biomedical field aimed at regenerating or replacing human cells, tissues, or entire organs for the restoration of the native functions of impaired part [1–3]. A synergistic partnership of different elements may be required in order to regenerate body parts: the interplay among suitable natural or artificial scaffolds for cells growth, molecular/physical signals and cells, eventually engineered with different techniques (Fig. 13.1).

In the last two decades, tremendous breakthrough has been achieved in the field, due to a better understanding of cell biology and the advances in material science, chemistry, and engineering strategies, all integrated within a multidisciplinary context and boosted by the revolutionary discovery of stem cells and the development of their applications. Plastic surgery practices have nowadays incorporated a large variety of the above-cited key elements of regenerative medicine [4]. Natural or artificial material scaffolds, standing at the core of regenerative medicine technologies, are devised to modulate cells behavior, and can be macroscopically designed to mimic natural tissue mechanical properties (i.e., hardness or elasticity for bone or bladder, veins and arteries, or cartilage respectively). Recently, biomaterial design has reached the nanoscale level, granting the possibility to fine-tune their properties, better mirroring the interactions between cells and the extracellular matrix (ECM) within living organisms, generating more suitable and functional architectures, thus increasing the chances of obtaining concrete biomedical benefits. For instance, a good enhancement of cell adhesion, proliferation, and expression of matrix components has been achieved through simply increasing the nanoscaled roughness of the scaffold pore walls [5]. This represents the ground level of tuning of biomaterials, and a variety of parameters can be modulated to influence cells behavior, comprising physical ones, such as morphology (i.e., fibers, sponges), roughness, topology, and topography (both at the micro- and nanoscale), and mechanical ones (i.e., stiffness). Upon these premises, new smart biomaterials and biopolymers can be created, such as elastic degradable polymers or polymers with shape memory [6]. Significant improvements have been made in recent years in understanding how physical properties of biomaterials affect cellular biochemical responses. This is principally carried out through mechano-sensing, an active cellular process involving dynamic interplay between cells and their physical environment, and several studies have shed light on how physical signals potently guide cell fate [7].

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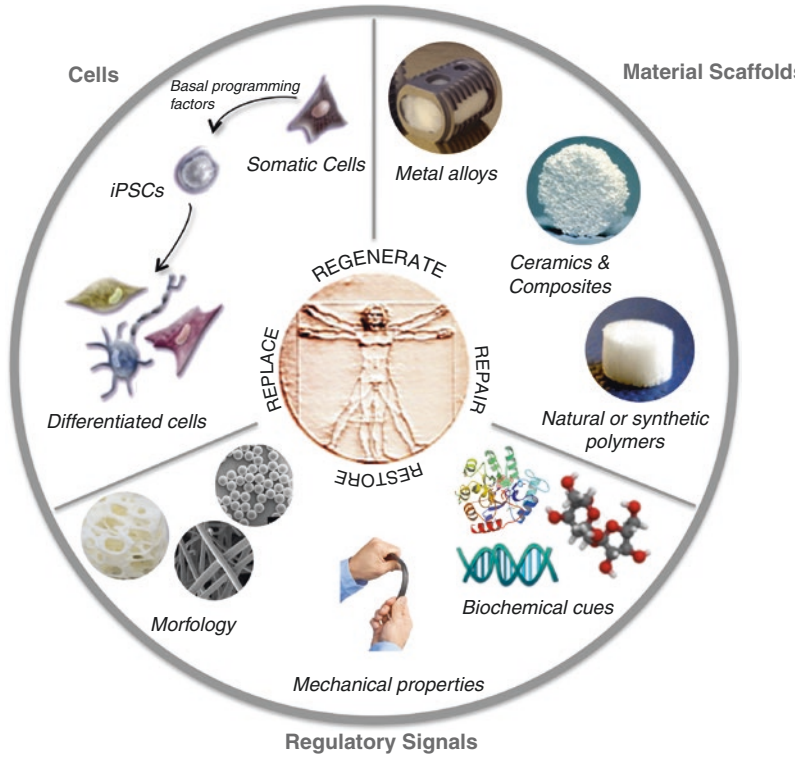

Fig. 13.1 Key elements interplay in regenerative medicine

In addition to the tuning of nano- and micro-scaled physical, topographical, and mechanical properties, biomaterials can be functionalized with biological signals in order to obtain bioactive scaffolds. In this respect, the creation of a biologically active surrounding puts cells in a naturally mimicked or engineered environment towards which they are inherently sensitive and can respond with different and specific behaviors triggered even with epitopes at the molecular level. Thus, the incorporation of bioactive cues may foster the desired cellular response towards tissue regeneration. Several signaling (macro) molecules can be used towards this aim, such as bioactive peptides, whole proteins, or even carbohydrates. In fact, a cell outer membrane is usually covered by a forest of carbohydrate structures (known as the glycocalyx) and at least six different receptor systems, which can be activated by interactions with adjacent cells, secreted signaling molecules, and specific ligands within the ECM, triggering a plethora of biochemical events, comprising cell adhesion, proliferation, migration, organogenesis, and wound repair. Carbohydrates in particular, in the form of complex polysaccharides or smaller epitopes, represent a tremendous resource for regenerative medicine applications that can be exploited directly as building blocks for natural scaffolds or as signaling cues added to the scaffold to better drive cells behavior.

Beyond scaffold bioengineering, enormous advances in cell biology, above all the discovery of human embryonic stem cells (hESCs) [8] and induced pluripotent stem cells (iPS) [9], have strongly broadened the horizons of regenerative medicine. Plastic surgery may benefit from regenerative medicine advancement in several fields of applications, such as burn care, nerve regeneration, breast reconstruction, wound healing, scar treatment, hand and face transplantation, bioprosthetic interfaces, bone regeneration, deformities treatments, and skin regeneration. In this chapter we will review recent advances of regenerative medicine based on carbohydrates, from scaffold design and bioactivation, to cell glycoengineering aspects, towards the modulation of cellular responses and behaviors.

13.2 Carbohydrate-Based Scaffolds

13.2.1 General Issues in Scaffold Design

Regardless of the tissue type that needs to be restored, a number of key issues (such as biocompatibility, biodegradability, and mechanical properties) should be considered in the design of implantable scaffolds for regenerative medicine.

13.2.2 Biocompatibility

First of all, any scaffold for tissue engineering needs to be biocompatible; cells must adhere, maintain their functions, and migrate onto the surface. After implantation, the scaffold or tissue-engineered construct must elicit a negligible immune reaction, in order to prevent severe inflammatory responses that might reduce healing capacities or cause material rejection by the body.

13.2.3 Biodegradability

Scaffolds and constructs are often not intended as permanent implants. The scaffold must therefore be biodegradable, while cells produce their own renewable extracellular matrix and renovate tissues. The by-products of scaffold degradation should be nontoxic, in order to ensure safety of the material, and waste products should be degraded through endogenous cellular metabolism.

13.2.4 Mechanical Properties

Ideally, the scaffold should have mechanical properties consistent with the anatomical site to be restored and, from a practical perspective, it must be strong enough to allow surgical handling during implantation. Producing scaffolds with adequate mechanical properties is one of the great challenges, for example, in bone tissue regeneration, since strong materials resembling our bone are necessary, which is a nontrivial task, while they have to be at the same time biocompatible, and porous, in order to allow vascularization. Many materials have been produced with good mechanical properties but to the detriment of retaining a suitable porosity. It is clear that a balance between mechanical properties and porous architecture, required for vascularization and sufficient to allow cell infiltration, has to be considered in the design of suitable scaffolds.

These issues are crucial in biomaterial design because mechanical properties, due to the nature of material and its chemistry, need to merge with biocompatibility and suitability for cellular life.

13.2.5 Scaffold Architecture

Scaffold architecture used in tissue engineering is a critical issue for surgery applications: generally these materials should have an interconnected pore structure and suitable porosity to ensure cell invasion and diffusion of nutrients towards cells. Moreover, a porous and interconnected structure is required to allow diffusion of waste products.

The issue of core degradation, arising from lack of vascularization and waste removal from the center of the engineered tissue constructs, is a major concern in the field of tissue engineering. Therefore, for any scaffold, a critical range of pore sizes exists which may vary depending on the cell type used and kind of tissue being engineered [10, 11].

13.2.6 Manufacturing Technology

In order to obtain a particular scaffold or engineered tissue construct which can be clinically and commercially viable, creation procedures should be cost effective and scalable up to small batch production [12]. The development of scalable manufacturing processes to good manufacturing practice (GMP) standard is critically important in ensuring successful translation of tissue engineering strategies to the clinic [13, 14]. Another key factor is determining how a product will be delivered and made available to clinicians. This will determine how either the scaffold

or the engineered tissue construct will be stored. Clinicians typically prefer off-the-shelf availability without the requirement for extra surgical procedures in order to harvest cells prior to a number of weeks of in vitro culture before implantation. However, for some tissue types, this is not possible and in vitro engineering prior to implantation is required.

13.2.7 Material of Choice

The final point in scaffolds design, and the one which all of the criteria listed above are dependent upon, is the choice of material from which the scaffold should be fabricated.

Different materials have been proposed to be used as both three-dimensional porous scaffolds and hydrogel matrices for distinct tissue engineering strategies. Typically, three groups of biomaterials, ceramics, synthetic polymers, and natural polymers, are used in the fabrication of scaffolds for tissue engineering [15]. Each of these individual material groups has specific advantages and, needless to say, disadvantages, so the use of composite scaffolds comprising different phases is becoming increasingly common.

Among materials, polymers of natural origin are one of the most attractive option, in some cases due to their similarities with the extracellular matrix (ECM), chemical versatility, as well as good biological performance. An important aspect is the processing of natural materials into porous matrices, a task that usually needs other technologies rather than those commonly employed in the processing of conventional synthetic polymers. There are also clinical needs for processing biomaterials into several different shapes, including nano/microparticles (for controlled release application), or into two-dimensional structures (e.g., membranes as wound dressing).

It should be noted that natural-derived materials may result in undesired or unexpected immune reactions while, more generally, inflammatory responses may arise from chemical impurities due to production processes.

13.3 Carbohydrate-Based Scaffolds

Being carbohydrates widely distributed in nature and performing different biological functions, their use in scaffolds preparation is particularly attractive, offering a variety of potential applications in regenerative medicine. Polysaccharidic materials can be isolated from different sources (plants, animals, microorganisms, algae); they show different biological and physical properties (i.e., solubility, mechanical features, gelling behavior, surface, and interfacial properties) as a function of their monosaccharide composition, chain length and tridimensional conformation, molecular weight, glycosidic bond stereochemistry, and regiochemistry [16, 17].

Several polysaccharides are already used as scaffolds in plastic surgery (Table 13.1) [18].

Some of them are constituents of the ECM, such as hyaluronic acid, and for this reason in the last decades gained widespread applications in regenerative medicine. In fact, as ECM plays an instructive role in cell functions, the hypothesis is that such biomolecules would maintain the biological information and other physicochemical features, which would preserve the potential of new tissue development after cell seeding. This would help to overcome one of the main drawbacks in the use of synthetic and inorganic materials: lacking of cell recognition signals.

In the following section, a brief overview of polysaccharides and their applications in regenerative medicine will be given.

13.3.1 Polysaccharides from Animal Sources

13.3.1.1 Hyaluronic Acid (HYA)

Hyaluronic acid (or hyaluronan) is a nonadhesive non-sulfated glycosaminoglycan, found mostly in connective, epithelial, and neural tissue [19]. Hyaluronic acid is a linear polysaccharide composed of 250–25,000 β(1 → 4)-linked disaccharide units, consisting of D-glucuronic acid and N-acetyl-D-glucosamine (GlcNAc) linked by β(1 → 3) bond. The repeating units of HYA form

Table 13.1 Commercial polysaccharide-based biomaterials

Products, commercial availability, or sources	Materials	Uses	Advantages/ disadvantages
VivoDerm (ER Squibb and Co, Princeton, New Jersey)	Hyaluronic acid	Partial-thickness burns; venous and pressure ulcers; vitiligo treatment	No apparent rejection; 2-d shelf life; delay in preparation because of graft cultivation
Hyalomatrix PA (Addmedica Paris, France)	Partial benzylester of hyaluronic acid	Partial-thickness burns; deep burns in children	No animal or allogeneic human-derived components
Hyalo graft 3D (Fidia Advanced Biopolymers Padova, Italy)	Esterified hyaluronic acid	Full, partial-thickness wounds; scleroderma cutaneous ulcers	May be combined with Laser Skin technologies for treatment of deep wounds
Integra (Integra Life Sciences Corp., Plainsboro, New Jersey)	Collagen and chondroitin-6-sulfate	Deep partial-thickness and full-thickness burns; post surgical wounds; diabetic ulcers	Bilayered; good barrier function; long shelf life; may be applied over bone; removal of silicone layer and auto graft required; possible fluid entrapment beneath construct
Algicell Calcium Alginate (Derma Sciences, Princeton, New Jersey); AlgiDERM (Bard Medical, Covington, Georgia); KALTOSTAT (ConvaTec, Skillman, New Jersey); Tegagen (3 M, St Paul, Minnesota)	Alginates and derivatives	Deep wounds; autolytic debridement; rope form to pack deep or tunneling wounds; infected wounds	Conformable and allows gas exchange; draws out contaminates and excess exudate in heavily draining wounds. May dehydrate wounds with minimal exudate; contraindicated in third-degree burns; need to be changed daily
Allevyn (Smith &Nephew, London, United Kingdom); DuoDERM (ConvaTec, Skillman, New Jersey); Hydrocol (Bertek, Rockford, Illinois); InvacareHydrocolloid (Invacare Supply Group, Elyria, Ohio); Tegasorb (3 M, St Paul, Minnesota)	Polycarboxymethyl cellulose and derivatives	Light to moderate exudate in shallow full-thickness defects; wounds requiring moisture, such as granulation tissue; used under compression dressing; autolytic debridement, especially with necrotic, dry eschar	Reduce pain; its property allows patients to continue daily activity; may leave residue or adhere to wound surfaces; not recommended with heavy exudate, active infection, or sinus tracts; highly occlusive property can promote anaerobic infection
Restylane (Medicis, Scottsdale, Arizona)	Stabilized Hyaluronic acid gel,	Mid-dermal applications, such as deeper wrinkle reduction, lip augmentation, nasolabial folds, and glabellar creases; also used in treatment of tear trough deformities	Advantages include minimal hyper sensitivity reactions, easily injected with nice flow through small-gauge needle, long persistence after injection; but include higher cost, higher incidence of bruising, and potential for severe swelling and pain from lack of anesthetic mixture
Hylaform/Hylaform Plus (InamedAesthetics, Santa Barbara, California)	Cross-linked molecules of hyaluronic acid	Approved for injection into the mid-dermis to deepdermis for correction of moderate to severe facial wrinkles and folds, subdermal injection lead to inferior results, and if injected too superficially	No skin test is necessary, thus can be used at initial consultation; disadvantages include shorter longevity than other hyaluronic acid products; cannot be used in patients with hypersensitivity to avian proteins, most notably eggs

(continued)

Table 13.1 (continued)

Products, commercial availability, or sources	Materials	Uses	Advantages/ disadvantages
Radiesse (BioFormMedicalInc, San Matteo, California)	Gel carrier comprising cellulose	Indicated for correction of nasolabial folds, vertical lip lines, acne scars, marionette lines, and restoring volume in and around the cheeks; also being studied for use in treatment of vocal cord insufficiency, radiographic marking, vesicoureteral reflux, stress urinary incontinence, and HIV-associated facial lipoatrophy	No risk of antigenic or inflammatory reaction, implants do not calcify and remain soft and flexible, and because of very low particle solubility, volume retention may theoretically persist for years; disadvantages include potential for aggregate of scar contracture around collection of the particles
Juvederm (Allergan, Santa Barbara, California)	Hyaluronic acid	Suitable for use in any wrinkles, moderate or deep, as well as scar correction; approved for nasolabial folds, off-label use for lip augmentation, marionette fold correction, prejugal sulci, and volume filler for atrophy and acne scars; will absorb water after injections and thus slightly expand within 24 h after correction; effects typically lasts 9–12 months	Advantages include longevity with better initial and sustained correction than bovine filler; disadvantage include more volume injection required, lack anesthetic, thus patients feel pain during injection

a rigid macromolecule with several repelling anionic groups able to coordinate cations and water. Hyaluronic acid is easily obtained in large scales through microbial fermentation. HYA scaffolds can be designed for both hard and soft tissue regenerations. A common form of HYA is as a hydrogel, for encapsulating cell and bioactive molecules. HYA may also be fabricated as fibrous scaffold by electrospinning techniques or blended with other materials and shaped into porous scaffolds. A promising technique for the fabrication of HYA scaffolds appears to be 3D printing. The wide scaffolding properties of HYA allow this biomaterial to be used for nearly any tissue regeneration application.

13.3.1.2 Chitin/Chitosan

Chitin is a hard, nonelastic, hydrophobic polysaccharide, main constituent of exoskeleton and of the internal structure of invertebrates, is the second most abundant natural polysaccharide after cellulose. Chitin can be obtained from many natural sources, including the exoskeleton of arthropods and insects. Chitin consists of (1 → 4)-β-N-acetyl-D-glucosamine repeating monosaccharide units. Chitosan is a partially deacetylated form of chitin, thus consisting of a mixture of N-acetyl-D-glucosamine and D-glucosamine units; it is usually insoluble in neutral or basic aqueous solutions, soluble in weak aqueous acids (pH < 6), due to amino groups protonation, and possesses gelling properties at low pH. Chitin and chitosan are known for their excellent biological properties, among which are the biocompatibility with human cells, the ordered regeneration of wounded tissues, the immunoenhancing activity, the induction of immediate hemostasis, the radical scavenging activity, and antimicrobial activity [20].

Chitosan and its derivatives are being extensively used for bone tissue engineering and central nervous system-related applications. Chitosan in vivo is biodegradable, probably by lysozyme through hydrolysis of acetylated residues. Furthermore, extracellular lysozyme activity was found enhanced in in vitro cultures of several

mammalian cells in the presence of chitin and its derivatives; as a result, connective tissue formation was stimulated and the self-defense function against microbial infection was also enhanced.

The partial absence of N-acetyl groups in chitosan allows exposed amino groups to react with different derivatization chemicals, such as acyl chlorides, anhydrides, or carbonyl compounds, by reductive amination. Of great importance are chemical derivatives of both types, formed through reaction with bi- or polyfunctional reagents, thus allowing further chemical reactions. On the basis of chitosan chemistry, several chitosan dressing materials have been developed commercially for the healing treatment of human and animal wounds [21].

As for HYA, chitosan-based biomaterials can be produced in different shapes, such as sponges, nanofibers, films, membranes, hydrogels with wide applicability especially in wound healing [22], and orthopedic tissue engineering [23–25].

13.3.2 Polysaccharides from Plant Sources

13.3.2.1 Cellulose

Cellulose is a linear polysaccharide of up to 15,000 D-glucose residues linked by β-(1 → 4)-glycosidic bonds, with good biocompatibility, excellent thermal stability, and interesting mechanical properties for biomedical applications. Cellulose is regarded as a semiflexible polymer; the relative structural properties as rigidity or stiffness are favored by the β-glucosidic linkage that bestows the linear form of the chain. A huge number of intermolecular hydrogen bonds participate to its structural properties, reflecting on its high viscosity in solution, a high tendency to crystallize, and its ability to form fibrillar strands [26, 27]. Cellulose can be converted into several different derivatives (oxidized cellulose, carboxymethylcellulose, cellulose nitrate, cellulose acetate, cellulose xanthate) [28] that can be easily molded or drawn into fibers.

While cellulose is typically obtained from wood, several algae, bacteria, and other organisms in culture can produce cellulose microfibrils in their cell wall. There are considerable structural differences between the various organisms due to different biosynthetic pathways; however, bacterial cellulose (BC), produced through fermentation processes, is gaining more interest in the last decades, resulting from its unique nanostructure, compatible with a wide range of biomedical applications [29]. For example, BC synthesized by Acetobacter xylinum shows great biocompatibility, high water-holding capacity, high crystallinity, a fine fiber network, and high tensile strength in the wet state so that it is gaining considerable potential as a novel wound healing system and skin tissue repair tool [30–32], for micronerve surgery, and as material for making artificial blood vessels suitable for microsurgery, for cardiac applications [33], and cartilage [34] tissue engineering (TE) [35].

13.3.2.2 Starch

Starch is a mixture of α-amylose (normally 20–30%) and amylopectin (normally 70–80%) synthesized by plants. α-Amylose is a linear polymer made up of several thousands of glucose α(1 → 4) linked. Amylopectin adds to the amylose structure α(1 → 6) branches at every 24–30 glucose residues. The α-glucosidic bonds of starch render this glucose-based polymer completely different from cellulose in terms of structure, properties, and applicability, sometimes inadequate to surgery devices.

Starch can be easily modified (cross-linked, oxidized, and acetylated) [36]; it can be converted into thermoplastics [37] or blended with synthetic polymers to improve its mechanical properties [30] towards bone and other TE applications [31].

13.3.3 Polysaccharides from Algae

13.3.3.1 Alginates

Alginate is a linear polymer made up of β(1 → 4) linked D-mannuronic acid (M) and α(1 → 4)-linked L-guluronic acid (G), which can be randomly arranged or alternated. Alginates are found combined with calcium and other bases as main structural component of cell walls of brown seaweeds [32]. The M and G ratios and the polymer lengths

can be extremely different, depending on the source of the alginate. The polymer undergoes ionotropic gelation in the presence of divalent cations, and gelling depends on the ion involved (Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}). Alginate hydrogels find application for cell encapsulation, cartilage TE, and injectable cell delivery vehicle, while alginate scaffolds have been proposed for hepatic and cardiac TE [38, 39].

13.3.3.2 Agarose and Carrageenan

Agarose, belonging to the galactan polysaccharide family, is one of the components of agar, the major polysaccharide of red algae. Agarose is a linear polysaccharide based on the β(1 → 3)-D-galactopyranose-(1 → 4)-3,6-anhydro-α-L-galactopyranose repeating unit. This characteristic composition makes the chains to join together and adopt a double helix structure. The two chains result wrapped together tightly, trapping water inside the helix. Agarose gels/sponges have been proposed for cartilage and disc TE, along with nerve regeneration [40].

On the other side, carrageenan polysaccharides are linear polymers consisting of chains of (1 → 3)-linked β-D-galactose and (1 → 4)-linked α-D-galactose units, which are variously substituted and modified into the 3,6-anhydro derivative, depending on the source and extraction conditions.

All carrageenans are known to be highly flexible molecules, forming double-helical structures at high concentrations, able to form hydrogel at room temperature, in some case thermoreversible [41].

Another key feature of carrageenans is the ability to show thixotropic behavior, i.e., they thin under shear stress and recover their viscosity once the stress is removed. Carrageenan-based gels find application for cell encapsulation [42].

13.3.4 Polysaccharides from Microbial Sources

13.3.4.1 Dextran

Dextran is a hyper-branched polysaccharide obtained by bacteria from sucrose through the action of dextransucrase enzyme [43], consisting of α(1 → 6)-linked D-glucose moieties with branching via α(1 → 3) linkages. The main features of dextran are its extensive solubility, from water to organic solvent and variable physicochemical properties depending on molecular weight and branching extent (0.5–60%), as a consequence of dextran source. As in the case of other polysaccharides, dextran can be chemically modified. It finds applications as porous hydrogels [44].

13.3.4.2 Gellan

Gellan gum is a high molecular weight bacterial anionic exopolysaccharide, composed of (1 → 4)-L-rhamnose-α(1 → 3)-D-glucose-β(1 → 4-D-glucuronic acid-β(1 → 4)-D-glucose tetrasaccharidic repeating units. Gellan gum can be obtained as native or in high acylated form in which D-acetate and D-glycerate are present on the same glucose residue of the repeating unit. This highly acylated derivative shows up as transparent, soft, elastic, and flexible gel, resistant to heat and acids; however the presence of cations, including Ca^{+2}, Mg^{+2}, Na^{+1}, K^{+1}, and H^{+1} may form hard, nonelastic, and brittle gels. Native gellan gum is usually nonelastic, brittle gels. Due to the high versatility of gelling conditions, gellan gums find a wide range of applications in plastic surgery and TE, such as intervertebral disc TE [45] and in cartilage TE [46].

13.3.4.3 Pullulan

Pullulan is an extracellular microbial homopolysaccharide with repeating units of maltotriose (α(1 → 4)-linked glucose trimer) joined by α(1 → 6) linkages, produced by fermentation from starch. Some advantages of pullulan are its nontoxicity and lack of immunogenicity; it dissolves readily in water to form a stable viscous solution that does not gel; it has adhesive properties and can be used to form fibers, and ECM mimics for TE applications [47].

13.3.4.4 Xanthan

Xanthan gum is an anionic polysaccharide and is fermented from glucose by the bacterium Xanthomonas campestris [48]. The structure of xanthan has a cellulosic backbone of β(1 → 4) -linked glucose branched at C3 of every second

glucose unit with the D-mannose-β(1 → 4)-D-glucuronic acid-β(1 → 2)-D-mannose. Some terminal mannose units can also contain a pyruvate group, while mannose residues are variably acetylated. It has unique rheological properties (high viscosity even at low concentrations) and forms hydrogels.

In addition to polysaccharidic materials outlined in this section, a number of other interesting carbohydrates remain unexploited, such as laminarin, gluco- and galactomannans, exudate and mucilage gums, levan and curdlan, among many others.

13.4 Carbohydrates as Biocues

In recent years both research and market of biomaterials for regenerative medicine applications are undergoing an unprecedented growth; in this framework, materials are considered not only as scaffolds with adequate mechanical properties to support cells growth but also as cell-instructive microenvironments specifically driving their behavior [49]. An efficient biomaterial for tissue engineering should firstly support cell adhesion and spreading, as implanted prostheses/scaffolds should promote colonization by primary cells. Cell adhesion may vary upon the nature of the material and the specific cell line under consideration, so it is fundamental to properly choose it. Secondly, the material should be bioactivated with accurately adopted biocues, rendering it cell-instructive for the desired application. Carbohydrate polymers have been considered for long time only as energy sources (i.e., glycogen and starch) or structural materials (i.e., cellulose); nowadays, carbohydrates are known to cover a huge variety of biological roles well behind energy or structural ones.

The sugar code (the Glycocode), given by complex carbohydrates or glycans, which are designed upon only ten monosaccharides [50, 51], reaches an unsurpassed number of possible combinations with specific structural features, coupled to different signaling events within and between cells, specifically bringing about a wealth of physiological and pathological cellular behaviors (i.e., adhesion, differentiation, migration, cell-cell, cell-matrix, and cell-pathogen recognition events, immune response, disease progression). Carbohydrates located on cell surface dispatch their biological signals by means of specific receptor proteins, such as lectins, widespread upon a variety of biological targets. In virtue of this, glycans represent unique candidates as biocues for bioactivating materials for regenerative medicine purposes. Despite carbohydrates might be included in biomaterials by non-covalent linkages, the most interesting approach is their covalent immobilization on the material of choice. A covalent linkage between carbohydrates and materials impedes diffusion and enhances stability towards enzymatic degradation [52]. The covalent approach may guarantee the control over the spatial orientation of the glycan on the material, ensuring more efficient recognition processes by cell receptors. Towards this end, several methodologies have been designed for the covalent and chemoselective bioconjugation of carbohydrate epitopes to biomaterials [53, 54]. In the next section, example on how carbohydrates can influence cell adhesion, differentiation, and homing are reported.

13.4.1 Carbohydrates Promote Cell Adhesion to Materials

While specific adhesive peptide sequences, such as the tripeptide RGD [55], have been widely used to promote cell adhesion, to date systematic studies on the adhesive properties of carbohydrates are still lacking. Keeping in mind that the bioactivation mechanism mediated by glycans is yet not completely clear and that great efforts are still needed for its elucidation, one should be aware that promising glycans for tissue regeneration must be experimentally validated for any specific cell lineage. Small carbohydrate epitopes (mono-, di-, and trisaccharides) have been bioconjugated to several materials as simple signaling cues [56]. Even carbohydrates which are not naturally involved in the adhesion phenomenon may be pressed into that service, as for instance galactose, used in material functionalization to promote hepato-

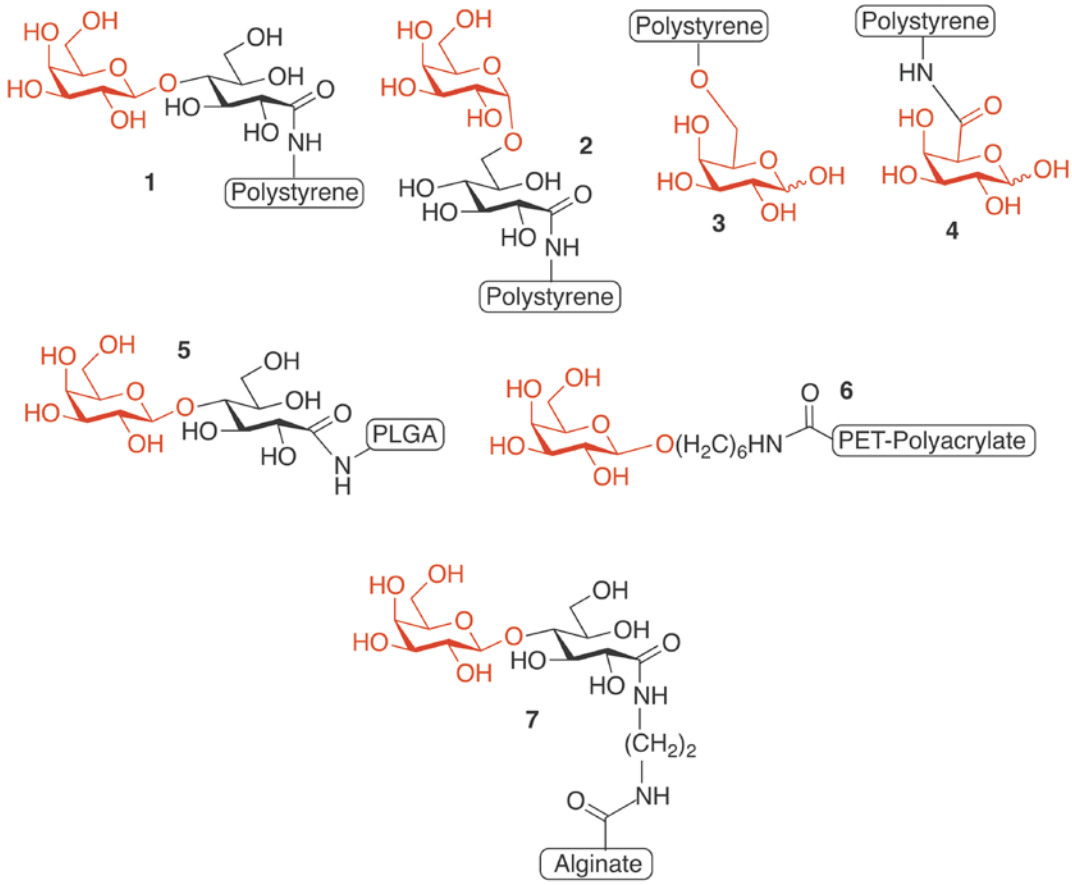

Fig. 13.2 Galactose (red) in different forms linked to different materials

cyte adhesion [57]. However, it should be noted that a variety of molecules can bind to cell surface components, either specifically or nonspecifically, allowing in many case a certain degree of adhesion; thus, care must be taken in interpreting results [58].

Galactose has been grafted to several natural or synthetic materials in different forms (Fig. 13.2) and detailed studies regarding exposed galactose residues orientation and density have been conducted [59, 60].

Studies on scaffolds 1 and 2 (Fig. 13.2) showed that galactose is able to support hepatocytes adhesion and spreading depending on the glycosidic stereochemistry: β-galactosides were found more effective than α-galactosides. In addition, it was demonstrated that galactose grafted to material surfaces shows preferential adhesion of hepatocytes when cocultured with fibroblasts, the attachment of which was essentially inhibited [61]. As glycosidic bond stereochemistry and epitope spatial presentation are the outcomes of a chemical reaction, biomaterial functionalization strategy is relevant in determining the final orientation of the carbohydrate epitope on the scaffold surface and, thus, its biological effectiveness. Another example of this is given by glucose-functionalized polystyrene for erythrocytes adhesion. Polystyrene was bioactivated with glucose moieties in different spatial orientations (scaffolds 8 and 9) (Fig. 13.3) and type-1 glucose transporter (GLUT-1), a mammalian cell transmembrane protein, was found to be implicated in mediating erythrocytes adhesion [62] only when glucose is exposed in the proper spatial configuration, enabling recognition. Only when glucose was grafted to polysty-

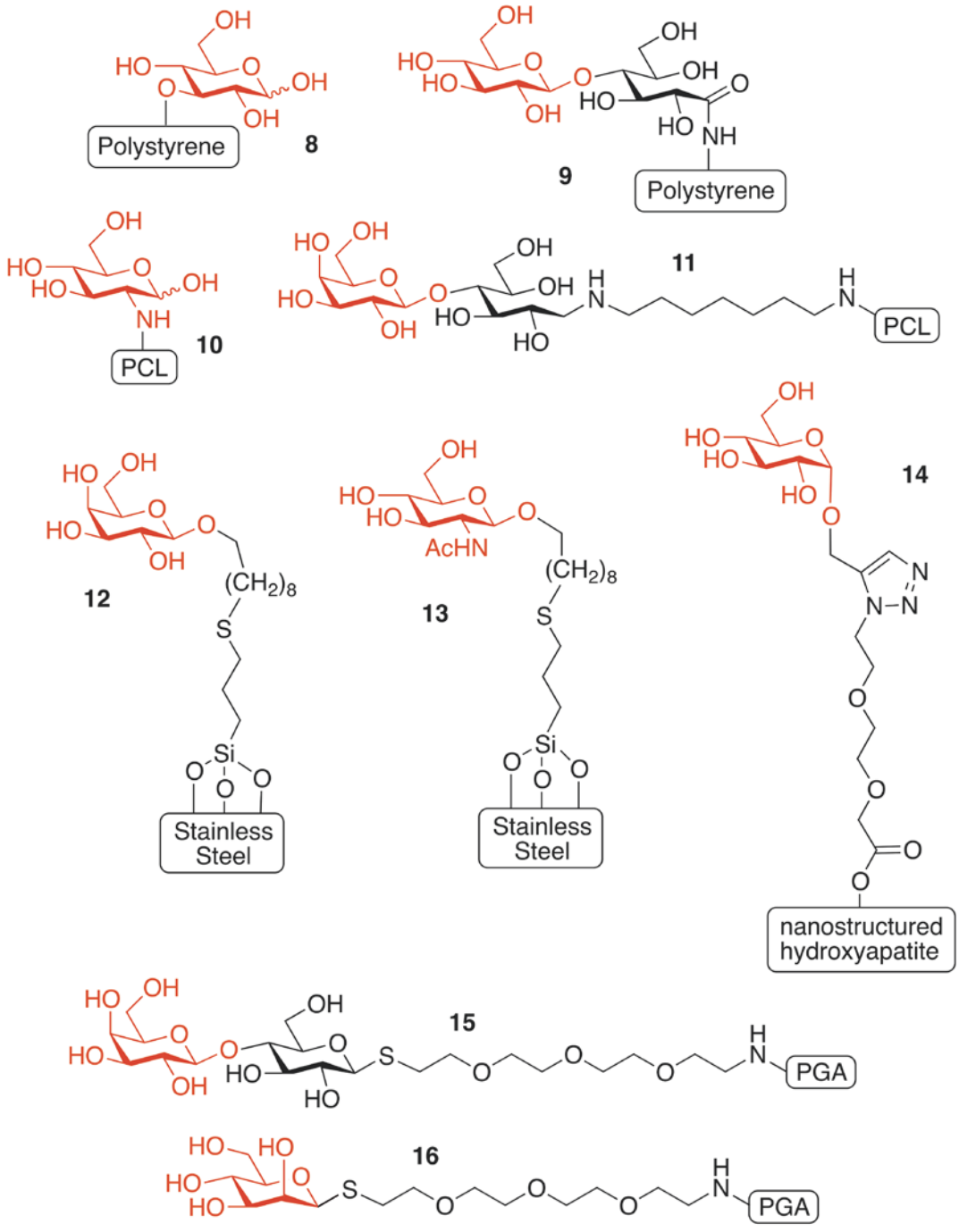

Fig. 13.3 Scaffolds based on different materials, exposing different carbohydrate epitopes (red)

rene through the 3-OH, thus being presented to the receptor in its reducing form (scaffold 8) (Fig. 13.3) [63], the interaction with GLUT-1 occurred. This observation evidences once more the extreme relevance of choosing the correct chemistry beyond biofunctionalization when dealing with carbohydrates or generally with any polyfunctional biocues.

Small carbohydrate epitopes may improve cell adhesion and spreading of human mesenchymal stem cells (hMSC) on synthetic polymers such as poly(ε-caprolactone) (PCL). PCL is a promising polymer for tissue regeneration, due to a unique combination of biodegradability and biocompatibility features. However, as any other synthetic polymer, it does not promote cell adhesion, due to its hydrophobicity conflicting with cell's outer membrane polar environment (the Glycocalyx above all) and to the lack of suitable molecular motifs. When PCL-based scaffolds were grafted with N-acetyl-α-D-glucosamine (scaffold 10) (Fig. 13.3) and non-reducing galactose moieties (scaffold 11, Fig. 13.3) adhesion, spreading and cell viability were increased in comparison to unfunctionalized scaffolds [64, 65].

Even inorganic materials like stainless steel or hydroxyapatite can be functionalized with small carbohydrates to improve their biological interaction potentials. Stainless steel can be used for medical implants, while bioceramics are useful biomimetic composite materials for bone tissue regeneration, but none of them possess bioactive/bioadhesive properties. Stainless steel has thus been functionalized with N-acetyl-α-D-glucosamine or D-galactose, via a suitable glycoalkyl trimethoxysilane, after activation of the metal surface by silanization (scaffolds 12 and 13) (Fig. 13.3) [66], while α-D-glucosides were conjugated to nanostructured carbonated hydroxyapatite, through a click azido-alkyne reaction (scaffold 14) (Fig. 13.3) [67]. In both cases, the reaction was effective; thus, these examples envisage new strategies for stainless steel and ceramic bioactivation by carbohydrates.

Coculture systems, in which multiple and distinct cell types are cultured within the same environment, are gaining much interest in tissue engineering methods [68]. Usually, in these strategies, there are target cells (which will compose the engineered tissue) and assisting cells (guiding target cells to obtain desired behaviors), interacting synergically to create the proper environment for tissue reconstitution. Another way of using coculture strategies resides in putting together different cell types, like, for example, healthy and cancerous cells, to study preferential adhesion on a given surface, in order to test biomaterials efficacy in complex environments better resembling in vivo conditions. In this context, a few studies highlighted how mono- or disaccharides may play a role.

A monosaccharide, mannose, and a disaccharide, lactose, once grafted to poly(L-glutamic) acid (PGA, scaffolds 15 and 16) (Fig. 13.3) were applied to multilayer film deposition in combination with poly(L-lysine). The adhesion of chondrocytes as primary cells versus a chondrosarcoma cell line in coculture was compared [69]. It turned out that lactose only slightly affected adhesion of both cell lines, while mannose sustained adhesion and proliferation of chondrocytes, whereas cocultured chondrosarcoma cell growth was very limited. In virtue of this, it is possible to speculate that mannose receptors might be present on chondrocytes [70].

D- and L-glucose grafted on glass surfaces were evaluated for chondrocyte adhesion as well; only D-glucose-functionalized glass surfaces were able to maintain the chondrocytic phenotype, in contrast to the unnatural L-enantiomer [63].

A series of phytoglycans (glycans not belonging to the human glycocode) such as N-acetylchitobiose, cellobiose, cellotriose, mannotriose, and maltotriose, once grafted to poly(methyl methacrylate) (PMMA) coatings, were characterized for their interactions with human fibroblasts [71]. Surprisingly, cellobiose and cellotriose improved fibroblast adhesion if compared to other saccharides or to pristine PMMA surfaces. On the contrary, when cellobiose and cellotriose were added in soluble form to the culture medium, fibroblasts adhesion to polystyrene plates was inhibited in a concentration-dependent manner.

It is noteworthy that these glycans from plant sources, not belonging to the human glycocode, are able to induce significant responses in human cells, highlighting how glycoengineering boundaries lie well beyond the sole natural mimicking.

13.4.2 Carbohydrates Can Promote Cells Differentiation

Biomaterials may boost regenerative medicine applications if suitably bioactivated through signaling molecules able to promote and drive cell differentiation towards the desired phenotype. In this respect, small carbohydrate epitopes have been shown to act as differentiating cues.

Once again, galactose was studied for its ability to drive mouse embryonic stem cell (mESC) to functional hepatocytes. Scaffold 1 (Fig. 13.2) exposing nonreducing galactose moieties on polystyrene surfaces together with E-cadherin was able to induce early expression of asialoglycoprotein receptor (ASGPR), sustaining differentiation of mESC and maintaining hepatocytes functionality [72]. Moreover, it was observed that hepatocytes cultured on galactose-containing layers expressed high levels of liver-specific genes compared to the cells grown on unfunctionalized polystyrene. From these results, it appears clear that galactose may be a key monosaccharide for the development of bioactive materials for liver tissue engineering.

Nonreducing glucosides and sialic acid containing disaccharides were studied for their adhesive and differentiating properties on different cell lines; when covalently bound to collagen films (scaffolds 17–19) (Fig. 13.4), 3′-sialyllactose (scaffold 17) and 6′-sialyllactose (scaffold 18) were characterized in vitro for their influence on mesenchymal stem cells' (MSCs) viability, proliferation, and induction of osteogenic and chondrogenic related genes [73]. Sialoside epitopes displayed on collagen surface promoted MSCs adhesion and proliferation; moreover, it was observed that the two different sialosides provide MSCs with different, specific, and saccharide-type dependent signals (despite their close structural similarity), in terms of expression of osteogenic or chondrogenic related genes. In particular, 3′-sialoside (scaffold 17) significantly upregulates the expression of osteogenic markers RUNX2 and ALP, while 6′-sialoside (scaffold 18) increases the expression of chondrocyte marker ACAN. This again underlines how structural details can be crucial to bring about different effects on cells. It should be also stressed that the increased gene expression is unambiguously attributed to the carbohydrate signals exposed on collagen surface, since no osteogenic or chondrogenic supplements were used in the culture media. Sialic acid-functionalized materials may open new perspectives towards osteochondral tissue regeneration.

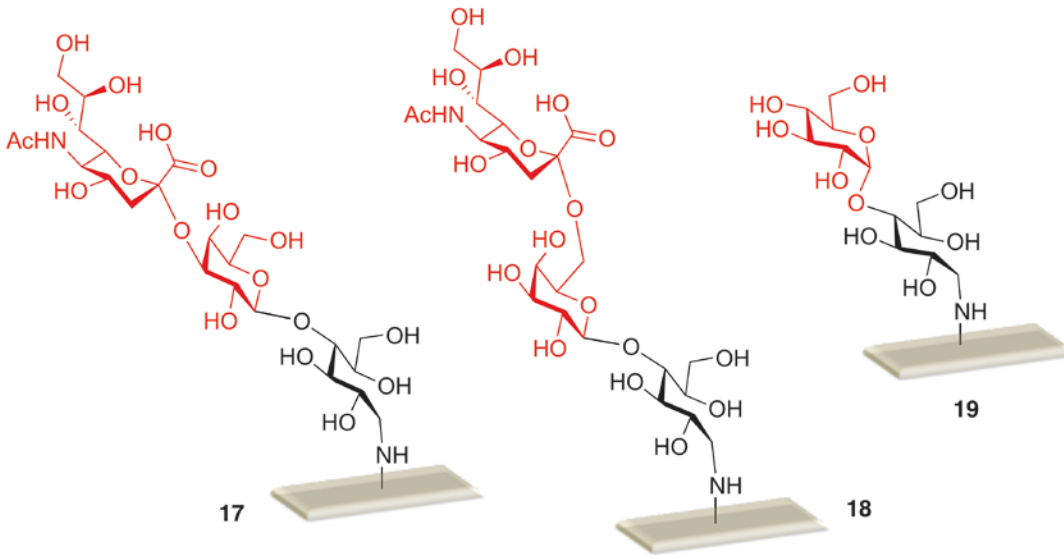

Fig. 13.4 Collagen films functionalized with glucose, 3′-sialosides and 6′-sialosides moieties (red)

Glycans have been shown to have crucial roles in nervous system development, regeneration, and synaptic plasticity [74, 75], and biomaterials are experiencing an increasingly relevant role in nervous system repair and regeneration [76, 77]. Thus, glycoengineering of materials with functionally active glycans might add another dimension to neural tissue regeneration [78]. As proof of concept, collagen films functionalized with glucose residues (Scaffold 15) (Fig. 13.4) were investigated towards neuroblastoma F11 cell lines behavior, in the absence of any differentiating agent [79]. Morphological and functional analyses of cells grown upon glucose-functionalized collagen revealed neuritic-like processes, the expression of the late differentiation neuronal marker β-tubulin III, and neuronal electrical activity, demonstrating the ability of grafted glucosides to drive neuroblastoma cells towards differentiation into active neurons.

Considering again neuronal applications, several others saccharidic epitopes, such as L-fucose (usually found as terminal residue in N- and O-linked glycoproteins and glycolipids), D-galactose, and N-acetylglucosamine, were tested towards neuronal tissue engineering. Fucose **α**(1 → 2)galactose (Fuc**α**(1 → 2)-Gal) and fucose **α**(1 → 3)N acetylglucosamine (Fuc**α**(1 → 3)-GlcNAc) epitopes were covalently linked to polyacrylamide (PAA) scaffolds and evaluated for their ability to stimulate hippocampal neurons [80]. Neurons grown on Fuc**α**(1 → 2)-Gal-PAA exhibited significant morphological changes, with a 50% increased neurite extension compared to the untreated control. PAA exposing N-acetylglucosamine, galactose, L-fucose, and Fuc**α**(1 → 3)GlcNAc displayed no significant neuronal processes, highlighting how neuritogenic activity was specific for Fuc**α**−(1 → 2)Gal and suggesting a key role in neuronal activation of its galactose unit.

13.5 Cell Glycoengineering

Cells functioning and fate are determined and controlled by the molecular interactions taking place between cells and their microenvironment. Detailed knowledge about (macro)molecules involved in these processes and mechanisms lying at the bases of their functioning, combined with the emerging possibility to remodel cell surfaces, promise to be golden tools for regenerative medicine developments. In this section, a different and up-to-date approach to tissue engineering is considered: while modification of substrate/biomaterials can be explored to accurately modulate cells adhesion and differentiation, as explained in the previous section, more recently many research groups have been focusing their work on the modification of cell surface in order to control cell behavior through biointerface interactions [81]. Thus, cell surface engineering has been explored for the enhancement of in vivo cell survival or function and differentiation control [82]. Nowadays, we can rely upon a promising cell surface bioengineering toolbox, built on key methodologies allowing surface modification of living mammalian cells [83]. Since diverse subtypes of glycans are present on cell surfaces, site-specific remodeling of the glycocalyx provides an attractive approach to understand glycans activities outside the outer membrane and to modulate them, promoting novel function and driving cells towards the desired responses. Remodeling of cell surface glycosylation is usually referred to as cell surface glycoengineering. Recent achievements in this field will be outlined in this last section of the chapter, highlighting most relevant examples for regenerative medicine.

Modifying glycoconjugates in cells and organisms can be achieved using monosaccharide analogs [84] via metabolic glycoengineering (MGE) approaches [85–87], pioneered by Wratil et al. [86] with the synthesis of sialylated glycans using N-acyl-modified mannosamines as precursors for unnatural sialic acids. After entering the cytosol, modified carbohydrate analogs are metabolized and subsequently expressed within newly synthesized glycoconjugates on cells.

Since then, the MGE strategy has been applied to several sugar analogs and exploited for different applications. For example, it is known that poly-α-2,8-sialic acid (PSA) is involved in synaptic plasticity, thus in memory and learning pro-

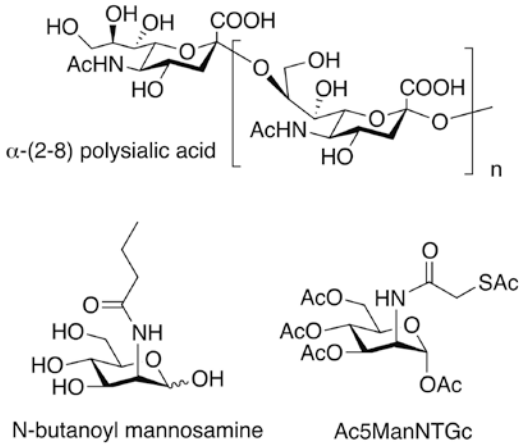

Fig. 13.5 Mannosamine analogs as precursor of sialic acid and polysialic acid biosynthesis

cesses, and is a marker of several tumors including neuroblastoma, small cell lung carcinomas, and Wilms tumor, implicated in their growth and metastatic development. In virtue of these roles, modulating PSA expression could be significant in contrasting neurological and cancerous diseases. Metabolic glycoengineering within this context was carried out by using N-butanoylmannosamine (ManBut) (Fig. 13.5) as a precursor of sialic acid, which acts as a biosynthetic blocker, resulting in the expression of truncated polysialic acid chains [88]. This metabolic glycoengineering approach granted transient disruption of PSA expression through reversible and temporally controlled ManBut administration, opening possibilities for the study of PSA roles in cell adhesion, synapses formation, tumor metastasis, and organ development, suggesting ample room for tissue engineering applications towards regenerative medicine and maybe tumoral confinement.

The possibility to modify glycans by metabolic glycoengineering can be exploited for the introduction of carbohydrate moieties that might influence cell differentiation. The sialic acid analog Ac_5ManNTGc (Fig. 13.5) allows the expression of thiol-modified sialic acids on cell surfaces. When expressed in human embryoid body-derived (hEBD) stem cells, Ac_5ManNTGc induced β-catenin expression and a change in cell morphology, suggesting neuronal differentiation. These results once more indicate that small carbohydrate epitopes are effective cues for tissue engineering, able to direct stem cells fate [89].

One limitation of metabolic glycoengineering related to its putative in vivo applications is that delivery of saccharidic analogs is not specific, making it difficult to confine sugar modifications only to desired cells. In this context, an interesting upgrade of MGE was achieved by the delivery of monosaccharide synthetic analogs to target cells in a cell surface receptor-dependent manner, via ligand-targeted liposomes [90]. Liposomes including monosaccharide analogs are decorated with ligands which bind specifically to cell surface receptors, expressed or upregulated only in target cells. This allowed a cell-selective intracellular delivery of the analogs, which were then included in cell metabolism and displayed within glycans on specific cell surface. In addition to helping cell/tissue-specific imaging applications and detection of glycosylation in vivo, enhancing potentialities of important biomedical techniques such as magnetic resonance imaging (MRI) and positron emission tomography (PET), this approach enriches the toolbox for tissue engineering applications by granting more selective ways to perform glycoengineering.

Chemically modified liposomes were also used in order to remodel cell surfaces with specific sulfated chondroitin sulfate (CS) glycosaminoglycans. Sulfation patterns in glycosaminoglycans (GAGs) are known to modulate recognition events in a sulfation-dependent manner, modulating cellular signaling cascades. In this example, embryonic (E18) rat cortical neuron cell membranes were glyco-engineered with polysaccharides built up with roughly 100 disaccharidic repeating units, with three different sulfation patterns, called CS-A, CS-C, and CS-E (Fig. 13.6), via a liposomal fusion strategy. Neurons displaying CS-E glycans exhibited increased activation of neurotrophin-mediated signaling pathways and increased axonal growth. On the contrary, neurons displaying CS-C glycans showed phospho-Akt levels similar to those of untreated neurons. These results highlight the relevance of CS-E polysaccharides in the activation of neuronal signaling pathway.

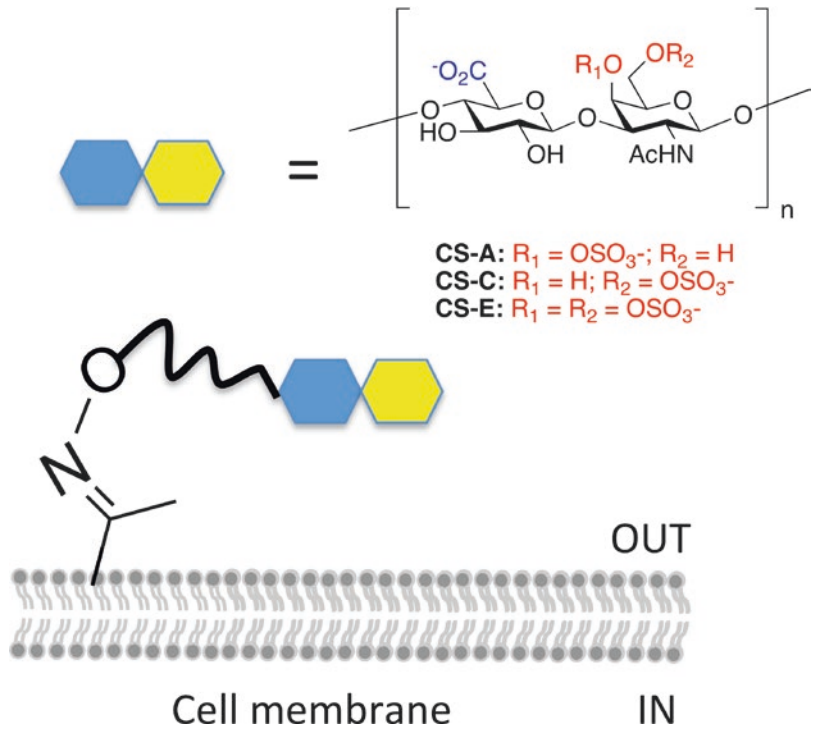

Fig. 13.6 Modified cell membrane with GAGs

A different lipid-based strategy was proposed by Woods et al. [91]: a glyco-engineered lipid anchor based on cholesterylamine was synthesized, in order to display synthetic glycans on plasma membrane with improved residence times, emulating natural mucins, to shed light on the role of mucins overexpression within cancer. Glyco-engineered cholesterylamine was durably displayed on cell surface after internalization, exposing linked glycopolymers for up to 10 days. The presence of the engineered glycans on cell surface mimicked the effect of mucins in focal adhesion and cell survival, both in vitro and in vivo. More strikingly, the engineered glycolipid was expressed also in daughter cells derived from a labeled mother cell. The same group proposed an additional strategy to expose mucin mimetics on cell surfaces. Synthetically challenging mucin-like domains were chemically synthesized and conjugated through a site-specific bioorthogonal strategy to a genetically engineered membrane protein on live mammalian cells [92]. These strategies could be adapted to replace native glycodomains in mucin proteins with synthetic analogs, systematically varying their chemical and biological properties potentially delivering great benefits to tissue engineering.

One of the greatest challenges in cell therapy for tissue engineering is the efficient delivery of viable cells to the tissue to be restored. Inefficient homing of systemically delivered mesenchymal stem cells (MSCs) is probably the major limitation of existing MSC-based cell therapies, probably caused by poor expression of adhesion molecules on cell surfaces. In order to improve MSCs homing, their surface was modified with a nanopolymeric structure bearing the tetrasaccharide sialyl LeX (sLe^X). This trisaccharide is usually overexpressed on leukocytes and is responsible for cell rolling during inflammation processes. sLe^X glyco-engineered MSCs exhibited a robust rolling response on inflamed endothelium in vivo and homed to inflamed tissue with higher efficiency than native MSCs. This approach highlights an effective glycoengineering strategy for cell homing through circulation [93].

Following a similar line of research, homing of human mesenchymal stem cells (hMSCs) to bone was obtained by glycan engineering of

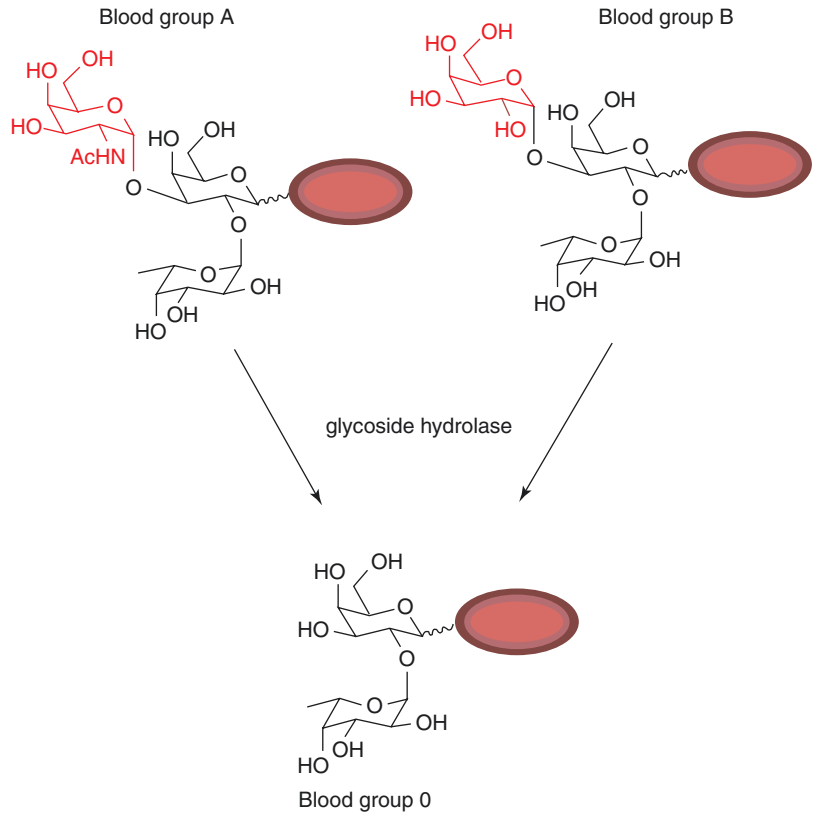

Fig. 13.7 Blood group antigenic determinants

CD44 [94]. The native CD44 glycans of MSCs bear α-2,3-sialylosides, but cellular recruitment to bone occurs via E-selectin, a lectin recognizing sialofucosylated determinants. As native CD44 lacks the key α-1,3-fucosyl hematopoietic cell E-selectin/L-selectin ligand, an enzymatic α-1,3-fucosylation of MSCs CD44 was performed under physiological conditions specifically designed for treating live cells. This modification conferred potent E-selectin binding and tropism to bone, without detrimental effects on cell viability or multipotency. This study unveiled a great potential to program cellular trafficking on through membrane glycoprotein engineering for directing cellular migration, a key issue for cell therapies.

An example of cell surface glyco-engineering is related to blood group antigenic determinants. The antigenicity of the ABO blood groups is defined by the structure of carbohydrates units (Fig. 13.7) expressed on red blood cells surfaces (RBCs). Conversion of type A, B, and AB RBCs to universal 0 type by enzymatic removal of galactose or N-acetylgalactosamine antigenic carbohydrate residues by specific glycosidases has emerged as a useful strategy to convert all blood donations of different types into the 0 universal one. Although cell surface modification may be accomplished by suitable enzymes, in many practical applications, their poor association with cell surfaces due to the repulsion of two hydrophilic entities hamper this technology, because of the need of high concentrations of reagent that may be toxic for cells and making the process costly. Thus, the development of a general process improving enzymatic reactions on cell surfaces are particularly attractive. In this example, the enzymatic reaction on blood group determinants was performed with good efficiency by a strategy based on macromolecular crowding with biocompatible and cheap neutral polymers, such as dextran and ficoll [95], granting over 440-fold increase of enzymatic glycoengineering activity on cells surface. This research empha-

sized the relevance of discovering new and efficient methods for glycan-specific cell surface engineering that hold promise for clinical applications.

13.6 Conclusions

We showed several examples highlighting the involvement of carbohydrates in the modulation of cell adhesion, function, homing, and differentiation. The ability to exploit and mimic glycan roles in a tissue-specific manner will afford new opportunities in regenerative medicine, which in the next future will rely on the development of innovative and efficient glycoengineering technologies. However, research is still needed, requiring continued crossing of disciplines, heavily including chemistry, along with complementary contributions of medicine, biotechnology, biology, engineering, and material science. In addition, a better understanding of glycobiology of stem cells and signaling within complex extracellular matrix interactions is still needed in order to fully exploit the potentialities given by the glycocode.

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Biomimetics: A New Abstraction for Bone Implant Design

14

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14.1 Introduction

Biomimetics and biomimicry are thought processes applied to biomaterial design, where biological implants have properties which mirror closely those of natural material. Designing implants with this mindset may elegantly circumvent some of the roadblocks in synthetic biomaterial design and function [1]. Bone regeneration is a particularly attractive frame for this work, given the tissue heterogeneity and mechanical role of bone. However, in recent years, problems with physical shape, surface chemistry, and mechanical properties have been identified in implants. Implant failure through peri-implantitis, peri-mucositis, peri-implant disease, or infection can lead to pain, mechanical loosening, and eventual need for extraction [2]. Similarly, some literature has outlined concerns with implants, specifically showing unfavorable physical remodeling over time [3]. Eliminating the variability associated with implants would mean a huge decrease in patient morbidity and cost burden. These concerns have ushered biomimetics and new material design techniques to the forefront.

14.2 The Recipient Site

Types of bony reconstruction range from structural nonload-bearing bone (e.g., calvarium) to larger compact load-bearing bone (e.g., femur). Host bone can be challenging, for instance if it is osteoporotic [4] or osteomyelotic [5, 6]. To ensure a fit of the implant in the host bone, underreaming is performed; a drilling process designed to facilitate the implant, sometimes known to fracture the host bone further [7]. It is especially problematic in the context of osteoporotic bone, which can exhibit low "pull out strength" of an implant once in situ [8]. Another quite common issue is poor nutrient diffusion, borne of compromised blood supply. Diffusion distance of oxygen in vivo is 150–200 μm [9]. As such, certain materials may stimulate vascular ingrowth into the host bone. Inactivators of prolyl hydroxylase (e.g., cobalt ions) have been shown to stabilize HIF-1α, a potent proangiogenic factor, resulting in upregulated expression of genes such as GLUT1, erythropoietin, VEGF, and PDGF [10, 11]. Bone that has undergone irradiation falls in the same category of hostile recipient sites. Macroscopically, a threaded implant (akin to a

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screw) gives immediate stability and provides close, fixed contact between the graft and host bone.

14.3 Mechanical Stability of Implant

Issues with bone implants chiefly lie in materials destined for load-bearing bone healing. The Young's modulus of trabecular bone is 10.4–14.8 GPa, and cortical bone measures 18.4–20.7 GPa [12]. Naturally, stainless steel has a much higher modulus of 180 GPa, cobalt chromium (Co-Cr) at 210 GPa, and titanium at 110 GPa [13]. "Stress shielding" is the physiological response resulting from implanting a harder material into a softer host tissue. The result is fibrous encapsulation of implant, allowing for micro-movement of the implant unit. Mobility of an implant creates a specific wear, called fretting [14]. Fretting implants can gradually loosen and eventually fail within the host bone [15]. Moreover, fragments of the implant are frequently known to break off the body of the implant, causing local abrasion of the surrounding, softer bone tissue [16]. Debris of implants can sometimes be found in alternate locations in the body, such as the spleen, liver, and abdominal lymph nodes of arthroplasty patients [17].

It was Albrektsson et al. in 1981 [18] who first showed the complete "osseointegration" of titanium. During a fracture fixation experiment in a rabbit femur, Branemark discovered the removal of the titanium implant from bone was completely impossible. Further studies using transmission electron microscopy showed a new phenomenon of direct contact between bone and implant, without the surrounding fibrous capsule responsible for implant looseness and micro-movement [19]. Filamentous collagen type 1 was found to form fibrils at the implant–bone interface (resembling the strong Sharpey's fibers of the scalp), giving the tight coupling of metal and bone [18]. The phenomenon of the absence of fibrous encapsulation around titanium implants has inspired implant design for more than just bone. Tissue expanders used for breast reconstruction can include a titanium-coated mesh to reduce the fibrotic content of the breast [20]. In the context of bone, titanium is now being tested in advanced models of craniofacial bone healing, using titanium granules to stimulate maxillary sinuses [21]. However, in terms of biomimetics, titanium presents some mechanical challenges, specifically in terms of difference in stiffness between it and the host tissue, for example, femur. This hurdle is called modular mismatch [22] and can be dodged by using material whose bulk mechanical properties more closely resemble bone.

14.4 Grafts Based on Bone Mineral Components

The unique nature of bone is that it is largely mineralized. In its dry mass, bone is 60–70% mineral, which is non-immunogenic and ubiquitously found (Fig. 14.1) [23]. As such, the use of natural material already in existence presents an option for creating biomimetic implant matter. Nacre, or mother-of-pearl, is pure calcium carbonate produced by mollusks. Mixing pulverized

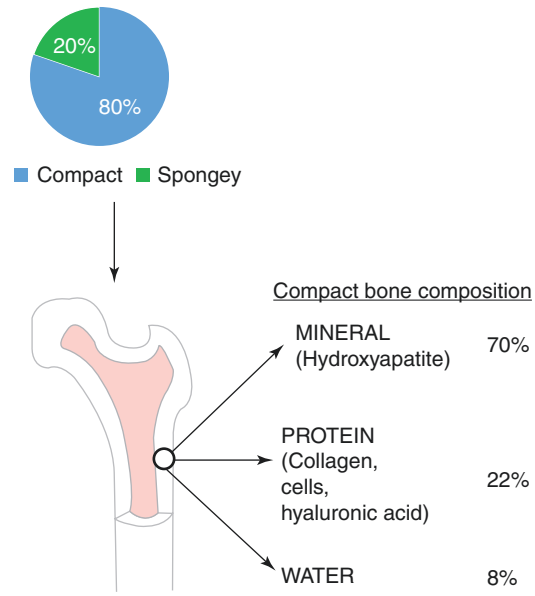

Fig. 14.1 Bone composition

nacre with patient blood and implanting the mixture into a human mandibular defect site was largely effective in closing the defect and stimulating regenerative cellular activity in the location [24]. Many successful bone biomaterials incorporate calcium or hydroxyapatite to help facilitate bone formation and graft "take." A study comparing biologic bovine-derived bone grafts (BioOss® Bone Substitute; Ed. Geistlich Soehne, Wolhusen, Switzerland), with a highly porous synthetic hydroxyapatite scaffold (IngeniOs™ HA Synthetic Bone Particles; Zimmer Dental, Inc., Carlsbad, CA), showed highly similar chemistry, morphology, and structure [21]. FT-IR spectra revealed high crystallinity (thus low resorption) of the synthetic IngeniOs Hydroxyapatite Synthetic Bone Particles, owing to the purity of its manufacturing processes versus natural variation [25]. The difference in purity between synthetic and biologic material by proxy of uniform industrial manufacture is seen throughout most implant forms.

Beta tricalcium phosphate (β-TCP) is a calcium salt abundant in bone, and has been shown to be highly and quickly reactive as part of a bone graft. This is because in an aqueous environment, TCP reacts to form hydroxyapatite [26]. Effects of TCP can be exaggerated with strategic addition of growth factors in vivo, as was seen throughout a series of randomized control trials using platelet-derived growth factor and β-TCP as agents to heal periodontal intraosseous defects [27]. In fact, β-TCP has been shown to contribute to bone healing faster than hydroxyapatite alone, secondary to its rapid rate of resorption [28]. Meanwhile, HAPEX is an amalgamation of the biologic mineral hydroxyapatite and synthetic high-weight polyethylene. This mixture makes for a bioactive polymer, which has been used in the reconstruction of orbital floor and middle ear defects [29]. Cerasorb is pure β-TCP, designed to be mixed with the patient's own blood or platelet-rich plasma and added to the defect site, primarily for periodontic healing. As a different oceanic source of bone mineral, coral holds properties which mimic bone. Coral forms hydroxyapatite on its surface due to its calcium carbonate core and also retains its native trabecular structure, having inherent biomedical value as a spongy bone substitute [30, 31].

14.5 Grafts Based on Structural Protein

An important component of bone structure is protein, both structural and supportive. Hyaluronic acid (HA) is a high-molecular-weight nonsulfated glycosaminoglycan, which is formed in the plasma membrane of cells [32]. It is a highly negative molecule capable of attracting proteoglycans, which in turn harbor water. Logically, ECM scaffolds containing HA have reached the market for dermal applications, given that hyaluronate is an antifibrotic, hydrating agent in a healing wound [33]. However, in the context of bone grafts, rabbit tibias which received HA showed increased healing 20 days after injury, exhibiting fibrocartilage formation, which later ossified, in comparison to the non-HA-treated bones, which formed purely fibrous unions [34]. More sophisticated, combinatorial approaches have been designed around HA, e.g., use of HA in a hydrogel with calcium sulfate hemihydrate, a bioresorbable, osteoconductive compound [35].

Composite biomimetic grafts over simple hydrogel injection are required for healing of large load-bearing bones. For instance, addition of protein to a hydrogel or impregnated into an implant introduces necessary cellular osteogenic mechanisms. In a canine femoral defect model, bone morphogenetic protein (BMP) in tandem with collagen type-1/TCP showed increased healing of the femur in the presence of bone marrow aspirate [36]. These studies suggest collagen type 1 as a logical and functional agent to match the modulus of the femur. Similarly, there is utility of bone marrow-derived cells stimulated by pro-osteogenic growth factors in bone healing.

A highly osteogenic component of bone is the periosteum, a stratified structure of an inner cell layer (cambium layer), and tough, fibrous outer layer [37]. Damaged bone which under-

goes delayed reconstruction may show heterotopic ossification, disorganized bony formations at the injury site due to disrupted periosteum [38]. As such, strategically placed periosteal grafts present a highly biomimetic solution of autografting onto damaged bone [39]. Due to the sheet-like structure of the periosteum, periosteal grafts have found their primary utility in dental and alveolar grafting, as opposed to long, load-bearing bone reconstruction [40].

14.6 Grafts Based on Cellular Implants

Despite orthopedic management of fractures becoming better and better, some healed injuries will persist with fibrous nonunions [41]. To address this, "The Diamond Concept" has been developed, which encompasses four different aspects of in vivo bone regeneration: an osteoconductive scaffold, a suitable mechanical environment, osteoinductive signals, and a pro-osteogenic cell population [42].

The ability to direct stem cells to an osteogenic pathway represents a huge regenerative role. Addition of cells to bone grafts is a concept based on isolating cell types, which will either immediately and directly add bone (osteoblasts/osteoclasts), or have the potential to differentiate, providing angiogenic and osteogenic factors (mesenchymal stem cells (MSCs) and adipose-derived stem cells (ASCs) [43].

Cell surface markers indicative of osteogenic behavior can vary from poorly defined CD markers to more well-known pro-osteogenic growth factor receptors. BMP receptor type-1b (BMPR-1b) binds BMP and has an important role in directing bone formation [44]. As such, using FACS to select for BMPR-1b-positive cells from ASCs results in a population with increased osteogenic gene expression and in vitro osteogenic potential [45]. Moreover, when coupled with a porous, osteoconductive scaffold coated in osteoinductive hydroxyapatite, rapid bone formation is observed in vivo [46]. Similarly, ASCs positive for CD90 (Thy-1) have been shown to significantly increase healing of bone defects when compared with their negative and unsorted control groups [47, 48]. Importantly, CD90 expression has been shown to vary dramatically between in vivo and in vitro settings, making use of this marker unpredictable [49]. Similarly, other markers also have difficult expression profiles to track and analyze, such as CD105 (endoglin), a bone marrow mesenchymal cell marker. However, it was discovered that isolating CD105-negative cells and waiting 36 h in culture yielded a subpopulation with enhanced osteogenic potential in vivo [50]. CD105 is especially nuanced, although in that it acts as a co-receptor for TGF-β1, which is a known antagonist of osteogenic differentiation [51], thus explaining a parallel decrease in bone formation with increased expression. These findings simultaneously highlight the promise and problems of using surface marker selection criteria in isolating heterogeneous stem cell populations for bone regenerative purposes.

There are multiple different bone diseases being researched under an autologous cell transfer lens. For instance, the efficacy of bone regeneration by autologous bone marrow harvested from the anterior iliac crest has been shown in atrophic diaphyseal nonunion. Here, a biomaterial was created by concentrating marrow via centrifugation, which could be loaded into a syringe and injected into the recipient site. Analysis of diseased tibias postmarrow transplant showed increased bone callus mineralization [52]. Similarly, osteogenesis imperfecta (OI) is a genetic disease of the mesenchymal cells, whereby a defective collagen type 1 is produced, giving rise to bone weakness and malformation. Unmanipulated bone marrow donations from healthy matched siblings or family members have been shown to increase trabecular bone formation in the recipient OI patient [53]. On the cellular level, the harvest, culture, and transplant of bone marrow-derived stromal cells have been found to be effective in repairing large bone defects in human [54]. However, these cells are most efficient when placed in situ seeded on a macroporous scaffold [55].

14.7 Conclusions

In order to medically complete bone regeneration, the current therapies must evolve in accordance with current research. Biomimetics drives creativity and innovation to emulate the healing mechanisms already in place. Due to the biological nature of much of the research mentioned in this chapter, product development still faces considerable regulatory hurdles. However, the baseline knowledge derived from experiments and research listed herein is a great platform upon which therapies can be built. The biomimetic boundaries to be pushed in bone regeneration are collagen or collagen analogs, soluble minerals, and active cell populations.

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The Role of Microbiota in Skin Regeneration

15

Angela Faga, Marco Mario Tresoldi, and Giovanni Nicoletti

15.1 Introduction

Regeneration can be defined as the regrowth of a damaged or missing organ part from the remaining tissue. It is a common feature in different animal species, as amphibians; on the contrary, in humans the wound healing process does not lead to regeneration but to a scar. Actually humans are not lizards; therefore, they pay their position at the endpoint of phylogeny with an almost total loss of the regenerative attitude, except for liver and, sometimes, for fingertips [1].

Everyday plastic surgeons face disfiguring or aesthetically impairing scars, huge loss of substance or difficult to heal wounds. Despite the current high sophisticated technical skills, the problem of regeneration is impairing severely the treatment of all of these clinical problems. Therefore, current plastic surgery is increasingly turning to regenerative medicine, an emerging interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs [2].

The wound healing process consists of highly integrated and overlapping phases, whose steps are clot formation, inflammation, fibroblasts recruitment, epithelization and scar remodelling.

Many types of immune and non-immune cells, including macrophages, neutrophils, platelets, fibroblasts, vascular endothelial cells and keratinocytes, contribute to wound healing. The inflammatory response begins immediately upon injury and leads to the secretion of a variety of growth factors and cytokines, which regulate the cellular and tissue movements that are required for repair [3, 4]. It is proven that the proliferative phase of wound healing is inversely proportional to the quantity of inflammatory post-traumatic reaction [5].

Multiple local and systemic factors can interfere with one or more of these phases, thus causing improper or impaired tissue repair; the main local barriers are necrosis, bacteria and exudates.

Regarding the negative role of bacteria, surgeons have slowly learned from Semmelweis' time [6] to fear and fight microbes by scrubbing their hands before surgery, treating the operating

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field with antiseptics and administering antibiotics to the patients. Actually, the infection of a wound turns to dehisce and to a clinical disaster.

In the past, the role of microorganisms was investigated only during pathologic events; however, the availability of upgraded technologies is currently allowing a more sophisticated and thorough analysis of their association with the host.

Microorganisms are present in every type of environmental niche and interplay with all its components.

The term microbiota is used currently to define the microbial community, composed of bacteria, viruses, fungi and Archaea, hosted by the human body [7]. Actually the microbiota might be considered as a 'superorganism' [8], ruled by specific inter-microbial communications and cell signalling associations with the human organism. Host-microbe interactions are essential for various aspects of normal mammalian physiology, ranging from metabolic activity to immune homeostasis [8–11].

Every area of the human body hosts a unique microbial community.

Microbial cells living in the human body outnumber the totality of cells of our organism by a factor of ten, and the genes of those microbial cells outnumber the human genes by a factor of hundred [12]; the term microbiome is currently used to indicate the whole genome of the microbial community.

From 2008, starting with the U.S.A. Human Microbiome Project (HMP), different studies were performed to establish any possible associations between the human microbiota and health and disease conditions [13, 14].

The difficulty of recreating the same conditions in vitro as in vivo is a major humper for the microbiota characterization [15]. Thanks to current metagenomics techniques, it is possible to screen the whole genes of an environmental sample and to create a genetic library of the proteins expressed by the community [16]. Moving from conventional cultures to metagenomics, a perspective change is occurring, focusing on the activity and products of a whole community, rather than of a single community member [17].

15.2 Skin Microbiota

Skin is the first defensive line of the human body and houses different populations of microorganisms. Every squared centimetre of skin (including hair follicles and sebaceous glands) contains ~1 billion bacteria [18].

The acquisition of the microbiota begins at birth [19], with the transfer of maternal microbiota, and differs according to vaginal or caesarean delivery. The skin microbiota evolves over the years, becoming similar to that of adults by the age of 12–18 months [20]. Basically, the microbiota composition is related to the skin structure and physiology [21] and to the adnexa-regulated microenvironment. Furthermore, microbiota composition changes according to ethnicity, geography and lifestyle [12]. Individuals modify their own microbiota through contact with other individuals, visiting different places, and eating food. The microbiota composition is dependent on the anatomical area too. Microbiota α-diversity expresses the difference in community composition, comparing a specific area of the body to other areas of the same individual. Microbiota β-diversity expresses the difference in community composition comparing a specific area of the body between different individuals. Notably, antecubital fossae have the highest β-diversity, but the lowest α-diversity [22]. The differences among different individual microbiotas are due to the less represented bacteria.

Most skin microorganisms are commensal or temporary passing members. The following four bacterial phyla are present on human skin: Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes. *Staphylococcus, Propionibacterium* and *Corynebacterium* are the dominant genera, and their inter-individual distribution is constant. Furthermore, on normal human skin viruses (human papillomavirus, human polyomavirus, circovirus and bacteriophages) and eukaryotes microbes (fungi and protists) are present [23]. Considering the community composition in different individuals, it is possible to state that the microbiota of an individual is as unique as a fingerprint [24, 25].

Microorganisms do not inhabit just the epidermis. Metagenomic techniques demonstrate bacterial DNA deep in the dermis too, although such a technology may not assess the viability of the associated microorganisms [12, 23]. Phagocytic cells might translocate the superficial microbiota to a sub-epidermal level and epidermal physical barriers or antimicrobial peptides (AMPs) would serve as key regulators in the maintenance of dermal microbiome homeostasis [26]. The action of microorganisms below the basement membrane might be in correlation with the immunological properties of the skin. The skin is an active immune organ where the keratinocytes can no longer be considered as the sole barrier against the external environment, but as components of a complex immune-regulatory network [27]. The resident skin bacteria provide the first line of defence against potentially dangerous pathogens, and produce small molecules that influence the growth and behaviour of their microbial neighbours. The major innate mechanism of the antimicrobial defence of the skin consists of AMPs, such as defensins, cathelicidin LL-37 and dermicidin [28]. These peptides are emerging as important tools in the control of skin pathogenic bacteria. It is proven that skin commensal bacteria have a promoting effect on T cell response, controlling nuclear factor-κB signalling and the production of cytokines, such as tumour necrosis factor (TNF)-α and interleukin-1β [29, 30] although they can act both in synergy and in opposition to the immune system [31].

Germ-free mice without commensal skin microbes have been demonstrated to produce abnormal cytokine and cutaneous T cell populations, as they were unable to mount an appropriate immune response against the intradermal *Leishmania major* infection; immunity could be rescued by allowing *Staphylococcus epidermidis* colonization on the mouse skin [30].

As skin microbiota is important in the development of a well-functioning immune system and in the modulation of the inflammatory processes, it may be significant in the wound healing process.

15.3 The Role of Microbiota in Wound Healing

Actually, although many animal species regenerate spontaneously in the wild life, this process does not take place in a germ-free environment.

While the role of microbiota in controlling the health-disease balance is being widely investigated as a source of novel therapeutic options, however the local application of bacterial products to enhance wound healing has been reported rarely.

Topical bacterial lipopolysaccharide is demonstrated to affect the wound healing process by accelerating the resolution of inflammation, increasing macrophage infiltration, enhancing collagen synthesis and altering the secretion of numerous mediators involved in skin regeneration [32]; moreover, inoculation with *Pseudomonas aeruginosa* is demonstrated to accelerate re-epithelialization and neovascularization in wound tissues through the production of TNF-α [33]. All of these effects might be related, although not exclusively, to some bacterial both anti-inflammatory and antibiotic-like actions, due either to microorganisms metabolites, or to a virtuous balance among strains, or both.

It has long been known that the recovery of skin lesions and scar maturation can be boosted by the topical applications of spring waters where a rich presence of non-pathogenic microflora is established [34–38].

In recent years, researchers have developed programmes to investigate the molecular mechanism underlying beneficial effects of spring waters. *Aquaphilus dolomiae* is a non-spore-forming bacterium belonging to the Neisseriaceae family, which is isolated from Avène thermal water (France), historically used in the management of chronic inflammatory skin diseases. The incubation of human keratinocytes with I-modulia, a biological extract from cultures of *Aquaphilus dolomiae*, showed an upregulation of the innate immune response [39, 40]. Similarly, the lysate of *Vitreoscilla filiformis* has been shown to enhance skin defence mechanisms and to decrease UV-induced sunburn cells in human

skin, possibly by the activation of cutaneous regulatory T cells. *Vitreoscilla filiformis* is a filamentous Gram-negative aerobic bacterium belonging to the Neisseriaceae family found in LaRoche-Posay thermal water (France), historically applied to manage chronic inflammatory skin diseases [41, 42].

Our research group is originally assessing the peculiar regenerative properties of a thermal spring water (Comano, TN, Italy). In an experimental animal (rabbits) wound model, the areas treated with this water healed faster than the areas treated with conventional medical dressings and demonstrated a network of collagen and elastic fibres comparable with the normal skin [37]. In an in vitro trial on human skin fibroblasts cultures, we observed that the vitality of cells maintained in DMEM (Dulbecco enriched conventional medium) 20% replaced with the Comano water, at 72 hours was 31% higher than the control cultures maintained in conventional DMEM [43]. Moreover, in an ex vivo human skin experimental wound model, used to simulate the physiological conditions in vivo, the adjunct of this spring water to the culture medium induced a faster repair of the wound, a more ordered reorganization of the newly formed collagen fibres and a higher number of actively DNA synthesizing epithelial cells [44].

In this spring water with conventional culture methods nine non-pathogenic different strains were isolated, whose common features are a very rare virulence, an antibiotic-like activity and the ability to control environmental pollution [38].The whole bacterial flora genoma is being investigated by the research group of CIBIO laboratories (Trento University, Italy) too, integrating culturomics and shotgun metagenomics. Preliminary results demonstrate that while the isolates are phylogenetically distributed among traditional phyla (Proteobacteria, Actinobacteria and Firmicutes), most of the metagenome-assembled genomes belong to phyla that are typically recalcitrant to cultivation, and many new species have been isolated or detected [45, 46]. The researchers are also testing specific strains to detect any immune modulating, proteolytic and anti-bacterial activity, likely related to the proven role of this water in the wound healing process.

Thus, the role of microbiota in the skin health maintenance appears to be of paramount importance and, therefore, indicates towards a hypothesis of exerting a positive effect in the skin healing processes.

15.4 Conclusion

The mechanisms on which the microbial community structure and the association between host and symbiont are based must become incorporated into the current definition of human health. Medical intervention must aim to minimize or avoid damage to health-associated homeostasis between humans and their microbiota; therefore, the therapeutic strategies to maintain healthy skin may require the inhibition of the growth of pathogenic bacteria, as well as the promotion of a balanced microbiota. Ignoring the association between host and microbiota in therapeutic planning is a shortsighted conduct, as demonstrated by the spread of antibiotic-resistant microorganisms [47]. Thus, the role of microbiota is resulting in novel and fascinating scenarios in regenerative medicine and surgery, promoting unexpected progress in the development of novel clinical proposals, not only for tissue regeneration and wound healing but for anti-ageing purposes, too.

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16 Erythropoietin: An Innovative Therapeutic Approach in Thermal Trauma

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16.1 Introduction

Erythropoietin (EPO), whose existence had been postulated already more than a century ago [1], is an endogenous hormone that is produced by fibroblasts of the renal parenchyma depending on the partial pressure of oxygen in the peripheral blood. It controls the differentiation of erythrocytes in the bone marrow. The first isolation of the protein succeeded half a century ago (1953) [2]. However, it took more than a quarter of a century (1984) to develop genetically modified hamster cells, which produced recombinant human EPO. Only thereafter the pharmaceutical mass-production could start. In the meantime, EPO has become indispensable in clinical routine: in anemia of end-stage renal failure, in tumor-induced anemia, and before donating autologous blood [3]. About 15 years ago, first papers were published on the non-erythropoietic effects of EPO [4].

16.2 EPO Effects After Trauma

Since then, numerous publications have demonstrated that EPO plays a key role in the response to both acute and chronic tissue damage. EPO inhibits the initial inflammatory response in a variety of tissues, thereby facilitating healing [5]. Also in the regenerative phases of wound healing, various EPO effects have already been published. These include, for example, the inhibition of apoptosis [6, 7], which has been described in particular for endothelial cells of the capillaries and other small blood vessels that run in the immediate vicinity of the trauma zone. These are often damaged by the reactions of the inflammatory phase and because of apoptosis. This in turn means that thromboses develop, and thus the trauma zone is ultimately enlarged.

The stem cell recruitment [8] is described, for example, for endothelial progenitor cells, which are recruited locally and from the bone marrow. Effects on mesenchymal stem cells have also been described. In vitro experiments confirmed the effect of EPO on human dermal mesenchymal stem cells cultured under hypoxia conditions. In addition, increased concentrations of IL-6 were added to the culture medium, which alone had a markedly antiproliferative effect. In addition, EPO added to the culture medium showed an increased proliferation rate [9].

The release of different growth factors [10] is also influenced by EPO. Angiogenesis is stimulated on the one hand by the release of relevant growth factors and on the other hand by the above-described effects of stem cell recruitment and inhibition of apoptosis. The increase in re-epithelialization is also caused by the secreted growth factors [10]. Comprehensive reviews of the acute and longer-term anti-inflammatory

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and pro-regenerative effects of EPO have been published by Brines et al. [11] and by Arcasoy [9] in 2008.

16.3 EPO Receptors

The different effects of EPO within the different organ systems (erythropoiesis, versus anti-inflammatory and pro-regenerative effects) can be explained by a different affinity for the individual receptor types and receptor subtypes [12]. The tissue-protective properties are mediated by the EPO hetero-receptor, whose affinity for EPO is lower than that of the EPOR2 receptor, which mediates the erythropoietic effect of EPO. The EPO hetero-receptor is usually not detectable in healthy tissue, but has been described post-traumatically [11]. Among other things, this could explain why EPO is only pro-regenerative and pro-proliferative after trauma [5]. As the proof of the EPO hetero-receptor has not yet been achieved beyond doubt, this approach is still controversial.

A major problem in the study of the non-hematopoietic effects of EPO is that the previous antibodies to the EPO hetero receptor are in all likelihood non-specific. It turned out that the antibodies used also bind to other cytokine receptors, which makes it difficult to interpret the results [13, 14]. It is therefore particularly important in the future to re-evaluate the present results with new, highly specific antibodies that are being intensively worked on. Ultimately, only with the help of a specific antibody it can be clarified whether a separate EPO hetero receptor exists and in which molecular signaling pathways it plays a role.

16.4 Adverse EPO Effects

In addition to the desired effects of EPO, undesirable effects are also described [3]. Three of these are known only for longer-term EPO therapy: increased risk of thrombosis [15, 16], antibody formation [17] against rhEPO, and an increase in blood pressure [18, 19]. Regarding the thrombophilia caused by EPO, however, the group around Corwin has shown that in patients with adequate weight-adapted thrombosis prophylaxis using low-molecular-weight heparin, the thrombosis tendency is not greater under EPO therapy than in the control group without EPO administration [20].

The blood pressure increase problem has so far only been described in patients who had previously exhibited blood pressure abnormalities. Here, an acceptable risk minimization should be feasible through careful evaluation and patient selection. An unprecedented problem is the treatment of tumor anemias with EPO. In principle, EPO is approved for the treatment of tumor anemia. In the meantime, however, numerous studies have shown that EPO therapy has a negative effect on patients' survival time [19–21]. The discussion of whether this is due solely to the optimized oxygenation rates after anemia correction or to the pro-angiogenic and anti-apoptotic or the pro-thrombotic effects of EPO, has not yet been completed. However, a direct, active oncogenic effect of EPO has not yet been demonstrated [21, 22].

16.5 EPO in Severely Burned Patients

As early as 1972, the concentration of autologous EPO in patient blood was investigated in anemia after thermal trauma [23]. In particular, it was noticeable that patients with burns below 30% TBSA generally had normal EPO blood values on the one hand and were not subject to transfusion on the other hand. In contrast, patients with burns above 30% TBSA were usually found to have markedly low EPO levels in the blood. In addition, these patients were usually anemic. It is also striking that patients with pronounced bacterial wound infections had even lower EPO and pronounced anemia values, and therefore all patients in these two groups were subject to transfusion [23].

In the 80s and 90s followed a series of publications, the aim of which was the anemia correction in severely burned patients by EPO

application. In none of the publications a significant reduction of the number of transfused blood products required could be detected; an effective increase in erythropoiesis, which had been intended, could also not be demonstrated. Effects on wound healing are not described, as they were not the subject of the investigations [24, 25].

It is now known that erythropoiesis is not likely to be increased by EPO administration in critically ill patients. In a mouse model, it could be shown that after an adequate thermal trauma a general depletion of the bone marrow occurs whereby the erythropoiesis and the lymphpectoris are more affected than the myelopoiesis. This depletion is refractory to EPO therapy [26]. However, this fundamentally undesirable effect could be a benefit for patients injured by serious burns, as this would make the feared complications of excessive erythropoiesis very unlikely, and the pro-regenerative effects of EPO could be exploited.

16.6 Pro-Regenerative EPO Effects in Burn Injury Animal Models

In 2006, Galleano [26] published the first work investigating the pro-regenerative effects of rhEPO after thermal trauma in the mouse model [27]. Three groups were formed: Verum group: rhEPO 400 IU/kgBW/d for 14 days, placebo group: distilled water, control group: they were previously passively immunized against rhEPO and given rhEPO 400 IU/kg/d for 14 days. Significantly faster wound closure was associated with faster re-epithelialization in the verum group compared to the other two groups. Wound healing in the immunized group (control) was again significantly delayed compared to the placebo group (distilled water). In the respective comparison, the verum group showed significantly better epithelial proliferation, a considerably more mature extracellular matrix and pronounced angiogenesis [27]. This was demonstrated in particular by the higher microvascular density in the histological sections, which also had a corresponding increased CD31 expression, as well as increased VEGF and NO values in the samples of these animals. The blood count changes were not statistically significant, but on day 14, there was a slight increase in erythrocyte and reticulocyte counts in the animals treated with EPO.

After standardized water vapor scalding, the effect of topically applied EPO was investigated in a mouse model. It showed that the deep dermal scalding in the treated with EPO hydrogel animals healed much faster, the re-epithelialization was completed earlier. Increased epithelial proliferation, faster formation, and maturation of the extracellular matrix, as well as marked angiogenesis induction and consequent higher capillary densities were also demonstrated with high CD31, VEGF, and eNOS levels [28]. In another work, the combined presence of EPOR and the EPO hetero receptor could be detected in both healthy and scalded mouse skin. In the healthy skin, a clear reduction of the EPOR expression after EPO application could be detected, in the thermally injured not, here the expression rate remains high. Likewise, a faster and higher-quality wound healing (dermis /epidermis papillae, maturity of the extracellular matrix) could be demonstrated by EPO application [29].

16.7 Experiences in Low-Grade and Severely Burned Patients

A pilot study for topical use was performed on 11 low-grade burned patients. In this project, EPO-hydrogel was applied locally to split skin donor sites or placebo hydrogel was used in the controls. A faster healing of the split skin donor sites in the verum group could be observed. For example, complete healing of the split skin donor sites treated with EPO hydrogel was observed after 7 days in 85% of patients [30]. Further healing attempts with topically applied EPO were carried out in pediatric scalding injuries. Complete healing of the affected areas within 10 days was observed in mixed 2a–2b scalding injuries.

To translationally review the promising results, hoping for a possible improvemend of wound healing in severely burned patients, a nationwide multi-center study funded by the Federal Ministry of Education and Research was

being carried out with systemically administered low-dose EPO (EPO in Burns, EuraCT Number: 2006-002886-38, Protocol Number: 0506, ISRCT Number: ISRCTN95777824) [31]. The objective of the original trial "EPO in Burns" was to demonstrate faster wound healing through the pro-regenerative and cytoprotective effects of systemic applied, low-dose recombinant EPO in thermally injured adult patients. Unfortunately, the results of "EPO in Burns" regarding the reepithelialization of the study wound did not show a conclusive result. A potential advantage in reaching the 100% re-epithelialization could be seen in the EPO group within the first ten days. Thereafter this trend changed into the contrary. Regarding results of several secondary endpoints, such as ABSI Score results the EPO group showed much better values and therefore a more positive prognosis, than the control group. ICH-GCP conform clinical trials are needed to investigate this findings more thorrowly [1, 2].

16.8 EPO Treatment for Prevention of Secondary Burn Progression

Further interesting reports on EPO effects in thermal trauma relate to the possibility that a "low-dose" EPO application can prevent the secondary burn progression of wounds within the first few minutes to a maximum of hours after trauma. In the animal model (rat), intraperitoneal administration of low dose EPO significantly reduced secondary burn progression within 45 min of standardized trauma. In the following days, the wounds also healed significantly faster. This promising approach is worth pursuing and, given sufficient positive data, performing ICH-GCP-compliant clinical trials.

16.9 EPO in Secondary Reconstruction

EPO may also play a role in secondary, reconstructive operations in patients with severe burn injuries, especially in the free microvascular tissue transfer. Based on the assumption that EPO could be tissue-protective in ischemic damage. The investigation required experimental flaps, which are designed to develop a zone of persistent ischemia with subsequent necrosis.

As early as 2003, Rezaeian et al. [32] published the first paper dealing with the use of EPO to improve flap survival in randomly perfused flaps in the rat model. On the one hand, the flap survival was investigated, on the other hand possible EPO-induced undesired side effects, such as hematocrit and blood pressure increase, were evaluated. Short-term low-dose and high-dose EPO was associated with statistically significantly improved flap survival [32]. A statistically significant increase in hematocrit and blood pressure could only be detected in animals that had received high-dose EPO for three weeks. This significant increase correlated with a statistically worse flap survival.

Harder et al. [33] developed a mouse model that integrates a randomly perfused, laterally pedunculated musculocutaneous flap into a dorsal skin chamber. Untreated, this flap developed partial necrosis of approximately 50% due to persistent ischemia. Using intravital fluorescence microscopy, it is possible to repeatedly examine both morphological and dynamic changes in the tissue and vasculature of the flap at the same localization in the chamber window [33, 34]. A first study examined the efficacy of recombinant human EPO first detected in two different dosages 24 h prior to flap elevation, before induction of ischemic stress and following, repeated over 4 days. The administration of rhEPO showed significant, dose-dependent anti-inflammatory (i.e., decreased leukocyte-endothelial interaction, impaired cell apoptosis), and pro-angiogenic effect (i.e., microvascular neovascularization). The lower dose resulted in significantly improved flap survival compared to the untreated animals, whereas the ten-fold higher dose only marginally improved flap survival. Administration of rhEPO maintained perfusion in the capillaries of critically perfused flap areas. The significant increase in hematocrit, which could only be detected in the high-dose EPO group starting on the fourth day after first administration, led to a significant worsening of the flow properties in the flap, and thus poorer survival despite rapid and strong abolition of the EPO-induced anti-flammatory effect characterized by a decrease in cell apoptosis and leukocyte-endothelial interaction [33].

Having demonstrated that EPO is dose-dependently protective of critically perfused flap tissue, Contaldo et al. [35] investigated in the same mouse model the optimal time of EPO administration for flap elevation, the induction of persistent flap ischemia. For this purpose, low-dose EPO was administered over a period of 48 h either before (preconditioning) or after (post-conditioning) the flap elevation. In a third group, the mice received EPO overlapping both 30 min before and after flap elevation and 24 h (perioperative treatment) after flap elevation. Both preconditioning and perioperative treatment resulted in a significant improvement in flap survival as a result of maintaining capillary perfusion in the critically perfused portion of the flap. This, in turn, results from a very early EPO-mediated up-regulation of inducible nitric oxide synthase (iNOS) [34], which leads to dilatation of the afferent flap vessels. If EPO is administered only after ischemia induction (post-conditioning), this iNOS-mediated maintenance of flap perfusion cannot be induced in a timely manner. In addition, a VEGF-mediated angiogenic reaction associated with de novo formation of functional capillaries was demonstrated [34, 35].

In another work, Contaldo et al. [36] also demonstrated in a murine model that persistent vasodilations, and thus improved flap survival after perioperative EPO administration, are not only mediated by iNOS, but also due to more than five days of prolonged up-regulation of endothelial NOS (eNOS). In this work, it was also investigated whether EPO-induced and VEGF-mediated angiogenesis is indeed involved in improved flap survival. The co-administration of rhEPO and bevacizumab, i.a., VEGF receptor inhibitor acting as an angiogenesis inhibitor led to a failure of the EPO-induced angiogenic response. Interestingly, there was no change in flap survival after EPO alone. The authors concluded that EPO-mediated angiogenesis is not involved in flap survival under these modes of administration. This is probably because of the time delay, as the newly formed capillaries are functional only five days after flap elevation. This period is beyond the ischemia tolerance of the tissue or the demarcation of necrosis [36]. In analogy to microvascular flap scans, Contaldo and coworkers investigated the efficacy of EPO on musculocutaneous tissue undergoing a 3-h ischemic phase, followed by reperfusion. High-dose EPO were systemically administered either 1 or 24 h before ischemia [37]. The animals treated with EPO showed an increased expression of both the EPO receptors in skin and muscle tissue, as well as the NOS. These flaps and the animals showed less reperfusion injury than untreated animals, resulting in maintenance of capillary perfusion, decreased hyperpermeability of the vessels, and less inflammatory response [37].

Lindenblatt et al. [38] investigated NO-mediated tissue protection after systemic EPO administration on a collateralized island flap on the hamster. For this purpose, rhEPO alone or rhEPO was administered together with a non-specific NO-blocker L-nitro-L-arginine methyl ester (L-NAME). The rhEPO application led to a significant improvement of the flap perfusion, as well as to a weakening of the inflammatory reaction and the apoptosis rate. As the co-administration of rhEPO and L-NAME led to a complete abolition of tissue protection, the authors concluded that the protective effect is primarily mediated via NO [39]. An implementation of the findings obtained in the clinic would be very desirable, in particular the use of non-hematocrit-effective EPO dosages or the short-term use of higher EPO dosages before they become hematocrit effective to a problematic extent. Here, the local EPO application, possibly also high-dose or alternatively the use of modified non-erythropoietic EPO molecules is of particular importance [40].

16.10 Summary and Conclusions

The results of the presented, animal experimental investigations on thermal injuries are promising. Unfortunately ICH-GCP compliant clinical trials for EPO as a therapeutic agent to optimize wound healing could not show positive results. This so called “EPO-Paradox” was discribed before by Steppich [3]. In the animal model, damage to the flap caused by acute persistent ischemia, as well as ischemia/reperfusion injury, can be reduced by EPO application. This shows both a time dependence with regard to flap elevation (ischemia induction), as well as a dose

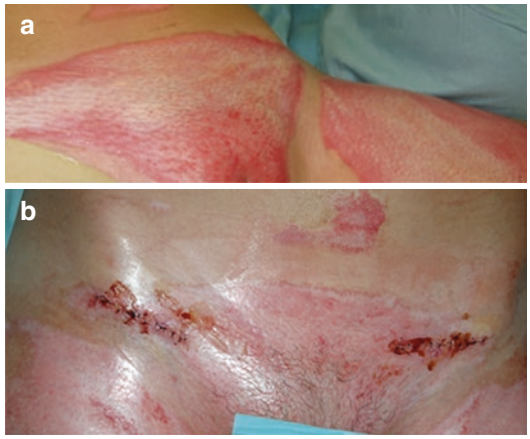

Fig. 16.1 (**a**) Scalding injury, 4 days after injury (2a°to 3°) First Treatment with EPO Hydrogel. (**b**) Result after 4x EPO Hydrogel Treatements

dependency of EPO. A significant increase in hematocrit, as observed in these models following repeated dosing of high-dose EPO, may worsen the flow of the blood, leading to thromboembolic complications [41], thus abolishing EPO-mediated protective, anti-ischemic effects. On the other hand, an anti-thrombotic effect of EPO applications has already been demonstrated in the mouse model [42], so that the scientific discussion is far from complete [43]. The tissue-protective effect of EPO appears to exist in ischemic tissue, e.g., flap models to be primarily NO-associated, in which with persistent ischemia, perfusion can be maintained in the critically perfused flap (area). Angiogenesis does not seem to play the crucial role, as the newly formed vessels are functional only after about five days, a time when the necrosis of the flap has already been irreversibly demarked.

For a possible individual use of EPO in the context of healing attempts in burn-injured patients, a very careful patient evaluation in the individual case must be advised. In particular, with regard to problematic pre-existing conditions such as hypertension, thromboembolic events or known malignancies, a particularly careful history-taking and consideration of the risk-benefit ratio must be ensured [44] (Fig. 16.1). The use of EPO as a routine therapy in the proregenerative field is not possible jet, as so far no clinical trial could demonstrate positive results. A possible alternative would be the further development and testing of non-hematopoietic EPO derivatives. Here, however, a longer time can be expected until they have overcome the hurdles of the necessary approval studies and other preconditions and are available for widespread clinical use [12, 45].

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Further Readings

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Targeting C-Reactive Protein in Inflammatory Disease

17

Johannes Zeller and Steffen U. Eisenhardt

17.1 C-Reactive Protein (CRP)

Despite extensive studies since CRP was first discovered and named by Tillett and Francis in 1930 [1], the exact role and mechanism of action of this prototypical acute phase reactant [2] has not yet been defined satisfactorily. Here, we try to give a brief overview of C-reactive protein as a major factor in physiological and pathological processes, and discuss its potential role as a rewarding therapeutic target.

C-reactive protein (CRP) is a member of the phylogenetically ancient and highly conserved pentraxin protein family. As such, it serves as a pattern recognition molecule in innate immunity. CRP production is evoked by the increase of circulating proinflammatory cytokines in plasma as a response to most forms of infection, inflammation, or tissue injury.

Interleukin-6 (IL-6) is the principal inducer of the CRP gene and regulates the expression through the activation of C/EBP transcription factors [3]. Additionally, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) potentiate the IL-6 effects, act synergistically and enhance the CRP expression at the transcriptional level [4, 5]. The CRP gene is located on the long arm (q-arm) of human chromosome 1 among other host protective genes. With a size of 2263 nucleotides, it extends from 1q21 to 1q23 with one single intron [6].

Although the main regulation of CRP gene expression happens on a transcriptional level, further post-transcriptional mechanisms have been reported. CRP is constantly synthesized at low rates and retained in the endoplasmic reticulum under physiological conditions [7]. However, during the acute phase response, the rate of secretion becomes more efficient, resulting in an acceleration of the CRP secretion, thus presenting a post-transcriptional regulation [8, 9].

The concentrations of circulating CRP in serum may therefore rise dramatically in a cytokine-mediated response from undetectable levels in healthy individuals up to 1000-fold and more within one to three days [10].

The hepatic synthesis is the predominant origin of CRP, and hepatocytes start with secretion of the acute phase reactant 6–8 h after the onset of inflammation or infection, while the inducing IL-6 levels rise within 1 h in conditions of tissue damage (Fig. 17.1) [11, 12]. Although CRP expression has been reported in various other cell types as well, e.g., neuronal cells in Alzheimer's disease [13], renal cortical tubular epithelial cells after inflammatory stimuli [14], arterial tissue, respiratory epithelium [15], adipocytes, and leukocytes [16–20], extrahepatic

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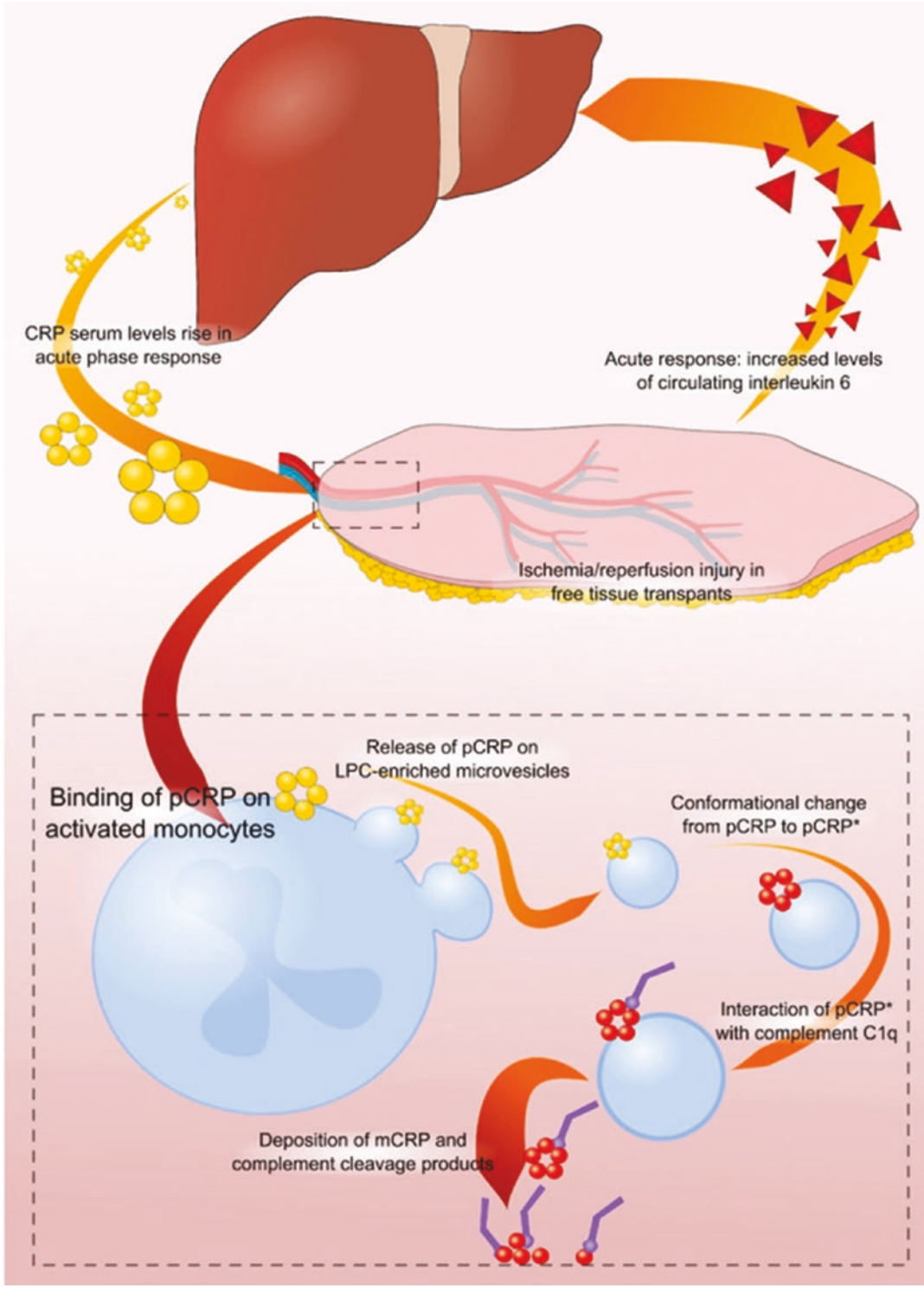

Fig. 17.1 Model of the genesis and the effects of CRP in ischemia/reperfusion injury

synthesis of CRP is not considered to affect plasma levels significantly.

The plasma half-life of CRP is about 19–24 h and it is cleared from circulation and catabolized by the hepatocytes. Plasma concentrations of circulating CRP remain unaffected by any physiological or pathological condition, and circulating CRP concentrations stable are solely dependent on synthesis rates [21, 22].

Therefore, CRP is an inflammation marker widely appreciated and extensively used in clinical practice. As the plasma levels are solely determined by the induction stimuli and synthesis rates, CRP conveniently serves in diagnosis and monitoring as a surrogate parameter for the intensity of tissue damage in trauma, inflammation, and infection [22]. To date, a plethora of studies have suggested that even slightly elevated CRP serum levels in apparently healthy humans, as measured with high sensitivity assays (hsCRP), are directly associated with an increased risk of coronary events [23–26]. Moreover, hsCRP may predict future clinical events among patients suffering various atherothrombotic syndromes [27–29]. In concentrations generally achieved during inflammation, CRP induces tissue-factor production in peripheral blood monocytes (PBM). The subsequent increased pro-coagulant activity may contribute to the development of thrombo-occlusive complications, as disseminated intravascular coagulation and thrombosis in inflammatory states [30, 31].

These studies suggested that CRP might have not only a predictive but also causal role in vascular disease. This sparked an interest of the role of CRP in perturbances of the microcirculation and in microsurgery. The wide distribution of CRP's main ligand phosphocholine (PC) as a constituent in pathogens (e.g., teichoic acid and lipopolysaccharides of bacteria) and apoptotic or necrotic cellular membrane [32–34], the conservation of its structure and the failure to detect any deficiency or mutation of this protein in human additionally suggests a pivotal physiological role of CRP in innate host defense [10]. While membranes of viable cells conceal the phosphocholine head groups, this major cell surface ligand of CRP comes accessible if cells undergo apoptosis and necrosis [32]. Therefore, CRP binds only to damaged or activated plasma membranes and promotes beneficial scavenging and host-defense functions [22, 35, 36]. Kaplan and Volanakis [28] were the first to describe the activation of the classical complement pathway by CRP-opsonized microbial polysaccharides. CRP mediates clearance by opsonization. CRP-opsonized particles can then be directly bound by Fcγ receptors [37, 38] and besides, CRP regulates the complement activation of the classical pathway commencing at complement C1q [39, 40], leading to phagocytosis by phagocytic cells [41, 42]. Further binding sites for other receptors on phagocytic cells have been suggested as well [43, 44]. The assumption of CRP enhancing the clearance of apoptotic cells appears even more likely as CRP also binds specifically to small nuclear ribonucleoprotein particles [45–47].

17.2 The Relevance of C-Reactive Protein in Plastic Surgery

17.2.1 Ischemia/Reperfusion Injury

In 1954, an innovative group around Plastic Surgeon Dr. Joseph Murray [48] performed the first successful kidney transplantation in history, a breakthrough in organ transplantation later honored with the 1990 Nobel Prize in Physiology or Medicine. Earlier attempts failed, inter alia, due to an ischemia-evoked inflammatory cascade [49]. Among the first to describe the common and relevant problem of ischemia/reperfusion injury (IRI) was Cerra et al. in 1975. IRI as a clinical phenomenon is of importance in a broad range of pathological conditions as myocardial infarction or stroke [50, 51] and describes an inflammatory reaction to reperfusion of previously ischemic tissue [52, 53]. In the pathogenesis, recovered blood flow subsequently to the ischemic period brings leukocytes into the area of impaired tissue and initiates pathologic leukocyte-endothelium interaction [54, 55]. In these conditions, the tissue damage is aggravated by the accumulation of activated white blood cells producing reactive

oxygen species (ROS) and the activation of the complement system [56–59].

In plastic surgery, reperfusion injury occurs primarily in the course of microsurgical free tissue transfer for reconstruction after tumor or injury, and after replantation of traumatic amputated limbs or their parts [53]. In a recent benchmark-study, we demonstrated that IRI in free human muscle tissue transplants launches molecular changes that lead to a significant upregulation of inflammatory parameters, transmigration of inflammatory cells, and angiogenesis [60]. In these pathological inflammatory conditions, CRP plays a causal role that leads to aggravation of inflammation and tissue injury [61]. We lately confirmed the significance of bioactive CRP deposits localized to myocardial tissue in a rat disease model of ischemia/reperfusion injury. The deposition of a tissue-bound conformer of CRP collocated with leukocytes was identified perivascular exclusively in the infarcted regions, but not in non-infarcted segments. Additional data demonstrated an agent-dependent stabilization of the circulating CRP and the prevention of an alteration to a conformer of CRP that exhibits strong pro-inflammatory properties. The blocking of the dissociation resulted in a distinct decrease of CRP deposition in ischemic perturbed muscle tissue and an inhibition of the CRP-mediated increase of leukocytic activity. This emphasizes the pivotal role of CRP alteration in the inflammatory exacerbation and concurrently the feasibility of this therapeutic approach [61]. Therefore, prolonged ischemia time during free flap reconstruction surgery may end in flap failure due to aggravating effects of the CRP deposits.

17.2.2 CRP in Severe Burns

Another clinical relevant occurrence of CRP in plastic surgery is in burn wounds/thermal injuries. In this form of critical tissue damage, numerous cells simultaneously undergo critical damage leading to apoptosis and necrosis [62]. Thereby exposed debris and apoptotic bodies function as danger-associated molecular pattern molecules (DAMP) and cause a rapid increase of acute phase reactants [63]. Serum levels of both the acute phase reactant CRP and complement have been shown to be elevated proportionally to the area and the depth of the skin involved in burn wounds [64–68]. If infection occurred in the burned area, the resulting acute phase response, and therefore the CRP production, was increased and prolonged [69]. Interestingly, however, a recent study conducted in our laboratories demonstrated that the deposition of C-reactive protein was restricted to burned areas, while healthy skin stayed unaffected. We found the deposited CRP co-localized with white blood cells and suggest a pivotal role of CRP in severe burns [62]. Regarding the clinical outcome, elevated levels of systemic complement have been linked to the progression of severe burn injuries and higher mortality [70, 71]. Furthermore, commonly encountered sequelae of major burn wounds as the intravascular hemolysis and thrombosis and deforming and disabling scar formation were reported to be related with increased complement levels [72–74]. Both the concordant rise of CRP and complement serum levels and the CRP-mediated and complement-driven inflammation are hinting at a significant causal role of CRP in the progression of pathogenesis in thermal injured patients. Thus, therapies that intend to lower CRP serum levels appear expedient to reduce the inflammatory response and improve prognosis [75].

17.3 Ischemia/Reperfusion Injury (IRI) and Allogeneic Transplantation

The transplantation of a functional composition of multiple tissues such as bone, muscle, nerves, and skin, summarized in the term Vascularized Composite Allotransplant (VCA), provides potential treatment solutions for complex tissue defects. The technology and knowledge for this upcoming branch of transplant surgery derive from clinical experience and research efforts in solid organ transplantations (e.g., heart and kidney), and became clinical reality with the first

successful human allogeneic hand transplantation in 1998 [76]. Unlike autologous transplantations, composite tissue grafts of allogeneic origin are subject to immune rejection by the recipient. Thus, clinicians face similar issues encountered in solid organ transplantation with the exception that VCA is usually not considered a life-saving treatment. The role of the innate immune system in recognition and eradication of microbial pathogens is relatively well understood [77, 78]. However, it remains elusive how sterile allografts trigger the maturation of antigen presenting cells (APC) in the absence of microbial-derived signals [79]. The so-called "danger hypothesis", a commonly accepted paradigm on the origin of APC maturation, holds particles of apoptotic and necrotic cells (damage-/danger-associated molecular patterns, DAMPs) in the transplanted tissue responsible for the activation of the innate immune response [80, 81]. Recently, it has been shown that innate recognition of allogeneic non-self by monocytes can initiate graft rejection in allogeneic transplantation that might represent another potential therapeutic target [79]. Furthermore, large retrospective studies on allogeneic organ transplantation demonstrated that an episode of acute graft rejection is a major risk factor for chronic rejection and a significant predictor of long-term allograft survival [82]. Consequently, solid organ transplantation and VCA are considered to be distinctively vulnerable to ischemia/reperfusion injury (IRI). Reflecting its novelty and therapeutic potential, the pathogenesis of vascularized composite allotransplants failure and the role of IRI- and CRP-induced activation of innate immunity are most in need of further investigation.

17.4 From Structure to Function

The pentraxin protein family is characterized by a cyclic pentameric assembly, sequence homology, and a calcium-dependent interaction with its ligands, respectively [83]. The interactions between the subunits in the CRP pentamer involve numerous electrostatic and hydrophobic bonding that arrange them symmetrically around a central pore forming a cyclic multimeric discoid wrath [84]. Each monomer of the pentameric protein is of identical molecular weight and structure [85]. Two antiparallel β-sheets of 206 amino acids in total length form one globular monomer of approximately 23 kDa molecular mass with a flattened Swiss roll typology [86, 87]. Electron microscopic and crystallographic data indicate that all CRP subunits show a specific orientation in the pentameric conformation. Therefore, the resulting two sides of the disc were suggested to have distinctive functions.

1. On one face, the so-called B face (or binding face), a hydrophobic pocket is presumably formed for the ligand-recognition of phosphocholine (PC) exposed on damaged or apoptotic cell membranes and pathogens. Two coordinated Ca^{2+}-ions adjected to the hydrophobic pocket mediate the major interaction between CRP and the phosphate head group of PC (Fig. 17.2) [84].
2. The opposite side is dominated by a single long α-helix and a deep and narrow cleft formation. Residues located or associated with this cleft enable the interaction with complement factor C1q and various Fcγ receptors, therefore, this face functions as the effector face (also "A" face) (Fig. 17.2).

The inaccessible location of these residues in the circulating pentameric configuration, however, suggests that the circulating pentamer is not the interacting form for host defense functions [32, 34, 84, 86, 88–91] and the pCRP is more a precursor form that does not significantly interact with the complement system [92].

17.5 Conformational Changes Alter the Function of CRP

A dissociation mechanism on activated platelets that causes a conformational change from the circulating native pentamer (pCRP) to the monomeric subunits of CRP (mCRP) has been identified [93]. Local environmental changes in pH (4.5–5.5) [94] and the accumulation of

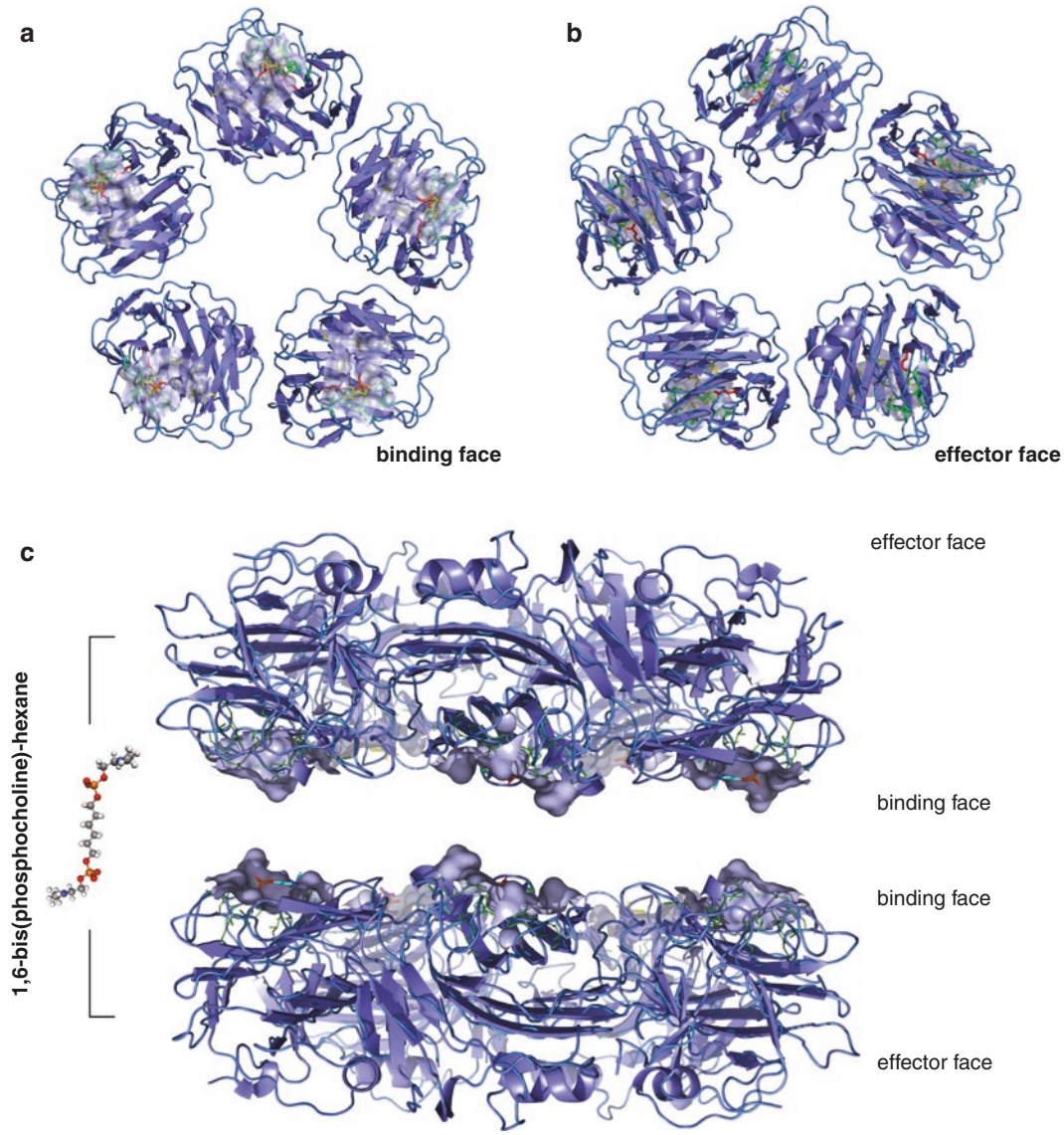

Fig. 17.2 Model of pCRP and its interaction with 1,6-bis(phosphocholine)-hexane

oxygen radicals [95] during inflammation, as in atherosclerosis, appear to facilitate the dissociation of pCRP in the site of inflammation. We demonstrated the process of pCRP dissociating to mCRP after binding to activated endothelial cell membranes in areas of acute inflammation in an in vivo rat model [61]. The emerging knowledge indicates that CRP can occur in vivo in at least two distinct conformers with distinct bioactivities, respectively. The pro-inflammatory capacity formerly attributed to CRP most likely is because of mCRP deposits. Under this new proposal, mCRP exhibits a significantly stronger binding potential to complement C1q compared with pCRP [96]. Accompanied with the alteration in structure, the functional properties of CRP in cell-interaction vary. Along with the dissociation to mCRP, CRPs obtain increased ability to activate monocytes. Activated monocytes show adhesion and transmigration, further, increased ROS activity, overall factors contributing in aggravating tissue inflammation [93]. In line with

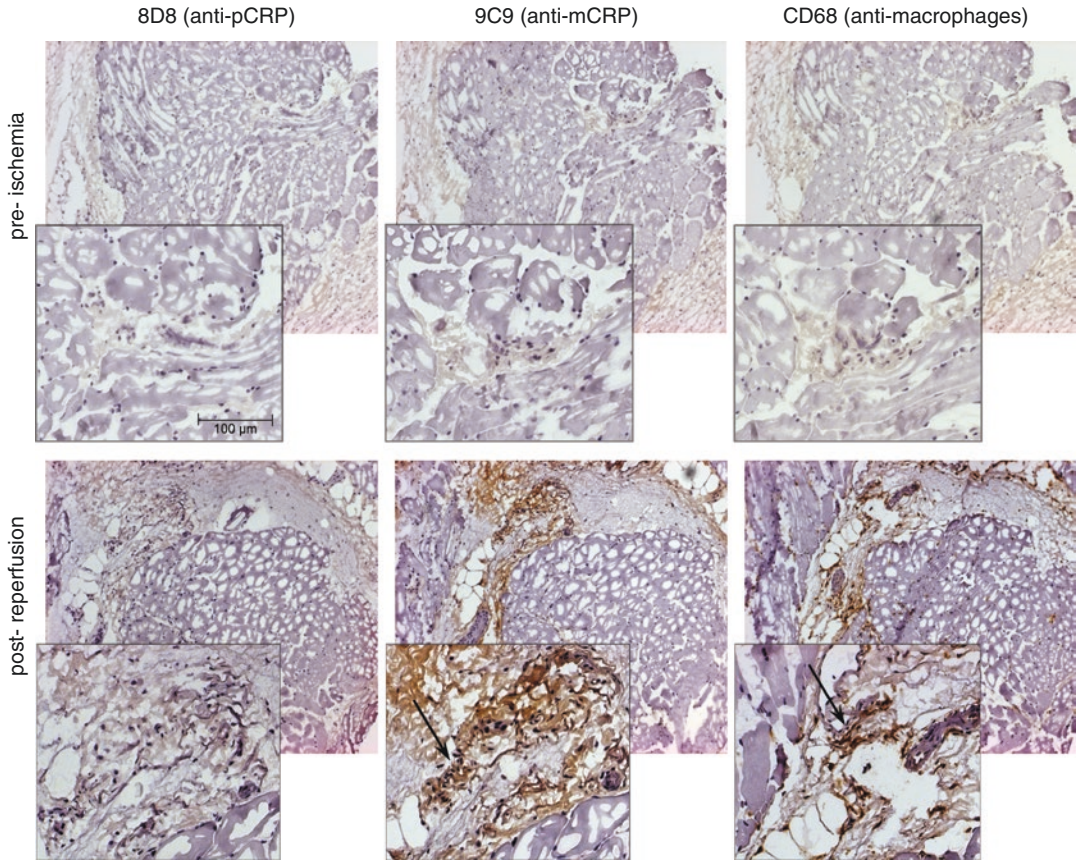

Fig. 17.3 Detection of mCRP and CD68+ cells in post-ischemic human striated muscle tissue. Immunohistochemical staining of muscle tissue with conformation specific antibodies. Antibody clone 8D8 was used to detect pCRP, and clone 9C9 was used for the detection of the monomeric conformation of CRP (mCRP). Right panel shows the co-localization of CD-68 positive monocytes/macrophages in the injured tissue. Reprinted with permission from "The Dissociation of Pentameric to Monomeric C-Reactive Protein Localizes and Aggravates Inflammation: In vivo Proof of a Powerful Pro-Inflammatory Mechanism and a New Anti-Inflammatory Strategy" by Thiele et al., Circulation, 2014 [61]

that, we detected in both human striated muscle biopsies of ischemia/reperfusion injury and biopsies of infarcted myocardium, mCRP, but not pCRP in the ischemia-affected tissue, which co-localizes with CD-68 positive monocytes/macrophages (Figs. 17.3 and 17.4) [61]. Recently, Braig et al. [97] identified a novel pro-inflammatory mechanism of CRP (Fig. 17.1). The findings suggest a cascade in which circulating pCRP undergoes a structural change by binding to cell-derived microvesicles without disrupting the pentameric symmetry (pCRP∗). In this in vivo and in vitro-observed process, circulating pCRP binds to perturbed plasma membrane of activated monocytes and is subsequently released on microvesicles. Microvesicles bud from cell membrane as lysophosphatidylcholine-enriched spheres of 100–1000 nm in diameter [98]. The microvesicle-bound pCRP undergoes structural changes and produce pCRP∗, a CRP isoform expressing the neoepitope of mCRP while maintaining an overall pentameric configuration. Thus, a CRP isoform, which, in contrast to circulating pCRP, exhibits a pro-inflammatory profile, is released into the surrounding tissue bound to plasma membrane-derived microvesicles [97]. In accordance with these findings, a prior study reported expression of neoepitopes on circulating microvesicles in patients following myocardial infarction [99].

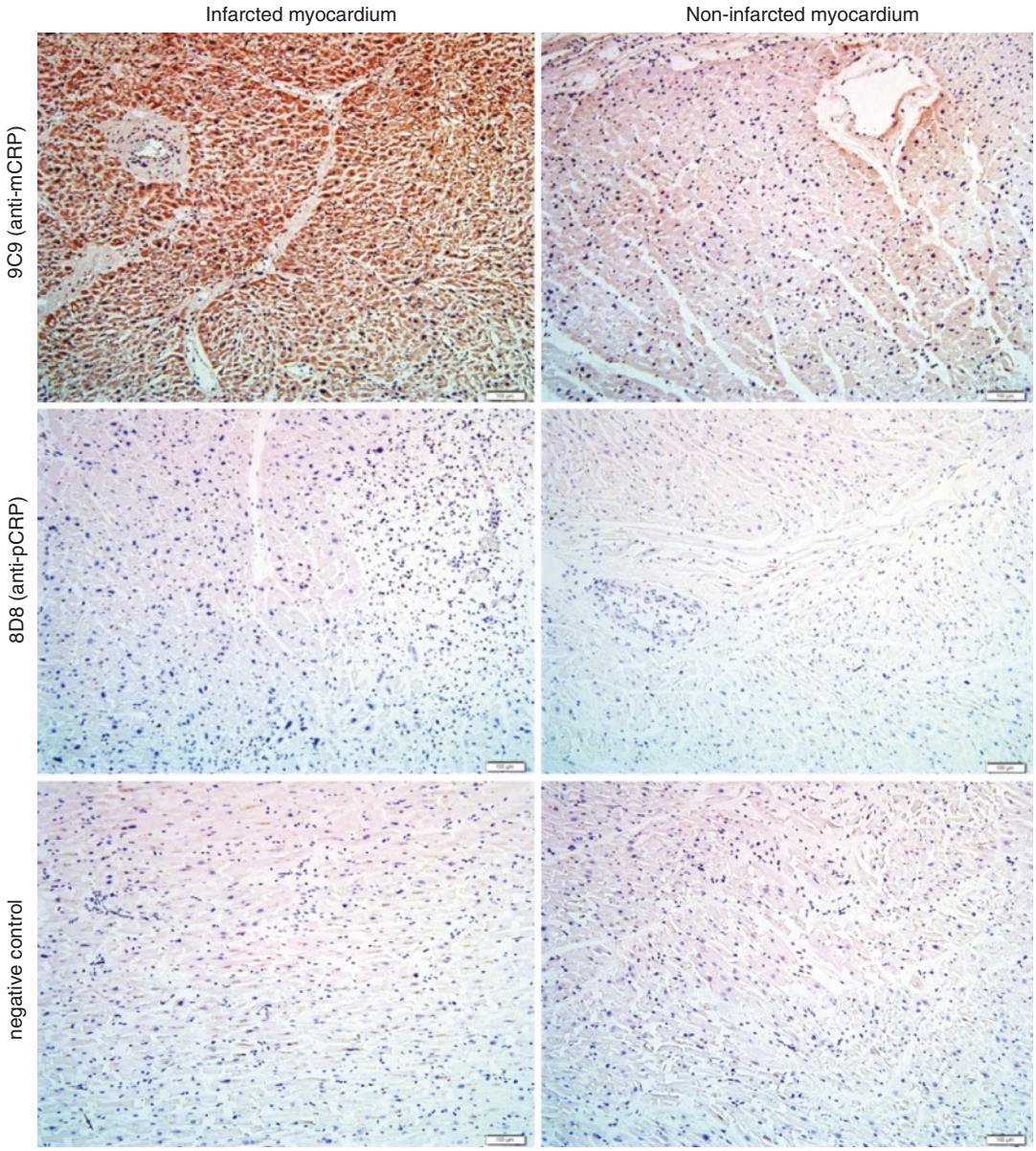

Fig. 17.4 Accumulation of mCRP in infarcted myocardium. Immunohistochemical staining of human post-mortem samples following myocardial infarction. The used conformational specific antibodies show significant deposition of monomeric CRP in the infarcted tissue, but only slightly (small amounts of peri-vascular staining) in the non-infarcted tissue. In both the pCRP staining and the control, there is no significant staining detectable. Reprinted with permission from "The Dissociation of Pentameric to Monomeric C-Reactive Protein Localizes and Aggravates Inflammation: In vivo Proof of a Powerful Pro-Inflammatory Mechanism and a New Anti-Inflammatory Strategy" by Thiele et al., Circulation, 2014 [61]

This novel understanding of the localized dissociating process of the inert circulating pentameric CRP to the distinctively pro-inflammatory monomeric derivative allows for a new view on CRP in inflammatory reactions. In addition, the findings emphasize the molecular structure-function relationships, and therefore highlight the dissociation process and mCRP as a potential therapeutic target of practical, as well as theoretical importance.

17.6 Therapeutic Targeting of C-Reactive Protein

Calor, dolor, tumor, rubor –a nearly 2000 years old attempt to characterize inflammation in four cardinal signs. Two millennia after Aulus Cornelius Celsus first recorded these words in his work De Medicina (De Medicina, AD25), our accumulated knowledge for the complexity of the processes in inflammation make such a simplification unreasonable and still much remains elusive. Yet, we know inflammation is a physiological and inevitable response to tissue injury and contributes to life preservation of the host, as reflected by the increased risk of severe infections in patients with deficiencies in principal components of the inflammatory process [100–102]. Therefore, anti-inflammatory strategies represent a two-edged sword and a sufficient inhibition must be ultimately purchased by a variety of considerable side effects. Currently, available anti-inflammatory drugs, e.g., non-steroidal anti-inflammatory drugs (NSAID), cytokine-inhibiting biologicals, or glucocorticoids exhibit systemic adverse effects even after local administration [103, 104], impair beneficial processes of inflammation as wound repair and angiogenesis [105–107], and increase vulnerability for infections and pathogens [108–112].

17.7 Why Is CRP a Rewarding Target?

Based on x-ray crystallography data [84], Pepys et al. [113] designed a proof-of-concept compound, 1,6-bis (phosphocholine)-hexane (1,6-bisPC), that inhibits the CRP-induced actions. The reported agent represents a specific small-molecule inhibitor of CRP. The concept utilizes the phosphocholine (PC) binding-site on the recognition face of CRP. It is suggested that the palindromic phosphocholine dimer crosslinks two pentameric CRP molecules, and thus make the ligand binding-site inaccessible for PC, thereby impairing pCRP activity (Fig. 17.2) [113]. In a recent study, we approved the presumed anti-inflammatory potential of 1,6-bisPC. The agent reduced the localized inflammatory response in a myocardial ischemia/reperfusion model by stabilization of pCRP and prevention of CRP dissociation. The localized deposition of mCRP to the site of primary tissue impairment was markedly reduced. 1,6-bisPC effectively inhibited generation of mCRP, and consequently its pro-inflammatory potential to exacerbate the local inflammation was abrogated [61]. Additionally, we demonstrated that 1,6-bisPC exhibits the capability to attenuate the CRP-driven exacerbation of inflammation by inhibiting the interaction between pCRP and cell-derived microvesicles. We were able to substantiate our hypothesis by intravital microscopic tracking of pCRP in inflamed muscle tissue. Preincubation of pCRP with the small-molecule inhibitor 1,6-bisPC resulted in an incapacity of pCRP to bind phosphocholine, thus no pCRP was detectable on transmigrated leukocytes [97]. The characteristics of the CRP dissociation as a local process and the following mCRP-driven exacerbation of inflammation emphasize their attractiveness as a rewarding therapeutic target (Fig. 17.5). After the application of 1,6-bisPC in animal models no significant adverse effects were notable, and 1,6-bisPC alone showed neither pro- nor anti-inflammatory properties [61, 113]. It is fair to assume that an anti-inflammatory therapy based on 1,6-bisPC has no major systemic side effects. It is more likely that anti-CRP therapy inhibits the exacerbation of pre-existing tissue-damage than the favorable inflammation in wound healing significantly [114, 115]. In the light of the reality that although CRP has been conserved throughout the evolution without any mutation, the potentially harmful effects of a therapeutic CRP inhibition cannot be excluded by now.

However, two already known shortcoming/deficiencies of this compound should be addressed beforehand.

1. 1,6-bis(phosphocholine)-hexane exhibits a relatively low affinity for pCRP. Therefore, higher quantities of the substance must be applied in vivo to achieve a sufficient effect (113).
2. 1,6-bis(phosphocholine)-hexane has poor pharmacokinetics. Due to its polarity, the bioavailability of 1,6-bis(phosphocholine)-hexane after oral administration remains unsatisfactory, and it is rapidly cleared from circulation [113].

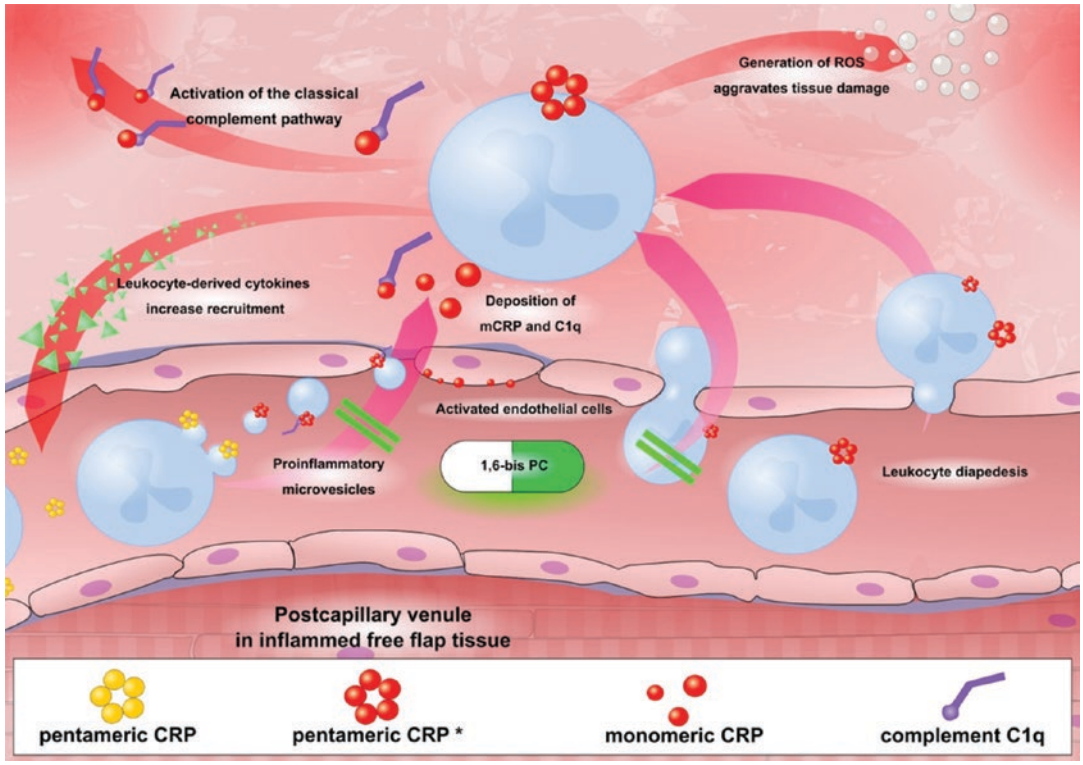

Fig. 17.5 Model of the therapeutic targeting of CRP using 1,6-bis(phosphocholine)-hexane (1,6-bis PC). 1,6-bis PC (white-green pill) inhibits the interaction of circulating pentameric CRP with recruited leukocytes. Subsequent release of membrane-derived pro-inflammatory microvesicles and leukocyte-endothelium interaction is inhibited. Therefore, 1,6-bisPC attenuates the inflammatory response to the ischemia/reperfusion injury and prevents an exacerbation of the local inflammation

17.8 Conclusions

Recent data identified and characterized the conformational change of CRP as the key event in a novel pro-inflammatory cascade. Proof-of-concept studies conducted demonstrated the implications and feasibility of therapeutic mCRP inhibition in various circumstances. In ischemia/reperfusion injury, mCRP exacerbates the local inflammation by activation of the classical complement pathway and massive leukocyte recruitment. In acute transplant rejection, mCPR mediates the innate immune response and considerably worsen the long-term allograft survival. The innovative small-molecule inhibitor 1,6-bis(phosphocholine)-hexane abrogates these pro-inflammatory capacities of mCRP. Yet, the therapeutic targeting of the CRP dissociation seems to exclusively affect the aggravation of local inflammatory processes, whilst beneficial responses of the immune system proceed widely undisturbed. Our new understanding of the pathogenesis in CRP-driven exacerbation provides the impetus to change the concept of post-surgical treatment in both autogeneic and allogeneic transplantation.

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18 Stem Cell Therapies for Tissue Regeneration and Wound Healing: Strategies to Enhance Therapeutic Effectiveness

Silvia Mihalceanu, Matthias M. Aitzetmüller, Hans-Günther Machens, and Dominik Duscher

18.1 Introduction

Wound healing is a complex process involving different cellular constituents of the skin compartments and its extracellular matrix (ECM). Extensive communication between all these factors is essential for efficient and complete tissue regeneration. Defect, loss, or dominance of one factor of this convoluted interaction can cause breakdown of the whole system, resulting in chronic wounds and consequently to significant limitations in every day's life. Chronic wounds are defined as barrier defects that have not proceeded through orderly and timely reparation to regain structural and functional integrity [1]. Vascular insufficiency, diabetes mellitus, and local-pressure are the major causes of non-healing skin wounds, although systemic factors, including diabetes, advanced age, inadequate nutrition, hypoperfusion, chronic mechanical stress, infection, malignancy, and obesity can contribute to poor wound healing [2].

Chronic wounds represent a major health care issue, potentially going along with significant social and economic challenges [3, 4], but research regarding this topic is limited by complexity of tissue regeneration, as well as many individual patient-related factors. The worldwide distribution towards an elderly population associated with multiple comorbidities markedly diminishing the healing capacity of all tissues, results in an increased prevalence of chronic wounds [5]. Current strategies to overcome impaired wound healing are based on [1] optimizing local wound management, consisting of debridement of the injury site, and providing an optimal environment through special dressings or negative-pressure-therapy [2], optimizing systemic factors such as blood sugar [3], administration of systemic antibiotic therapy in the setting of infection, and [4] reconstructive surgery of the defect [6].

Still, these efforts often remain unsuccessful and the clinical outcomes suboptimal [7]. Therefore, understanding the unique features of the wound environment is crucial in developing new therapeutic strategies. A variety of new treatment modalities such as bioengineered skin substitutes, growth factors, and stem cells are currently being assessed in both preclinical and clinical studies [8, 9]. Among these new therapeutic options, because of their strong cytokine profile, their immunomodulating properties, and their potential for multilineage differentiation, stem-cell based therapies hold particular promise in the setting of wound repair and tissue regeneration [10].

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18.2 Stem Cells in Wound Healing

Stem cells are a population defined by multipotency, self-renewal capacity, and long-term viability. They are able to differentiate into cells of multiple lineages by performing asymmetric divisions [11]. Hence, such properties could be useful for the development of regenerative therapies for skin tissue defects.

Essentially, these cells can be broadly classified in embryonic stem cells (ESCs) and adult stem cells, according to their evolutionary origins. Embryonic stem cells are derived from the blastocyst, a hollow structure in the early mammalian embryonic development. While the placenta will originate from the external layer, called throphoblast, the cells of the inner cell mass (ICM) are capable to form any fully differentiated cell of the organism [12]. Although theoretically appealing because of their pluripotency, the widespread use of the ESCs is still limited by ethical considerations and regulatory issues. Additionally, problems derived from their vast differentiation, such as the potential for immunogenicity and tumorigenicity, must be addressed before clinical application becomes feasible [13, 14].

Adult stem cells, however, are easier to harvest and their usage is hardly limited by ethical issues. Beginning of the adult stem cell research can be set in 1963, when Becker et al. [15] first described the hematopoietic stem cells (HSCs) by reporting the presence of self-replicating cells within the bone marrow of mice. Since then, multiple progenitor cell reservoirs of the adult organism have been discovered and utilized for research and clinical applications. There is evidence that adult stem cells may be found in most tissues and organs, predominantly at sites with rapid cell turnover, such as bone marrow, skin, liver, and intestinal mucosa, but also in skeletal muscle tissue, pancreas, heart, and central nervous system [16–18]. The most prominent part of these cells turned out to be from mesenchymal origin [19]. Subsequent to their identification as fibroblasts precursors in bone marrow in the 1950s, mesenchymal stem cells (MSCs) have been isolated from several sites, including adipose tissue, skin, peripheral blood, dental pulp, endometrium, postnatal tissues (i.e., umbilical cord blood, placenta) [20–25], and even tumorous tissue [26].

Generally, MSCs are defined by The International Society of Cellular Therapy (ISCT) by several abilities. Their ability to adhere to a plastic surface, their expression of certain surface markers CD73, CD90, and CD105, their lack of expression of hematopoietic markers CD14/CD11b, CD34, CD45, CD79a/CD19 and HLA-DR class II, and their ability to differentiate along osteoblastic, adipocytic, and chondrocytic lineage when treated with established lineage-specific differentiation factors in vitro characterize MSCs as a unique cell population [27]. Initial studies have shown that the MSCs are beneficial for the treatment of Crohn's disease, graft-versus-host disease, and diabetes mellitus, as well as for reducing cellular damage after heart, lung, liver, kidney, brain, and skin injuries [28]. These findings indicated that MSCs-based therapy could represent a major breakthrough in the healing of chronic wounds [2, 29].

Extensive research on the regenerative potential of mesenchymal stem cell showed that the treatment with MSCs interferes with different stages of the wound healing process. MSCs can contribute to the repopulation of the wound bed by differentiating into a variety of skin cell types, including keratinocytes and endothelial cells [30]. In the setting of chronic inflammation, which is considered as a hallmark of wound healing disorders [31], MSCs have also been shown to exert immunomodulatory effects on the immune cells of the host, such as B lymphocytes, T lymphocytes, and Natural Killer (NK) cells, decreasing the inflammation and preventing fibrosis by attenuating the scar formation [32].

Mesenchymal stem cells may serve as a powerful tool to promote tissue regeneration due to two main abilities: secretion of paracrine factors and differentiation [33]. Several studies suggest that the positive effect of stem cell transplanted to the wound site mainly relies on the secretion of pro-regenerative trophic factors, rather than via differentiation and direct replacement of damaged cells [34–37]. The secretome of the

stem cells leads to activation of different signaling cascades and provides the microenvironment of the wound with growth factors, cytokines and chemokines. Consequently, wound healing is accelerated through enhanced proliferation and migration of fibroblasts, keratinocytes, and epithelial cells, [33, 38] as well as promoted angiogenesis [39]. MSCs have also been identified to have antimicrobial effects [40, 41]. Thus, their enormous therapeutic potential for applications in regenerative medicine and tissue engineering has put them in the focus of many researchers. Bone marrow-derived MSCs (BMSCs) and adipose-derived MSCs (AdSCs or ASCs) are the ones used the most in preclinical and clinical research in the setting of impaired wound healing.

18.2.1 Bone Marrow Derived Stem Cells

Derived from the mesodermal germ layer, the bone-marrow compartment consists of hematopoietic stem cells (HSCs), supported by a heterogeneous mesenchymal stroma. First isolated in 1966 by Friedenstein et al. [42] as part of the stroma, the bone marrow-derived mesenchymal stem cells (BMSCs) have been originally identified as a source of osteoprogenitor stem cells [43–45]. Since then, extensive research has shown that BMSCs are capable to undergo differentiation towards osteogenic, chondrogenic, adipogenic, and myogenic lineages [46–50]. Therefore, bone marrow-derived stem cells (BMSCs) have been proposed to be a promising source of stem cells for mesodermal tissue engineering. Following harvesting and in vitro selection based on their ability to adhere to plastic culture dish and expression of surface markers, BMSCs can be expanded in culture and applied topically to wounds or injected to promote tissue repair.

The regenerative potential of BMSCs has extensively been researched in the setting of diabetic wounds and critical limb ischemia [51–53]. A study compared the therapeutic effects of intramuscular and intra-arterial delivery of BMSCs in patients with advanced critical limb ischemia and found that they both represent effective strategies [54]. Another clinical study used a topically delivered composite graft consisting of BMSCs and a collagen sponge scaffold to treat skin wounds. This approach was shown to be effective in 18 of 20 patients [55]. Therefore, BMSC-based therapies are a promising therapy for difficult-to-heal wounds.

Nevertheless, bone marrow procurement has potential limitations. The harvesting procedure is invasive, may lead to significant donor site morbidity, and often requires general anesthesia and intensive patient care [56]. To achieve clinically significant concentrations, BMSCs require an ex vivo expansion [57]. This step is expensive, time consuming, and harbors the risk of possible cell contaminations. For this reason, such procedures should only be performed in special facilities. Because of these factors, many researchers have been utilizing MSCs of alternate sources to investigate their potential for tissue regeneration.

18.2.2 Adipose Tissue as a Valuable Source of Stem Cells

For a long time, the function of the adipose tissue was believed to be limited to the storage of excess energy, and the surplus of tissue was discarded by liposuction or rarely used as a soft-tissue filler. In addition to plastic and reconstructive surgical applications, adipose tissue has become essential to an increasing number of translational efforts involving the understanding of cellular, molecular, and immunological mechanisms. Adipose tissue is a heterogeneous entity derived from the mesodermal germ layer, consisting of various cell population [58, 59]. According to the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT), the stromal vascular fraction (SVF) can be defined as a heterogeneous mesenchymal cell population separated from the mature adipocytes by processing the adipose tissue [60].

Usually, the SVF is obtained through enzymatic digestion with collagenase, followed by

centrifugation and resuspension [56]. The SVF contains adipose stromal stem cells (ASCs), hematopoietic stem cells (HSCs), and progenitor cells, as well as endothelial cells, fibroblasts, erythrocytes, lymphocytes, monocyte/macrophages, and pericytes, among others [60, 61]. The progenitor cells, which are responsible for the turnover of the adipose tissue, show resemblance to fibroblasts and have been referred to as preadipocytes [62]. In 2001, researchers from UCLA found that the adipose tissue cell population contains not only such progenitor cells, but also a population of mesenchymal stem cells capable of multilineage differentiation, termed adipose-derived stem cells (ASCs or AdSCs) [20].

As the first one, Zuk et al. [20] isolated the ASCs from human lipoaspirates, proposing that the stromo-vascular fraction contains a niche of multilineage stem cells. Their findings revealed that the ASCs present similar properties to the previously characterized BMSCs: they are capable of mesodermal differentiation towards the osteogenic, adipogenic, myogenic, and chondrogenic lineages, upon specific in vitro induction with established specific factors. Several studies that compared these two types of stem cells showed a morphological resemblance, and a similarity of majority of their expressed cell surface markers. Transcriptomic and proteomics analysis also showed that adipose-derived stem cells and bone marrow-derived stem cells pose a similar profile [63, 64]. In addition, it was found that regardless of the heterogeneity of the adipose tissue, the ASCs might be able to cross the germ layers and undergo differentiation towards ectodermal and endodermal lineages [56, 65–67]. Based on this, adipose tissue was proposed as an additional source of adult stem cells with a possible far-reaching regenerative potential.

The concept of using ASCs as cellular vehicles for mediating the therapeutic repair of chronic wounds became very appealing. Adipose tissue, like bone marrow, is a mesodermal derivative and the stroma containing the ASCs can be easily isolated by liposuction under local anesthesia and with minimal discomfort [56]. A large body of medical literature investigated possible ACSs mechanisms of action in wound healing. Kim et al. [68] suggested that the ASCs promote the proliferation and migration of human dermal fibroblasts, not only by direct cell-cell-contact, but also indirectly via secretion of soluble factors. Another study found that the delivery of autologous ASCs in a murine model of hind limb ischemia stimulates angiogenesis. Conditioned media obtained from culturing the ASCs under hypoxic conditions significantly increased the endothelial cell growth and reduced their apoptosis. Enhanced rate of multiple pro-angiogenic growth factors secretion has been explored as underlying mechanism [69]. Independently, a preclinical study showed that the ASCs significantly accelerate wound closure in normal and diabetic rat model, increasing epithelialization and deposition of granulation tissue. Moreover, ASCs also can differentiate into epithelial or endothelial cells and secrete pro-angiogenic cytokines, promoting neovascularization of the wound bed [70]. All of this data suggests that ASCs promote the re-epithelialization of the wound bed largely through their paracrine effects.

An increasing amount of literature describes the trophic effects of ASCs on a variety of endogenous cells. It has been shown that ASCs' secretome contains molecules such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), keratinocyte growth factor (KGF), transforming growth factor ß (TGF-ß), fibronectin, and collagen 1 [34]. In a different study, it was found that the ASCs also secrete granulocyte and macrophage colony stimulating factors, interleukins (IL- 6, IL-7, IL-8, and IL- 11), tumor necrosis factor-α (TNF-α), brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), adipokines, and others. Moreover, some of these factors have also shown to possess an immunomodulatory character, and thereby might positively affect wound closure [71].

Therefore, it has been hypothesized that ASCs can initiate and enhance wound healing by two main mechanisms: (1) migration and differentiation into skin cells in response to injury and (2) paracrine-mediated activation of fibroblasts and keratinocytes and down-regulation of the

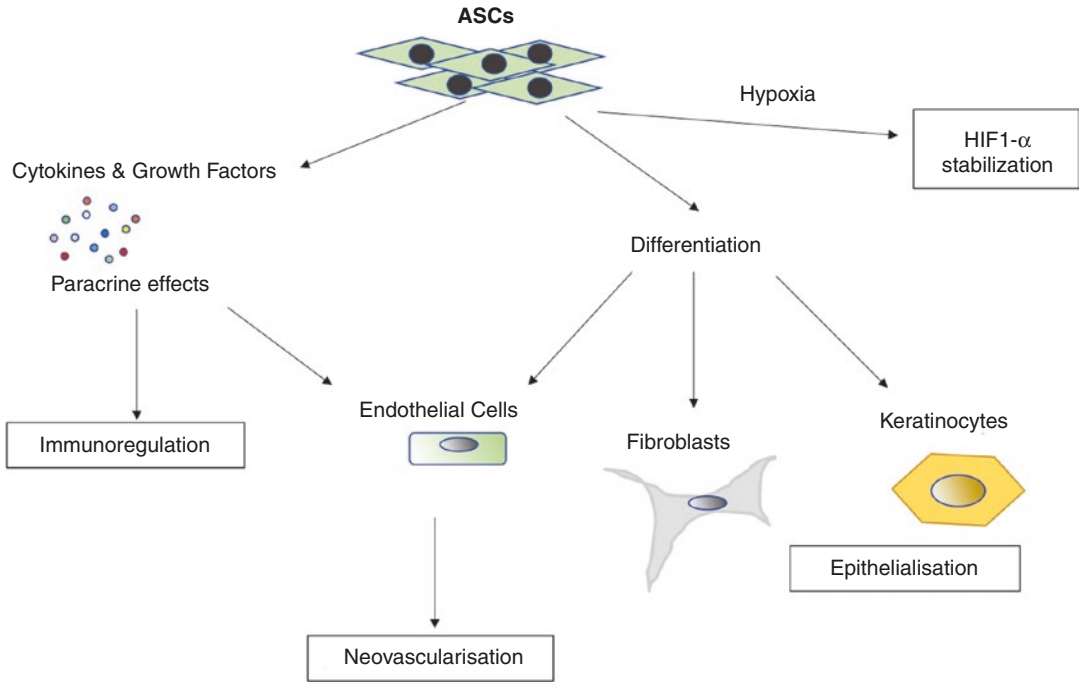

Fig. 18.1 Wound healing mechanisms of ASCs: ASCs can initiate and enhance wound healing by migration and differentiation into skin cells in response to injury, paracrine-mediated activation of fibroblasts, and keratinocytes and down-regulation of the inflammatory response

inflammatory response (Fig. 18.1) [72]. The beneficial effects of ACSs were also confirmed in a phase I clinical trial: Bura et al. [73] evaluated the safety of the intramuscular injections of autologous ASCs in patients with non-revascularizable critical limb ischemia and reported an improvement of the wound healing, as well as the absence of complications.

These findings suggest that the ASCs could represent a promising alternative population of adult mesenchymal stem cells, possibly more feasible that the BMSCs for clinical translations.

The advantage of using the ASCs as an alternative stem-cell source resides in the availability of adipose tissue, as well as in the reduced donor site morbidity compared with bone marrow harvesting. The extraordinary high cell yield from lipoaspirates, as compared with bone marrow aspiration, can be used directly without culturing in vitro [74]. Thus, in vitro manipulation of the stem cells and regulatory issues such as the need for good manufacturing practice (GMP) facilities could be avoided. Nevertheless, more clinical evidence is required to prove the safety and efficacy of ASC-based therapies for tissue repair applications.

18.3 Challenges and Potential Strategies to Enhance the Therapeutic Effectiveness of ASCs

In a physiological setting, acute injury of the cutaneous tissue is followed by the activation of multiple well-orchestrated cell and cytokine signaling pathways [75]. Wound healing in healthy individuals initiates several overlapping phases: inflammation, cellular migration, proliferation, extracellular matrix (ECM) deposition, and tissue remodeling [1, 76]. However, advanced age and underlying disease states such as diabetes and vascular insufficiency can significantly affect these physiological processes, leading to appearance of chronic wounds. There is a need for therapeutics that maintain efficacy in the clinical

setting, despite obstacles such as hypoxia, ischemia, oxidative stress, and bacterial infection [77]. To effectively modulate the complex mechanisms that are involved in wound healing and to overcome the harsh wound microenvironment, stem cells need to be delivered in a favorable route. Therefore, the whole process, beginning with the selection of the cell source to the administration to the patient, can be addressed with the aim of improving the yield and the effects exerted by the stem cells.

18.4 Effects of Pathology and Aging on ASCs

Although therapeutic approaches utilizing ASCs for wound healing have been quite promising in preclinical studies, clinical translation remains difficult. There is a continuously increasing need for stem-cell based therapies especially in the treatment of chronic wounds. Unfortunately, these wounds most likely occur in aged and multi-morbid patients. These systemic factors have shown to be responsible for a multitude of pathological modifications in the microenvironment of the wound site, as well as for impairment of the regenerative potential of stem cells of these patients [78].

Several studies have postulated that non-healing wounds are characterized by lack of oxygen, chronic systemic inflammation, and increased matrix metalloproteinase (MMP) activity, resulting in degradation of ECM and growth factors [31, 79, 80]. These characteristics of chronic wounds have shown to have a negative impact on the functionality of ASCs. Koenen et al. [81] evaluated the influence of acute wound fluid (AWF) and chronic wound fluid (CWF) on the ASCs. He found out that the CWF significantly impairs the proliferation and migration of the ASCs, in contrast with the stimulating effect of the AWF.

The effect of diabetic disease state on the ASCs has also been studied extensively in both in vitro and animal model: by using single-cell analytical approach there was examined possible effects of diabetes on the intrinsic properties of ASCs and demonstrated that prolonged hyperglycemia alters some subpopulations of the ASC niche [78]. Another study analyzed ASCs from diabetic mice for their cytokine profile and concluded that the secretion of growth factors is diminished [82].

Advanced age plays a major role: it is not only associated with increased incidence of comorbidities, but also impairs by itself the regenerative abilities of the organism, including the progenitor cells. Aged stem cells have shown to decrease their functionality and differentiation capacity, as well as their ability to support the formation of new vessels in vitro and in vivo [83, 84]. Duscher et al. [85] investigated the effect of advanced age on the dynamic of the ASCs populations and identified an age-related depletion of certain pro-regenerative subpopulations. These data suggest that the translational potential of ASCs derived from patients with diabetes may be limited. Nonetheless, the age- and morbidity-related differences regarding the characteristics of the ASCs and the consequences of their therapeutic use remain incompletely understood. Thus, interventions to improve the function, application, and survival of the ASCs used as therapeutics are needed (Fig. 18.2).

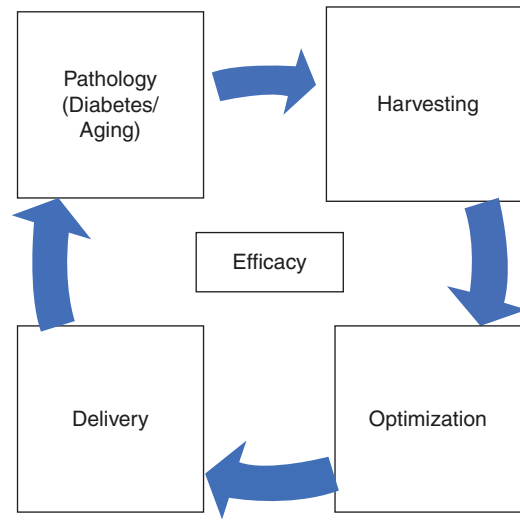

Fig. 18.2 Strategies for enhancing the efficacy of ASCs in the setting of impaired wound healing: to address challenges from advanced age and comorbidities such as diabetes, optimal harvesting and delivery of the ASC-based therapies are required

18.5 Harvesting

The harvesting method may have an impact on the functionality of the adipose-derived stem cells. Currently, various liposuction methods such as suction-assisted liposuction, power-assisted liposuction, ultrasound-assisted liposuction and laser-assisted liposuction are used in clinical routine [86, 87]. Although suction-assisted liposuction has been found to provide significantly fewer ASCs when compared to excised fat tissue, the yielded stem cells were viable and capable of multi-lineage differentiation [88]. Another study assessed the impact of ultrasound-assisted liposuction on ASCs yield, viability, and differentiation capacity and suggested that ultrasonic energy allows safe and efficient harvesting of the ASCs and does not pose negative effects on their regenerative capacity [89]. Still, a clinically feasible harvesting procedure where the adipose tissue can be collected, and the stem cells isolated and processed for immediate application in a single session is not available. Further research is required to establish a harvesting approach that is a safe, reliable, and cost effective.

18.6 Optimization

As the functionality of unselected autologous ASCs from elderly and highly morbid patients has shown to be likely reduced, selection of certain cell-populations with a higher regenerative potential may be a solution to improve their therapeutic potential. Researchers have been able to develop a method to identify and isolate distinct subpopulations via single-cell transcriptional analysis and screening of the cell surface markers [90, 91]. Although extensive investigations are required to implement this selection method in the clinical setting, the concept of using only the cell subsets with superior functionality is very attractive and opens the possibility of allowing therapies with autologous ASCs in a broad cohort of patients including aged and diabetic individuals.

Another approach that may be beneficial for adjusting the characteristics of the ASCs is culturing the stem cells under certain conditions that resemble the in vivo wound environment. Several studies demonstrated that the exploration of hypoxic conditions to modulate the secretome of mesenchymal stem cells increase their therapeutic potential [92, 93]. Hypoxia or low oxygen level has shown to increase the proliferation of ASCs and enhance their regenerative properties by up-regulating the VEGF and basic fibroblast growth factor (bFGF) [93]. Another study utilized a Matrigel implant model and showed that hypoxia-preconditioned ASCs promote angiogenesis, as well as maturation of the newly formed blood vessels [94].

18.7 Delivery

An additional challenge for the use of stem cell-based therapies is to provide effective cell delivery to the site of injury. As the engraftment and survival of the stem cells at the wound site are limited by the harsh environment of the chronic tissue injuries, different ways of delivering MSCs have been developed: topical/spray, scaffold-loaded, subcutaneous injection, or intravenous delivery [2]. Given the less predictable delivery of the systemic administration, localized delivery has been widely accepted as the optimal approach for treatment of wounds [95]. The local application facilitates the effective tissue targeting and avoids systemic side effects. However, chronic wounds present a highly unfavorable environment for the delivered cells. The direct topical application has the disadvantage of delivering the cells unprotected and not uniformly dispersed in the wound bed. Moreover, the engrafted cells may differentiate to fast, which could be detrimental for the full development of their paracrine activity [84].

One possible alternative delivery system that may work synergistically with the stem cells, protecting them and prolonging their viability, is the scaffold-based delivery. A study locally administered ASCs to murine excisional wounds using a biological non-immunogenic extracellular matrix patch material. Increased viability and significant reduction of the wound area were

observed (LAM). Altman et al. [96] also showed acellular dermal matrices as carriers for ASCs to be effective for targeting in vivo soft tissue regeneration. In another study, enhanced engraftment of MSCs and higher levels of pro-angiogenic growth factors were demonstrated in wounds treated with a MSC-seeded hydrogel [97]. A novel protocol for optimizing the hydrogel cell seeding has been investigated by Garg et al. [98] who developed a capillary-force based seeding approach able to rapidly engraft the ASCs into a collagen-pullulan hydrogel at the point of care, with promising results. All these represent important steps towards the clinical translation of progenitor cell therapy.

18.8 Outlook

Autologous cell-based approaches may be limited by the difficulties in obtaining sufficient autologous stem cells from aged patients suffering from multiple pathological conditions. Therefore, the research focus shifted toward investigating the possibility of isolating the ASCs from healthy individuals and delivering them in allogeneic fashion to the recipient [99–101]. Recent research has shown the MSCs may have a privileged immunological profile and do not induce significant immunological responses in the host [32]. This may represent a valuable strategy to overcome age-and disease-related impairments in stem cells. Allogeneic cells may be isolated from one or more donors, expanded, and cryopreserved for future use as an "off-the-shelf" therapy to meet emergency requirements such as burns and large wound areas. Nevertheless, the preclinical and clinical studies using allogeneic ASCs are still limited, and further research is required to prove the immunological safety of allogeneic stem cell-based therapies.

In 2006, researchers made another breakthrough, by discovering that somatic adult cells can be genetically reprogrammed to revert to the previous state of an embryonic stem cells, preceding their initial differentiation status. These so-called iPSCs (induced pluripotent stem cells) were firstly generated by Takahashi and Yamanaka by reprogramming embryonic and adult fibroblasts to create an immature, pluripotent state in a mouse model [102]. Through retroviral transduction of the fibroblasts with transcription factors like OCT4, SOX2, Klf4, c-Myc, NANOG, and LIN28, Takahashi et al. [102] and Yu et al. [103] showed that some cells exhibit certain characteristics of embryonic stem cells. Human iPSCs were also reported to express stem cell markers and are capable of generating cells characteristic of all three germ layers [104, 105]. The use of iPSC technologies has the potential to combine advantages of both stromal cell types, MSCs, and ESCs. Ethical issues that go along with the use of human ESCs can be avoided and autologous iPSCs are highly available and pose a high chance of immunological compatibility. Recent studies have already proven the potential use of iPSCs for cutaneous repair: in vitro 3D skin equivalents composed of human iPSC-derived keratinocytes or fibroblasts have been generated and used for dermal regeneration [106–108]. Moreover, epidermal appendages like the hair follicles could have been generated [109], suggesting that the iPSC-derived skin substitutes are promising for the generation of complete cutaneous equivalents. However, further research is needed to determine the ideal methods to completely and reproducibly commit the iPSCs to appropriate cell lineages. The transition of iPScs into the clinic is still hindered by the missing evidence regarding safety and reliability of used reprogramming technologies. Furthermore, issues like the risk for enhanced tumorigenesis and the need for an in vitro genetic manipulation and a possible iPSC-mediated immune response need to be addressed before widespread clinical adaption is possible.

18.9 Conclusions

Stem cells-based therapies remain at the forefront of tissue regeneration, since they represent a unique source of cells endowed with remarkable healing potential. The therapeutic use of progenitor cells for treating chronical wounds has showed promising results in basic science

research and preclinical studies. Given their extraordinary characteristics to differentiate and to release cytokines and soluble growth factors, these cells are promising therapeutic sources.

Nevertheless, our current understanding regarding the mechanisms of stem cells-based therapies with regard to complex signaling cascades, environmental influences, or epigenetic modulation is still limited. Therefore, there are still major hurdles to be overcome to achieve clinical translation of these novel therapeutics.

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Iron Chelators & HIF-1α: A New Frontier for Skin Rejuvenation

19

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19.1 Mechanisms of Skin-Aging

The human skin belongs to the integumentary system and represents the largest organ in the human body, comprising about 15% of the body weight. The total skin surface of an adult ranges from 12 to 20 square feet, and the skin is composed of 70% water, 25% protein and 2% lipids. Different cell types like fibroblasts, keratinocytes, and melanocytes build the external (epidermal), the middle (dermal), and the inner (subdermal) layer. The skin derives from the ectodermal tissue, interfaces with the environment, and thereby acts as the first line of defense against microbiological invasions, physical aggressions, and chemical assaults. In addition, this particular composition plays an important role in insulation, temperature regulation, sensation, and is key to the production of vitamin D [1]. All of these functions are based on physiological tissue homeostasis and require an intact epidermis, dermis, and hypodermis [2]. The thickness of skin significantly depends on body location. In humans, the skin located around the eyes and eyelids is the thinnest in the body (0.5 mm thick) and is one of the first areas to show signs of aging, colloquially known as "crow's feet". In other parts of the body like palms and soles of the feet, the skin can be up to 4 mm thick. Skin is a multifunctional organ and, like any other organ system subject to different stress factors, leading to specific impairments in its composition and functionality over time [3, 4].

In the last decade, healthy-aging principles and longevity pathways were studied [5]. At a cellular and molecular level, aging is characterized by the accumulation of damage such as DNA oxidation and progressive loss of physiological integrity, leading to impaired function and increased vulnerability, and subsequently death. At present, progressive aging in humans cannot be successfully and satisfactorily stopped. Researchers are only just beginning to understand the biological basis of aging even in relatively simple and short-lived organisms such as yeast [6]. Derived from these studies, numerous hypotheses have been formulated with the aim to explain the aging phe-

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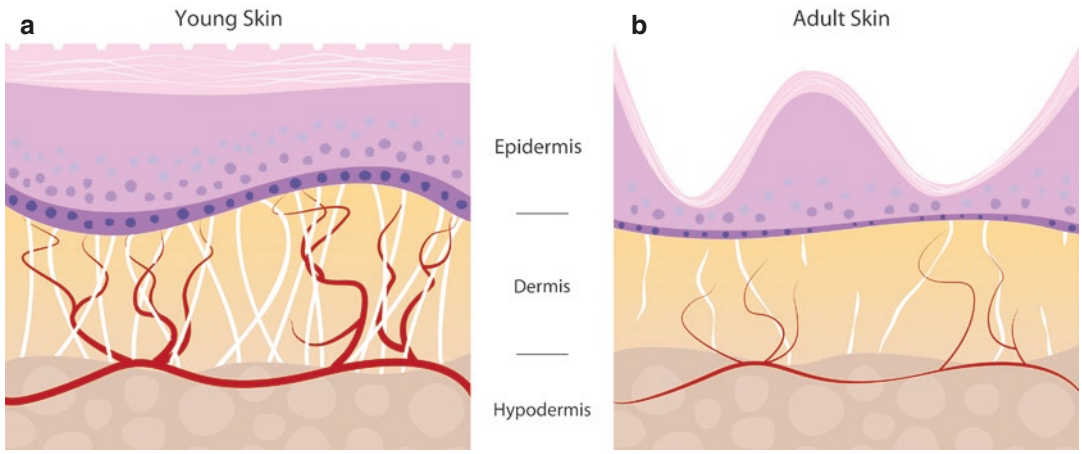

Fig. 19.1 Differences between young and aged skin. (**a**) Youthful healthy skin presents thick epidermis, a strong dermo-epidermal junction, normal collagen content, and healthy vascularity. (**b**) Aged skin contains several signs of degeneration, such as uneven epidermis, thinner dermo-epidermal junction, inadequate collagen content, and compromised vascularity [71]. Reprinted with permission

nomenon. Aging could be the natural result of entropy on the cells, tissues, and organs [7]. However, evidence is accumulating showing that aging is in part genetically regulated. In parallel, other approaches show that the regulation and the subsequent breakdown of cellular processes represent a programmatic decision by the cell to either continue or abandon maintenance procedures with age [8]. Regarding the skin, it is widely accepted that advanced age brings changes to all components of the integumentary system with consequent signs of deterioration on epidermis, dermis, and hypodermis. During the aging process, skin gets progressively thinner and the blood capillaries of the dermis become sparse and more fragile, resulting in wrinkles and a paler, translucent appearance [9] (Fig. 19.1).

Due to increasing understanding of genetic pathways and biochemical processes, research about the cutaneous aging process has experienced an unprecedented advance within the last years [10]. Generally, age-related skin changes are triggered by a combination of intrinsic factors and extrinsic ones (e.g. ultraviolet/infrared light exposure, smoking). Intrinsic or innate aging is a degenerative process, which affects the skin in the same way as it affects all other organs. Intrinsic skin aging represents the biological clock of the human body and reflects the reduction of function that is extensively described to affect internal organs [11]. This loss of function is mainly characterized by the decreased ability of response to exogenous and endogenous stress [12]. Three main protagonists of innate/intrinsic cutaneous aging are: telomere-loss, oxidative stress, and DNA-damage [13–15]. Several studies indicate that telomere length is able to modulate the pace of aging and onset of age-associated diseases [16, 17]. However, there is emerging evidence showing that lifestyle factors (obesity, smoking, and alcohol) may influence health and lifespan of an individual by directly affecting telomere length [18] demonstrating the strong interplay between the triggers of aging. Recent studies from our group and others, involving free radicals (Reactive oxygen species - ROS), suggest that oxidative stress may damage not only the lipid bi-layer in cell membranes but also connective tissue components, particularly elastin fibers and collagen [19]. Additionally, ROS also interact directly with the DNA leading to base loss, DNA modification or breakage of strains, making DNA lesions an important factor involved in the aging process [20].

The second main variable of cutaneous aging, also known as "photoaging", is a result of the extrinsic capacity of the environment to damage the skin surface. This "extrinsic aging" is the result of skin exposure to external factors, most importantly ultraviolet (UV) radiation [12]. Age related changes are able to impair the two most important features of the skin: strength and elasticity [21, 22].

The so-called "elastosis" represents a progressive accumulation of elastic fibres in the upper and mid-layers of the dermis and is heavily driven by sun-exposure (therefore also named "solar elastosis") [23]. Altogether, the cutaneous aging phenomenon manifests as an observable change in the external appearance of the skin with a loss of function of cells [24]. Considering both innate and exogenous factors, aging leads to degradation and the breakage of collagen fibers, microtextural impairments, and loss of connective tissue structures.

The intracellular and the extracellular machinery is heavily impaired in aged skin [25, 26]. Aged and senescent cutaneous cells have the ability to modify their biosynthetic network by the expression of different genes such as ID3, SMAD7, and FAM83G [27]. It has been demonstrated that the rate of collagen biosynthesis is markedly lower in aged skin than in infant or foetal tissue [28]. In addition to wound healing disorders, this reduced collagen production leads to the atrophy of the dermis effecting wrinkle formation. Similarly, the rate of elastin gene expression is markedly reduced after the fourth decade of life [29]. Elastin is of paramount importance for the connective tissues by allowing the skin to return to its shape after stretching or contracting. Lack of elastin explains the impaired pliability of aged skin. An imbalance between biosynthesis and degradation of elastin fibres clinically manifests as atrophy and loss of recoil. Recent evidence further identified matrix metalloproteinases (MMPs) as important mediators of this degeneration [30]. By destroying the endogenous collagen network, proteoglycans, fibronectin, and other components of the dermis, these enzymes are leading to a rapid, but not irreversible, cutaneous aging effect [31]. Altogether, the interplay of these mechanisms affects all three layers of the skin, with its biggest influence on the dermis result in significant impairments of the regenerative capacity of aging skin [32, 33].

19.2 The Role of HIF-1 in Skin Aging

Intensive study efforts are currently undertaken to develop agents capable of mitigating or reversing the signs of cutaneous aging. Nevertheless, no single approach has been identified to address all important factors, structural and physiological components, epidermal and dermal atrophy, and loss of connective tissue structure and vascularity. While most cosmetic products only provide adequate skin hydration, they lack the ability to actively support the biological processes, which are known to be diminished in aged population.

Similar to chronic wounds, skin-aging is characterized by the dysfunction of key cellular regulatory pathways. Recent evidence suggests that the same mechanisms, which hinder the physiologic healing response in chronic wounds, are the reason for impaired tissue homeostasis in aged skin [34–39]. The Hypoxia Inducible Factor 1 alpha (HIF-1α) pathway represents one key-mechanism in both conditions [34, 39, 40]. It is widely accepted that the physiological activation of the dimeric protein HIF-1α), representing the main transcriptional factor of the HIF-1 pathway, is significantly involved in tissue homeostasis and neovascularization. Therefore, activation leads to production of new collagen strains, elastin, glycosaminoglycans, and nutritive blood vessels [41, 42]. Slight modulation of the functionality of this pathway has been clearly demonstrated to significantly enhance tissue regeneration [35, 36, 43–46]. Advanced age, similar to diabetes and other degenerative skin diseases, has been shown to correlate with attenuated HIF-1α function [34–39].

In aging, HIF-1α is destabilized by enhanced activity of the oxygen-sensitive prolyl-hydroxylases (PHD) [34, 39] resulting in impaired release of growth factors, reduced neovascularization, and inadequate tissue quality and regeneration. As mentioned above, the Hypoxia Inducible Factor (HIF-1) is a dimeric transcription factor, composed of two subunits, HIF-1α and HIF-1β. These two proteins have different molecular characteristics. While HIF-1α is an oxygen sensitive subunit, which is activated under hypoxic conditions, HIF-1β is constitutively expressed. This special geometry is essential to allow heterodimer formation between the two proteins HIF-1α and HIF-1β, such as binding to DNA on the target hypoxia response elements (HRE). In addition, the HIF-1α subunit has two different transactivation domains (TAD): NH_2-

terminal [N-TAD] and COOH-terminal [C-TAD]. These two domains are responsible for the transcriptional activity by interacting with co-activators of HRE such as p300 or the cyclic-AMP binding protein (CBP) and stabilizing HIF-1α against degradation.

In normoxia, HIF-1α protein levels are low due to constant ubiquitination-dependent degradation via the Von Hippel-Landau (VHL) E3 ligase protein [47], which recognizes proline hydroxylated (Pro-OH) HIF-1α on both transactivation domains [48–50]. These hydroxylation reactions lead to degradation of HIF-1 α and are catalyzed by the oxygen-sensitive PHD. Another level of control lies within the oxygen-sensitive asparaginyl hydroxylase FIH, a factor inhibiting HIF. The oxygen-sensitive asparaginyl hydroxylase FIH hydroxylates the HIF-1α protein and inhibits subsequently the recruitment of transcriptional co-activators p300 and CBP, thereby the HIF transcriptional activity progressively decreases [51–53]. However, in addition to the absences of oxygen, lack of local free iron is also able to inhibit of HIF-1α degradation. The consequences are decreased HIF-1α hydroxylation, decreased pVHL mediated ubiquitination, degradation and increased HIF-1α protein stability [50] (Fig. 19.2).

HIF-1 alpha is essential for skin homeostasis and is mainly expressed in the basal layer of the epidermis [54–56]. Molecular pathways between fibroblasts and keratinocytes are crucial for the skin environment, especially in the basal layers of the epidermis. Therefore, slight modulation of HIF-1 activity could be strongly involved in novel approaches for skin rejuvenation. The possibilities of a therapeutical modulation of some of these networks are prom-

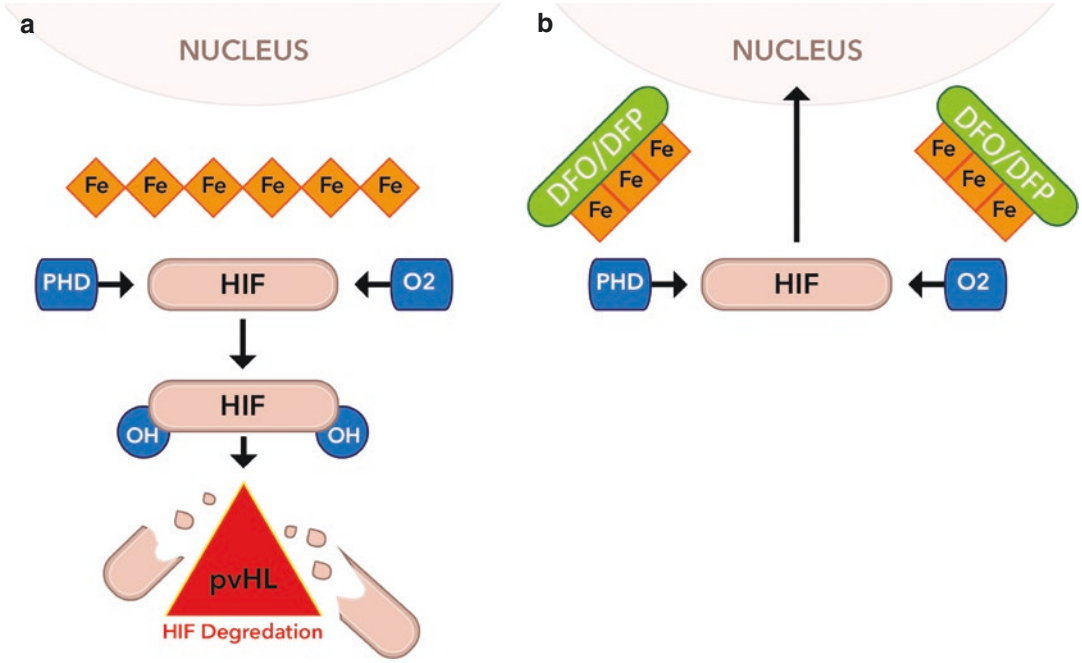

Fig. 19.2 Modulation of HIF pathway regulation. (**a**) HIF pathway activation in the presence of iron. Hydroxylation occurs by PHD, followed by ubiquitination by VHL, which facilitates enzymatic degradation of HIF1α. (**b**) Truncated HIF-1α breakdown pathway in the presence of an iron chelator. PHD is inactivated, allowing HIF1α to remain intact and free to dimerize for downstream HIF-1 pathway activation. (*DFO* Deferoxamine, *DFP* Deferiprone) [71]. Reprinted with permission

ising. The further activation of over 100 downstream genes of the HIF-1α pathway was proven to modulate angiogenesis, cell proliferation, migration, and glucose metabolism [57–59]. Regulation of HIF-1 has been demonstrated to be crucially involved in skin homeostasis [60] and wound healing [61, 62]. Therefore, localized control of tissue blood flow, or autoregulation, is a key factor in regulating tissue perfusion and oxygenation. Recently, it was demonstrated that the balance between the two transcription factor isoforms, HIF-1 alpha and HIF-2 alpha, is an essential mechanism regulating both local and systemic blood flow [63].

Recent studies using human epidermal cells showed that the controlled upregulation of HIF-1 substantially increases the growth potential of keratinocytes and fibroblasts improving the formation of viable and stratified epidermis [37, 38]. HIF-1 alpha overexpression expands dermal vasculature, suggesting a substantial influence on blood vessel formation by cutaneous cells through this pathway [64, 65]. HIF-1 has also been shown to drive the expression of Ln-332 [66], a high-molecular weight (400–900 kDa) protein of the extracellular matrix composed by an alpha-chain, a beta-chain, and a gamma-chain. Ln-332 is the major component of the basal lamina, a protein network foundation for most cells and organs, influencing cell differentiation, migration, and adhesion. The main role of Ln-332 is the maintenance of epithelial-mesenchymal cohesion in tissues that are exposed to external forces such as the skin [67]. An interaction with the heterodimeric cell surface receptors mediates adhesion of the extracellular matrix (ECM) to the cytoskeleton [68]. Notably, a diminution of keratinocyte growth potential following HIF-1 silencing was associated with a decreased expression of Ln-322 [38].

The concept of intrinsic and extrinsic damage can further be linked to age-related loss of epidermal HIF-1 expression [38]. Recent findings shown that cutaneous HIF-1 expression is modulated after UVB exposure, and that HIF-1α has an important role in the regulation of cellular responses to this type of genotoxic stress. Lastly, UVB induces ROS, which in turn influences HIF-1α expression affecting DNA repair and keratinocyte survival [69].

19.3 Iron Chelation for HIF-1 Modulation

Upregulation of HIF-1 reverses age-dependent functional impairments of the skin, and results in improved regeneration of aged tissues [70]. The biochemical reactions regulating HIF-1 signaling provide simple therapeutic strategies to promote HIF-1α stabilization and transactivation. PHD and FIH, the hydroxylases responsible for HIF-1 degradation, both belong to a family of iron-dependent dioxygenases that require iron, oxygen, and 2-oxaloglutarate (2-OG) as cofactors for the hydroxylation process [71]. Therefore, these enzymes are diminished in the absence of oxygen. Hypoxic conditions can be mimicked by the presence of iron chelators, such as deferoxamine or deferiprone, or in the presence of a 2-OG competitive inhibitor such as dimethyloxalylglycine (DMOG) [72, 73]. Our group has recently demonstrated certain advantages for utilizing iron chelators to stimulate HIF-1 and tissue regeneration [36, 70]. In this approach, the removal of iron to deprive HIF-1 degradation of a necessary co-factor is further complemented by reducing ROS stress via the binding of iron molecules. While iron is essential for cellular metabolism, an excess of iron can be toxic and accelerate the aging process through catalyzing the formation of reactive oxygen species (ROS), thus stimulating oxidative damage [60]. Well known as treatment option for Beta-Thalassemia and Hemochromatosis [74, 75], iron chelating drugs have shown benefits in the field of Plastic and Reconstructive Surgery. With their regenerative potential, they have the ability to increase the retention rate of fat grafts, the survival rate of free flaps, and the healing process of diabetic wounds [76, 77].

One of the first approaches to use iron chelation for regenerative medicine can be dated back to 1993. It was described that deferoxamine induces Erythropoietin gene expression and HIF-1 DNA-binding activity [78]. Deferoxamine

(DFO) and Deferiprone (DFP) are FDA-approved molecules with different molar masses (DFO = 560.69 g/mol and DFP = 139.152 g/mol). Because of its ability to chelate iron from ferritin and hemosiderin, hemoglobin and transferrin, Deferoxamine is already a first line therapy for Hemochromatosis. Deferiprone is an orally active agent firstly approved for use of treating thalassaemia. Due to the different molecular weight, scientists have successfully tried to exploit possible synergistic interactions to achieve a more relievable effect on iron chelation [79]. While DFO is hydrophilic, DFP belongs to the hydrophobic molecules. Despite these chemical differences, iron chelators typically contain oxygen, nitrogen, or sulfur-donor atoms that form bonds with iron. The donor atoms of the ligand affect the preference of the molecule to chelate either Fe(II) or Fe(III) oxidation states. Chelators that prefer Fe(II) contain 'soft' donor atoms, such as nitrogen and sulfur, and consequently retain a relatively high affinity for other divalent metals such as Cu^{2+} and Zn^{2+}. Iron chelators like DFO have an hexadentate arrangement, allowing to bind iron in a 1:1 ratio, and therefore show the highest affinity.

All molecules of the iron chelator family have been in clinical use for decades and have favorable safety characteristics promising for therapeutic HIF-1 signaling modulation. Using an iron-chelation approach for skin rejuvenation with an appropriate monitoring of the progression of the effects on aged skin aims to become a new paradigm in anti-aging medicine. Although numerous studies are suggesting a powerful role for iron chelators in the emerging field of regenerative medicine, thorough basic science studies and a clinical proof of principle is mandatory to investigate a possible application of these molecules as cosmeceuticals.

19.4 Conclusion and Outlook

The possibilities of a therapeutic modulation of hypoxia inducible signaling pathways by repurposing iron chelators are promising. An upregulation of HIF-1 alpha mediated by iron-chelation leads to the correction of age-dependent changes of HIF-1 expression. This directly results in improved cutaneous regeneration, as well as in resistance to exogenous stressors such as radiation and infection. These benefits are mediated through positive effects on all cutaneous cell types, the upregulation of pro-regenerative cytokines, growth factors and peptides, and the recruitment of circulating regenerative cells. However, the impact of such a modulation on skin homeostasis is not fully understood. A thorough investigation of the molecular effects of HIF-1α pathway alteration by iron chelators like DFO or DFP and their influence on human keratinocytes and fibroblasts is warranted. If supported by solid clinical trial data, this approach would have the potential to become a paradigm shift in aesthetic and regenerative medicine.

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Part IV

Translational Aspects

20 How to Overcome the Valley of Death from Basic Science to Clinical Trials

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20.1 Introduction

Biomedical research has led to an explosion of knowledge about the mechanisms underlying many diseases and physiological processes, yet fewer than 5% of all life science discoveries lead to change in clinical practice [1], and those that do may take up to 10–25 years before they are implemented in the clinical environment [2]. Similarly, scientific breakthroughs in the field of regenerative medicine are abundant, yet their clinical applications are scarce [3] . The inability of novel scientific discoveries and technologies to reach clinical application led to the birth of translational medicine, a discipline which bridges the gap between the basic scientist and clinician, facilitating innovation from the bedside-to bench- and back [3, 4]. This bridge is not easy to navigate, as it requires the expertise of the scientist, clinician, university technology transfer office, and an interested entrepreneur.

In the United States, the National Institutes of Health (NIH) has led the translational science effort with the development of the National Center for Advancing Translational Sciences (NCATs) [1]. NCATs provides support for translational research programs at academic medical institutions throughout the United States, and similar efforts have been made throughout Canada, the United Kingdom, and Europe [1, 3, 4]. Such programs are poised to streamline the translational process and bring an abundance of novel scientific discoveries to the forefront of clinical practice. However, each critical step of the translational process faces a unique set of barriers that hinders the transfer of academic knowledge to the clinic. This gap lies at the interface of academia and industry. This divide can figuratively and literally be separated by time and space. It requires at some point for these two unique entities, academia and industry, to interface into a mutually beneficial arrangement both regarding technology transfer, consisting of intellectual property and capital (money) infusions.

The first translational gap, coined the 'valley of death,' spans the period between preclinical studies and clinical trials. Innovations that fail in this phase of the process may be promising in in vitro and in vivo animal models but cannot obtain the resources to progress through Phase I and Phase II Food and Drug Administration (FDA) clinical trials. With adequate resources, technologies that successfully prove their safety and efficacy in early clinical trials may overcome the 'valley of death' and transfer to entrepreneurial opportunities with family and friend cash infusions during Seed rounds and Series A, B, C capital raises.

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However, inventions in this phase are plagued by yet another set of hurdles before commercialization into routine clinical practice [3].

Research universities worldwide have developed technology transfer offices to facilitate product commercialization [5]. These offices are created to protect the intellectual property created by employees of the university. Through common disclosure pathways and provisional patent applications, the university tech transfer office looks to place a fence around the idea, product, application, etc. that the individual or individuals comes or come up with. Without the protection of this intellectual property, maximal value creation cannot be realized if anyone else can duplicate a similar idea. Despite such efforts, however, many university inventions fail to commercialize and achieve their desired clinical impact. Additionally, academic researchers are encouraged to publish and present new findings without knowing that is a form of public disclosure, thereby precluding patentability. Increasing evidence shows that startup companies based on university inventions can act as the bridge between academia and industry, allowing the translation of scientific ideas into clinical applications [6]. While seemingly a daunting task, with appropriate planning, collaboration, resources, and institutional support, the translational scientist can successfully bring their ideas from their minds to the bench, and finally, to the bedside.

Every successful innovation begins with an idea. However, translating breakthroughs in scientific research into new clinical treatments and therapies is no easy task. There are known risks associated with every aspect of this process. Before embarking on this journey, it is important to assess such risks and decide whether the product is appropriate for the development of a biotechnology startup.

There are multiple key issues to address, beginning with the idea itself. Is the innovation truly novel, and would it significantly advance its respective field? The product must not only meet an unmet clinical need, but it must do so in a manner that is better than any existing products and competitors. If the product does in fact meet an unmet need, one must assess the market that the product would be entering. Industry is the barrier that stands between the bench and the bedside, and each market has its own inherent risks. Will the potential benefits of your product outweigh the risks of entering the market? A key factor to consider is the size of the market. The market must be large enough to provide a substantial financial return for potential investors, yet it must not be overflowing with alternative products or competitors. Furthermore, one must evaluate what it will take in terms of financial investment and development time to succeed in the target market. Can the product enter the market in a reasonable amount of time? How much will it cost to get there? Finally, it is necessary to consider the regulatory hurdles that your product will face before commercialization. What FDA pathway will the product take? For instance, the FDA will accept premarket submissions (510 K) to demonstrate that a device to be marketed is at least as safe and effective or equivalent to a legally marketed device. This would make the device not subject to premarket approval (PMA) by the FDA. If a device requires a PMA by the FDA, then clinical trials of Phase 1, 2, and 3 need to be organized and planned for. The PMA will require significantly different types of capital requirements and business and organizational structures than a 510 K device. What are the clinical trial requirements based on the FDA pathway? It is essential to assess each of these issues individually before entering the translational pathway to avoid wasted time and money. Once the decision is made to proceed forward with the translational process, the path from the bench to the bedside can begin [7].

20.2 The Unmet Clinical Need

A well-characterized need is the DNA of a good invention [8]. Identifying a clinical need that is a “pain point” for patients, providers, or the healthcare system facilitates the development of technologies to maximize success. These high-value needs spawn ideas that investors

will care to fund and health care systems will adopt. Even the best innovations that only address a weak clinical need will often lead to eventual failure. Objective assessment of needs that are worth pursuing solutions should be performed before generation of ideas. One must first understand disease mechanisms and fundamentals that underlie the need in question. An evaluation of the competitive landscape and current solutions that exist enable identification of gaps and trends and whether there is sufficient space for new treatments. Treatment pathways and workflows can show the innovator where additional opportunities exist, as well as value-based problems such as phases of care (home, outpatient, inpatient, operating room). A stakeholder analysis around the need can help identify champions and roadblocks to future solutions. Most importantly, an exhaustive market analysis, including size and growth of those affected by the need further helps validate the decision to invest effort in the area of interest. The market can be segmented into total available markets, serviceable available market, and target markets. Large growing markets are attractive to investors and solutions organically are able to gain traction.

20.3 Ideation

Once an unmet need has been selected, ideation can begin. This step often results in only incremental changes to existing solutions when constrained and influenced by traditional thought or clinician-driven ideas of what will or will not work. Truly disruptive ideas enjoy unconstrained brainstorming using design-thinking principles. Out-of-the-box ideas should be encouraged, and inspiration should be sought from other fields or even outside of medicine. After exhaustive ideation, idea selection should assess feasibility and the height of the hurdles of intellectual property, regulation, and reimbursement that may derail that particular concept. Early low-fidelity prototyping can be immensely useful during both concept generation and selection.

20.4 Intellectual Property

A crucial first step in the path to commercialization is to protect innovations via obtainment of intellectual property (IP). In an academic setting, this process begins with invention disclosure to the university technology transfer office (TTOs). Since the passing of the Bayh-Dole Act of 1980, the United States requires that all federally funded academic researchers disclose their inventions to their university. The university will then own and be responsible for protecting the product's intellectual property. Following disclosure, TTOs will subsequently work to protect IP through patents and university licensing to allow for future commercialization [5, 9].

Although often overlooked by academic scientists, it is crucial to work with the university's TTO to file a patent and protect your invention from competitors. Potential investors are hesitant to finance non-patented products. Consequently, many non-patented inventions fail to ever make it out of the laboratory. For an invention to be patentable, it must be useful, novel, and non-obvious. Early preclinical data must help show that the invention can function as described. Furthermore, the invention must not overlap prior art; that is, competitors must not previously describe it. Lastly, the invention cannot be an obvious variation or extension of a previously existing patented invention. If an invention is deemed patentable, the university TTO may file a provisional patent that is valid for one year; within one year, a standard patent application must be filed. While academics may face pressure to publish their data quickly, it is imperative not to publicly disclose any aspect of your invention in abstracts, presentations, or publications until a provisional patent has been filed [9]. Finally, it is essential that you have freedom to operate; that is, the commercialization of the company's product must not infringe on other existing patents. For example, if one was to have a patent on television and another on color-televisions, it would be to the inventor and the patent office on each individual's patent

to have freedom to operate within the confines of each other's IP. Working with the university to develop a clear, cohesive patent strategy will ensure protection of intellectual property and allow for successful technology transfer from academia to industry.

20.5 Regulatory

Regulatory approval is a major milestone for a novel drug, device, or combination device towards commercialization and clinical impact. In the United States, the regulatory process is overseen by the Food and Drug Administration (FDA). Medical devices are classified as class I, II, or III, in order of increasing risk and therefore requirements. Most Class I (low/minimal risk) devices obtain exempt status and only have registration and labeling requirements. Most Class II devices require 510(k) clearance, which requires identification of a predicate device and proof that the proposed device is safe, effective, and has substantial equivalence to the predicate device. Class III devices usually require a Premarket Approval (PMA) pathway to FDA approval. PMA devices generally pose the greatest risk or do not have a predicate device, and therefore require the most data to prove safety and efficacy. A newer pathway, de novo 510(k), provides potentially less stringent requirements for novel devices with no predicate that are not deemed to be of significant risk to the patient [10]. Consultation with an experienced regulatory consultant in the specialty the device is intended is recommended to develop appropriate regulatory strategy. For example, although pursuing the PMA pathway requires more time and money for the required submission, approval via this route provides strong defensive strategy against competitor devices. Engaging the FDA through pre-submission meetings generally is useful to help a new company find out what type of studies would be required to prove safety and efficacy for the likely regulatory pathway for that device, and helps plan out time and funding requirements to meet this milestone.

20.6 Reimbursement

An understanding of how the device will be reimbursed is important to assess early in technology development. The Centers for Medicare & Medicaid Services (CMS) generally sets reimbursement for treatment of patients and procedures via codes. Most insurance companies follow CMS reimbursement structures to provide reimbursements to providers and hospitals. An understanding of how the new technology will be reimbursed has significant impact on stakeholders and eventual adoption of the technology by individual providers or hospital value committees. The technology may fit into a current reimbursement code; payment in different settings of care (e.g., outpatient home, inpatient) may differ significantly and influence product design. If existing codes are not favorable, company strategy may dictate attempting to apply for a new code to be created by CMS. This is another milestone that takes time and money to reach over an existing code, but may be worth the effort if it provides strategic improvement in reimbursement for adopters of the technology. For certain markets (such as private pay or direct-to-consumer), this hurdle may not be applicable.

20.7 De-Risking Technology: From Basic Research to Clinical Trials

Innovations themselves have intrinsic risks. Fortunately, there are a number of steps that academic researchers can take to "de-risk" their technologies early in the translational process to help ensure an efficient and successful pathway from early-stage research to clinical application. In an academic environment, research resources are limited. Thus, it is important to perform vigorous yet efficient research to substantiate the concept of your invention, and gain the attention of potential investors. A common failure of basic science research is the use of test systems that cannot accurately predict the outcomes of preclinical studies, and later, human applications. It is essential to carefully select validated in vitro

and in vivo models to avoid inefficient resource expenditure early in the translational process. High-quality, repeatable in vitro and in vivo studies must be performed before progressing to early clinical trials. In the health care industry, data is critical and must prove to both scientific experts and potential investors that the product will succeed beyond the laboratory. Innovations with inadequate basic science evidence are prone to failure later in the translational process after a significant amount of investment from researchers and investors alike [11].

After leaving the laboratory, technologies must be further 'de-risked' during clinical trials. Careful planning must be performed for each phase of a clinical trial to ensure there are enough resources to progress to each subsequent phase. Clinical trials are typically divided into three phases. Phase I clinical trials often involve a small cohort of healthy volunteers to prove a product's clinical potential. Phase II studies are used to determine a product's safety and efficacy in patients to establish 'proof of concept.' That is – does the product do what it is intended to do? Strong data collection in early clinical studies is paramount to support further product development and attract the attention of investors. Phase III clinical trials are typically large, randomized controlled trials. De-risking technology all the way through late stage clinical trials is required for regulatory approval and for future purchase by a large biotech/pharmaceutical/medical device company [9].

20.8 Management Team

One of the most important variables of a new company is the team. In fact, team has been shown to be the most important factor for startup investment selection across all stages, all industries, fund sizes, and locations [12]. Investors want to know that the money they invest will successfully take the company through the expected milestones. To successfully create a startup on their own, one must identify a clinical need, form an idea, develop a product that addresses the idea, test the product, and commercialize. This requires great individual and collective effort. The most promising technology may fail to receive funding because of an inadequate management team. A successful team should be composed of experienced individuals with complementary knowledge and skill sets [6, 7]. While building a team with a strong scientific foundation is important, it is equally important to consider including individuals with previous industry experience with a good track record in execution. Finally, each individual on the team should be enthusiastic in working towards a common goal or vision of driving the innovation from the bench to the bedside where it can impact patient care.

20.9 Business Model/ Commercialization

An appropriate business model should be created early on to understand how and when the technology could generate revenues to self-sustain, rather than remaining reliant on external funding. Different business models exist for different industries and device types (e.g., capital equipment, subscription, disposable, razor and blades, etc), and each should be explored to find the appropriate fit. Designing device features around an understanding of the cost of goods (COGS) allows the company to forecast how to build product at various powers of scale to maximize profits. Lastly, milestones should be set towards strategic exits (acquisition, merger, initial public offering, etc.) where investors will be able to recoup initial investments and multiples over that. These dynamics are dictated by capitalization tables that provide an analysis of company percentages of ownership (shares) by shareholders. Sales and distribution should also be planned to facilitate sales once the device is ready to sell to consumers. Acquiring key opinion leaders (KOLs) within the target markets may help boost adoption of the new technology into common practice. In the end, usage and adoption by target consumers and hospital value committees will result in the technology reaching patients and the company generating revenue.

20.10 Funding

Money is an integral component of every successful biotech startup. Capital raise can be the most difficult part of forming a successful biotech company. Companies that fail to commercialize their products often do not have enough resources to progress from early clinical trials through the end of phase III clinical trials. A clear financial plan must be in place to obtain and maintain adequate financial resources throughout the translational process.

Non-dilutive funding is often a significant source of funding in the early stages of a biotech startup company. Non-dilutive funding refers to any sources of funds that are provided by an agency without sacrificing ownership to the company or intellectual property. Common sources of non-dilutive funds include university grants, governmental grants, or donations from charitable organizations. In the United States, for example, organizations such as the Small Business Innovation Research (SBIR) and Small Business Technology Transfer (STTR) Programs of the NIH have an allotted annual budget to support early innovative efforts. Obtaining grants is competitive and requires substantial time and effort. However, non-dilutive sources provide a critical source of early resources for research and development purposes. Early funding is essential to help 'riskier' technologies progress from preclinical studies to early clinical trials [7, 9].

Once a technology has been substantially derisked, it may become attractive to potential investors, including angel investors and venture capitalists. Angel investors are high-net-worth individuals (or a group of individuals) that are accredited to invest in private companies. Investments typically fall in the range of $10,000-$100,000 from individual angels (angel group pooling may result in larger investment sums). Venture capitalists, on the other hand, are individuals who invest a large amount of another individual's or institution's money in private companies. Venture capitalists tend to be knowledgeable and strategic investors that will ultimately plan to purchase (all or part of) the company, help take it public, and commercialize it into routine clinical practice.

Biotech investors are well aware of the risks associated with translational medicine and the 'valley of death.' As such, they are highly selective when choosing which companies they will invest in. Investors favor technologies with minimal risk and maximal financial reward. Key components that investors look for include large market size with minimal competition, technological proof of concept, intellectual property and freedom to operative, and a strong management team with a clear business model [9].

Individuals must also be highly selective when choosing a potential investor. It is essential to select reputable investors that are knowledgeable in the product's respective market. If chosen wisely, angel investors and/or venture capitalists can be valuable mentors that will help drive the company's product towards commercialization. In a university setting, the technology transfer office can help introduce individuals to the potential investors that are most likely to help their company succeed [9].

Funding cycles are the financing that keep pre-revenue companies running until exit, generally proceeding in the following order: friends and family, pre-seed, seed, Series A, Series B, Series C, etc. Each round of funding makes the "pie" bigger (increased valuation) but reduces the founder's ownership, known as dilution. Each subsequent round is less "risky" for the investor and generally require more funding. The amount of raise requested by the early stage company is a combination of burn rate (e.g., employee salaries, equipment, office rentals, etc.) and money needed to achieve the next particular milestone (e.g., cost of clinical trials for FDA approval). The shares offered for each round is a function of the amount of the raise and current valuation of the company. There are a variety of financial models to estimate a company's valuation, most commonly cash-on-cash multiples, internal rate of return, and net present value (Gompers). How many rounds a company raises depend on their particular exit strategy. Some early stage companies are acquired after seed funding. Others go through multiple Series rounds with increasingly higher investments (for example, SpaceX

is currently raising a Series F round, with a $1 billion Series E round led by Google (Crunchbase)).

20.11 Commercialization Process

Technology represents devices and processes that can be made for financial gain. This concept of bench to bedside or the commercialization of biotechnology is the valley of death that one as inventor tries to hurdle (Fig. 20.1). To progress to market, the scientific entity must define customers and users. Most inventions will potentially fail in their rush to commercialization and most patents are not commercialized. This is the reality of the biomedical commercialization process.

Challenges to technological commercialization can take the form of recognition of the device potential, technology push, market pull, regulatory hurdles, access to capital, and overall entity management. The commercialization process is akin to the garden metaphor where one plants a seed, nurtures the product, and then pulls the weeds or harvests. Overlapped with this metaphor is discovering the technology (planting the seed), developing (nurturing), and market development (weed or harvest). Commercial entities develop options when to access these different time points. Identifying the product, testing and developing the product, and then marketing the product coincides in parallel with regulatory phases depending on what country the product is developed in.

Accessing this value-chain is critical for commercialization success. The value begins by defining the product and its clinical need. Otherwise known as discovery, this phase will use research funds granted by federal, state, and outside investors. Moving forward into product development, which will be tethered to phase 1, 2 and 3 trials, commercialization exits will need to be decided upon. Licensing the product or selling the company must be considered. A license will maximize the likelihood of short-term success. However, the entity will lose the ability to be involved with discussion for future innovation, as the larger corporate entity will control innovation and direction of the technology. Self-commercialization can be a path taken but is not always feasible secondary to increased financial risk. Joint-commercialization can be a considered option. This enables the firm to acquire experience that can enable self-commercialization over the long term.

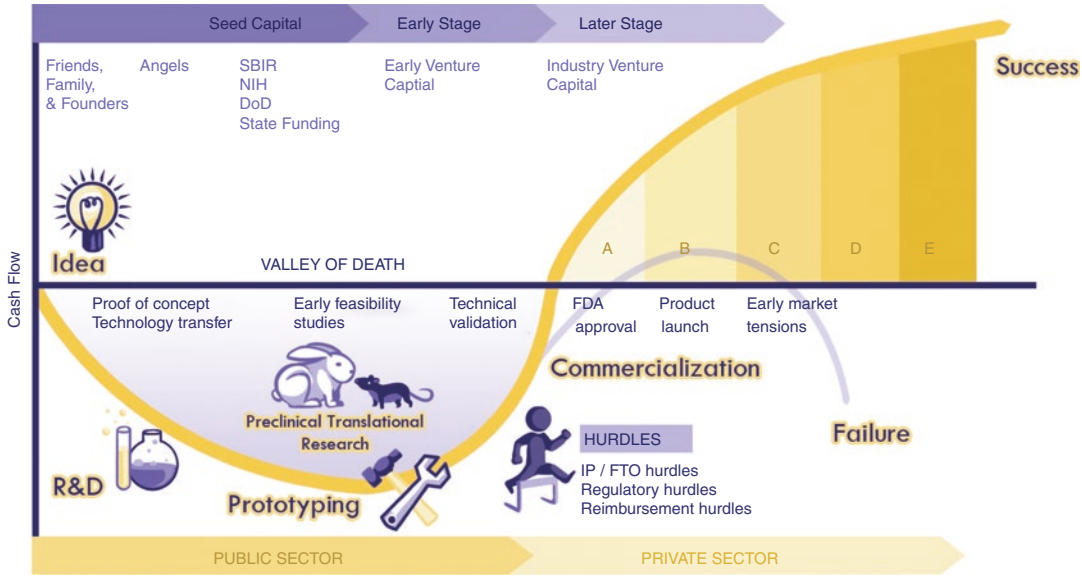

Fig. 20.1 Idea discovery, development, and market launch highlighting the role of capital infusions both pre and post regulatory approval

Taking factors into consideration such as amount of financing required to move through regulatory hurdles must be balanced along with the cost of capital raises. Dilution equity events for stakeholders must be considered. Risks that must also be factored when to commercialize are based upon scientific risk, operational risk, and whether there is a true market for the product. Financial return for the original inventors and developers must be considered versus incorporating and raising capital from outside sources such as venture capital, professional investors, and corporations.

Commercialization activities should be integrated into development plans. Market analysis, commercial planning, market cultivation, prelaunch, launch, and commercialization are all overlapped with phase 1, 2, and 3 development of regulatory development. These activities all begin the same time when conception of idea is formulated, and the path to customer is realized.

20.12 Conclusions

The success of any innovation is measured by whether it achieves its desired impact. In the case of a new biotechnology, pharmaceutical, or medical device company, the end goal is for the product to reach routine clinical implementation. Taking a basic science idea and turning it into a novel treatment or therapy for patients is a complicated process that requires a substantial amount of time, effort, and money. Not every idea or innovation can overcome the hurdles associated with the translational process. Those that are well suited to overcome these hurdles face a long journey ahead, however, the rewards of the journey could affect the lives of millions. Particularly high expectations surround the field of regenerative medicine. It is essential that the breakthroughs experienced in the laboratory reach our clinics. With the appropriate knowledge and planning, the potential clinical impacts of regenerative medicine are endless.

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21 Moving Your Results from Bench to Bedside: Protecting Scientific Findings

Dominik Thor

21.1 Introduction

Scientific research, especially in natural sciences, can be an exceptionally expensive and time-consuming process requiring the investment of millions of dollars before any potential profit and often before a technology's later aptitude for monetization can be guaranteed. This happens to be the case in most if not all medical research, where a plethora of different aspects influence the actual suitability of new scientific findings for clinical use. There are multiple challenges in the drug design process [1], such as selectivity and potency optimization for the intended target through changes in the physicochemical properties of the drug or the modification of functional groups, or drug formulation. This, together with the extensive pre-clinical and clinical testing required for the identification of the likelihood for metabolic interactions and significant side-effects in toxicology studies and thus a comprehensive benefit-to-risk evaluation of a new drug, result in huge financial efforts and therefore entrepreneurial risk. Additional efforts are linked to the obligatory review of new drugs by a regulatory agency, which is required before a new substance can be marketed [2]. The European Medicines Agency (EMA), the Food and Drug Administration (FDA) and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) are the biggest regulatory agencies, which are responsible for the regulation, approval, and oversight of drug products. The complexity of interacting with the regulatory agencies is significant and the challenges of fulfilling their specific requirements force pharmaceutical companies to dedicate funds and human resources entirely to dealing with regulatory affairs, thus increasing the costs and financial risks for them further.

For companies to accept such risks, the eventual monetization of their scientific research, most often in the successful launch of a new drug, has to offer an accordingly high potential income, and the prerequisite for that comes in the form of exclusive legal rights to sell the new drug for at least some years after it enters the market. This may be different for basic research, which requires less investment, and is often inspired by the scientist's wish to further the understanding of a certain aspect of a scientific field rather than monetary motivation or simply does not offer an obvious or feasible potential for monetization. As such basic research is often a domain of academic efforts [3], nonetheless, reaching the goals of both commercial and academic research requires the protection of scientific results or, in more general terms, intellectual

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property. This unfortunately still happens to be an insufficiently understood and inconsequently applied principle by many scientists especially in the academic sector.

21.2 Patents for the Protection of Intellectual Property (IP)

Intellectual property (IP) is a term used to describe a plethora of creations of the mind, such as inventions of all kinds, artistic and literary works, as well as symbols, names, and images. While some kinds of intellectual property are automatically safeguarded by copyrights and thereby belong to their creator, other types of intellectual property require legal protection that must be applied for. This legal protection comes in the form of intellectual property rights such as patents, trademarks, and copyrights, which are essential for monetizing or protecting innovation. The importance of intellectual property was officially recognized in two treaties, namely the Paris Convention for the Protection of Industrial Property (1883) and the Berne Convention for the Protection of Literary and Artistic Works (1886). Today, they are administered by the World Intellectual Property Organization (WIPO). These conventions recognized several reasons why intellectual property rights are vital to progress, and in today's world of quickly evolving technologies they are of increasing importance. Further progress of humanity requires the capacity to create and invent new works in the areas of technology and culture. Only legal protection, through its options for recognition or financial benefit, can sufficiently encourage the pursuit of innovation – which in turn spurs economic growth, creates new jobs, industries, enriches our cultural lives, enables us to lead healthier lives with increased life expectancy, and enhances the general quality of life [4].

From the perspective of the innovator, intellectual property rights offer many advantages:

1. They give control over a creation's commercial production, use, distribution, or sale.
2. They ensure that a superior product or process cannot be copied by competitors.
3. They can be sold or licensed, and thus generate revenue.
4. They are proof of a pioneering role in science.
5. They can be an essential part of marketing.
6. They are assets of economic value and can be used as collateral.

Intellectual property falls into two groups. First, types of IP that are under automatic protection and for which the author automatically has the copyright. This category consists of original literary and artistic works including photography, film and music, as well as design rights to three-dimensional products. Then there is the second group of intellectual property, which does not come with automatic legal protection. This group consists of inventions, novel products, or innovative processes for which patent rights must first be obtained.

Obviously, the results of medical research fall into this latter category of intellectual property, which has to be protected by patents. Medical inventions, including incremental inventions, first require patenting for the scientist to acquire the intellectual property rights to his or her creation [5]. Patents are intellectual property rights enforceable in court, which give a patent owner the right to decide on the use of the patented invention. Filing patents is the logical choice for commercial drug developers, who need the exclusive rights to sell a new drug at least for some years after it enters the market to compensate for the costly development. Once their strictly confidential research on a potential new drug shows enough promise and for security reasons, this decision often happens in early stages such as lead structure development but can also be postponed until the results of pharmacological and toxicological tests. The strategy here is to file a patent on the drug structure and potentially also its synthesis route. Most countries follow the legal concept called first-to-file (FTF), which means that the right to the grant of a patent for a given invention lies with the first person to file a patent application for protection of that invention, regardless of the date of actual invention. Given that trends exist not only in corporate but

also in the context of scientific research, not only is there competition between different scientists to come up with a suitable solution to the same problem, this race further extends to the subsequent filing of a patent, which is necessary to secure the rights of the inventor. This means that the ultimate financial benefit will be with that party who ultimately manages to file the patent first, not necessarily with the one who comes up with the solution first [6]. The downside of filing patents early on is a reduced time for the exclusive marketing rights of the fully-developed drug, which has to do with the fact of patents usually having a limited lifespan of 20 years. If a drug is patented during its early development and may require another 7–15 years to reach the market, then a pharmaceutical company would be left with only 5–13 years to make a profit on its new product, which often explains the high costs of new drugs [7].

21.3 Defensive Publications Offer Freedom to Operate

Medical research typically aims at finding solutions to complex medical problems in the form of innovative inventions or significant improvements to the status quo. Once the scientist has come up with a successful and unique solution, his or her decisions in the context of establishing proof of authorship and the publication of his or her invention will have severe implications in the future. Depending on the intentions and means of the inventor, he or she must decide between different options.

There are circumstances when patents are not practical for protecting intellectual property that results from scientific research. Filing a patent typically tends to be very expensive - primarily because of the complexity of inventions and the abundance of prior art in the respective field of science resulting in complex application documents, which usually must be prepared by an experienced lawyer to ensure that a patent has a greater chance of being successfully granted. Consequently, attorney fees can easily cost several tens of thousands of dollars in addition to the patent office filing fees. Considering that a patent will need to be filed for every single country in which the innovator wishes to posses IP rights, big companies have a major advantage over private innovators, who often lack the necessary funds to secure the global rights to potential high-revenue products with long-term marketability. The high costs of pursuing national or international patent rights may prevent private innovators from going this route altogether, while commercial inventors usually have the necessary experience and funds to do so. In some cases, however, the benefits afforded from patent monopoly rights are not sufficient to justify the cost of obtaining a patent. Either because the products lack long-term marketability or, and this happens frequently in the corporate environment, because the patent holder already has rights to another product that he or she only seeks to protect against the possibility of others patenting a technology that may make the product redundant. In other cases, the high costs of patenting may outweigh the benefits afforded by patent rights, irrelevant of the innovator's financial background.

Occasionally, e.g., in the case of academic research, when commercialization is not a priority or its potential not even recognized, obtaining intellectual property rights may not even be desired. However, the innovator might still be interested in retaining his or her freedom to operate by preventing others from filing a patent. This regularly happens in academic settings, where continued freedom to operate and to further refine a technology might be of more interest than financial benefits. Instead, the innovator may simply wish to secure the right to continue using and enhancing a product or technology to further research in his respective field. He or she might also pursue altruistic goals, as seen in open source projects, by wishing to put his or her invention into the public domain to make it freely available and prevent others from filing a patent.

Patents may be essential for incentivizing continuous scientific effort through their importance for eventual monetization but the costs associated with obtaining patent rights often prevent innovators from pursuing them, and some scenarios

profit from a different course of action than patent application. This alternative comes in the form of defensive publication, which is the intentional and purposeful publication of an innovation. As a cost-effective intellectual property strategy, it consists of disclosing aspects of an invention in a way that ensures that the invention gets the status of prior art, thus precluding others from obtaining a patent on the innovator's idea. Since the novelty of an idea is an essential precondition for obtaining a patent, the publication of an idea can be strategically used as an IP strategy, if the previous publication date can be proven in court and a patent office can readily obtain knowledge of existing prior art [8].

Current venues for publishing defensive publications range from traditional peer-reviewed journals to online publications. Publication in peer-reviewed journals, as desirable as it may be from a scientific point of view due to the additional validation by the journal, cannot be easily or quickly obtained. Submitted articles frequently take months to get published and the editors' criteria for the selection of content are associated with the possibility of eventual rejection by the journal. While academics learned to live with these disadvantages, this option renders too time consuming and not suitable for most private innovators or corporate researchers, as journals also typically avoid any content that could be interpreted as marketing. Unfortunately, many other forms of publication such as web sites, social networks, fairs, public demonstrations, or tradeshows are however insufficient proof in the eyes of courts, and are therefore not suitable for defensive publication. Neither would such methods of publication offer a guarantee that the published invention would be successfully found, identified, and considered by patent examiners and patent offices, as they are not listed in readily searchable databases. Therefore, a scientist should carefully consider his or her chosen venue for defensive publication and also consider all possible implications of such an act.

In many countries, the inventor might lose the right to file a patent following publication, as it turns an invention into prior art. The European Patent Office for instance regards early disclosure an absolute bar to an EPO patent. However, other important markets such as the United States, Russian Federation, Japan, Canada, South Korea, Australia, Brazil, Argentina, Malaysia, Mexico, and several others use the so-called first-inventor-to-file system (FITF). Following this system, the United States Patent and Trademark Office (USPTO) and other patent offices afford early disclosers a 'grace' period until they need to file a patent. This grace period starts at the time of publication and typically lasts 6–12 months during which the inventor can still file a patent, but this does not prevent someone else to file a patent for the same idea during that time [9].

21.4 Trade Secrets as a Common Alternative

With patents being frequently too expensive for academic researchers and defensive publications prohibiting the later exclusive rights to a new drug or medical product, valuable information that can provide a competitive advantage is instead often kept strictly confidential, as a so-called trade secret. Unfortunately, holders of such intellectual property are often exposed to misappropriation (the intentional and illegal use) of their trade secrets. While large companies possess greater resources to protect their intellectual property, including costly patents and funds for legally enforcing their rights, smaller companies, in comparison, do not have such financial means. Consequently, smaller companies rely on trade secrets to an even greater extent than large companies. The problem of misappropriation has increased so much that political institutions are now seeking new ways to prevent misappropriation. The European Commission has urged the European Parliament to standardize the existing divergent national laws against the unlawful acquisition, disclosure, and use of trade secrets. Such harmonization will give victims of trade secret misappropriation more protection and the means

to stop unlawful use and further disclosure of misappropriated trade secrets, as well as the right to compensation for any damages caused [10]. Thus, the EU Directive on the Protection of Trade Secrets will provide a legal framework to discourage unfair competition, and facilitate collaborative innovation and the sharing of valuable know-how. EU countries must bring into force the laws and administrative provisions necessary to comply with the Directive by June 2018. New and improved legislation to better protect trade secrets is not, however, limited to the EU. In fact, the Defend Trade Secrets Act of 2016 (DTSA) is a United States federal law that allows an owner of a trade secret to sue in federal court when its trade secrets have been misappropriated [11]. International efforts by the World Trade Organization to address this problem led to the conclusion of the Agreement on Trade-related Aspects of Intellectual Property Rights (the TRIPS Agreement) [12]. What all of these regulatory efforts have in common is the vital condition that companies must prove that a piece of information, the trade secret, has deliberately been kept secret, for them to be able claim protection under trade secret laws. Needless to say that academic researchers working in a university environment may find it much more difficult to keep such information confidential than scientists working in a strictly corporate environment.

21.5 The Right to Filing IP Rights

So far this analysis of ways to protect inventions in the academic (or corporate) environment highlighted different reasons for choosing a specific path, often linked to its economic and organizational aspects (Table 21.1). Complexity, costs, and revenue potential all have an important impact on the practical suitability of each path or instrument for protecting new innovations and ultimately decide whether such actions should be taken. It should be noted that in many cases the decision however does not lie with the scientist or inventor. Both in academic and corporate research, the contractual agreement between the employer and the employee doing specific research typically stipulates that any rights to new developments belong to the employer - may that be a university, a research center, or a pharmaceutical company. This also extends to any research done in the spare time of the employee if the company can prove that any of its assets or intellectual property have been involved and/or used or if there is any resemblance or relation to the scientist's work or the company's other activities. Even completely unrelated scientific work has to be very carefully kept separate from any professional endeavors. Patent applications for inventions developed in his or her professional capacity may be filed in the scientist's name but will identify the employer of said researcher as the holder of the patent rights. The failure to make any such potentially useful information known to the company or university could lead to the termination of the employment agreement and likely be linked to risks such as claims for both compensatory damages and punitive damages.

In the context of academic research, the right of the institution to new intellectual property often even transcends contractual agreements between commercial sponsors and the individual research unit. To avoid any disputes regarding the ownership of IP rights deriving from research projects sponsored by or involving third parties, such as pharmaceutical companies, the contract has to consider all applicable rules and laws.

Table 21.1 Comparison of Forms of IP protection

	Monetization	Costs	Complexity	Duration
Patent	Yes	High	Very high	20 years
Defensive publication	No	Low	Medium	Unlimited
Trade-secret	Yes	None	Non	Potentially unlimited

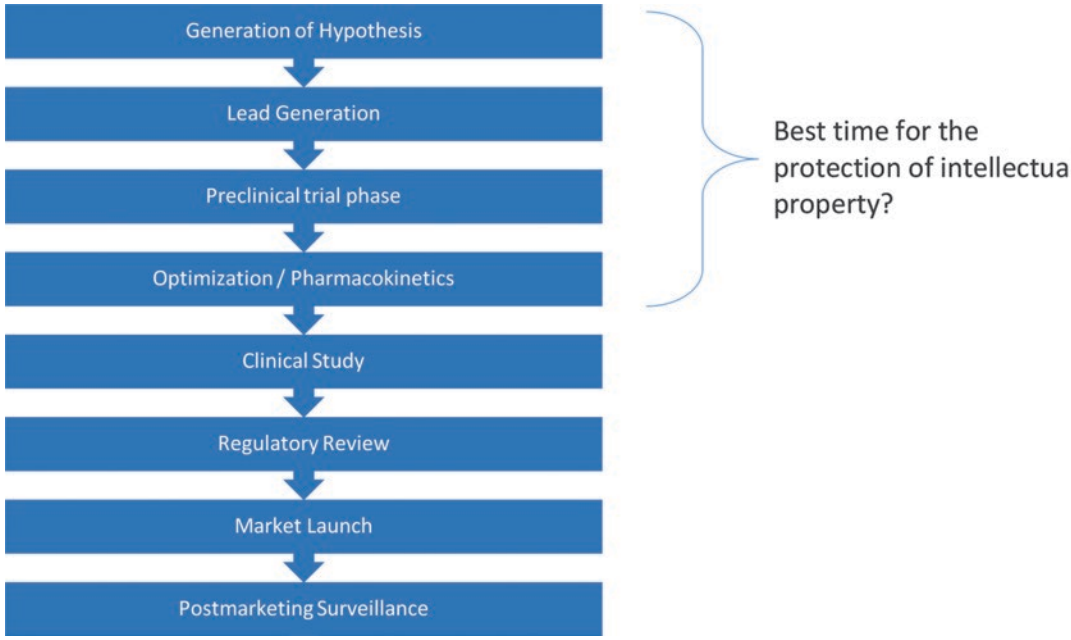

Fig. 21.1 Timing of IP Protection in Pharmaceutical Research

21.6 The Right Time to Decide (Fig. 21.1)

Protecting the results of scientific research at an early stage has been proven to be the best paradigm, if said research has scientific or commercial potential. Even if monetization and monopolization are not desired, retaining freedom to operate is often the decisive prerequisite for complex scientific undertakings. To decide on the best course of action, the scientist will however have to familiarize himself or herself with the legal consequences of his actions, the available methods of protecting his or her interests, and has to be capable of making qualified assumptions as to costs of such legal steps and the commercial potential of his invention. Needless to say, these decisions place a lot of responsibility on the shoulders of someone with often very little practical experience in such matters. Having internal or external expert advisors involved in the ongoing research efforts should therefore be regarded as essential.

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22 The Regulatory Landscape of Cell- and Tissue-Based Regenerative Medicine: Current Challenges and Emerging Issues

Sarah Khalid Alageel, Martin Hildebrandt, and Ana Valéria Gouveia de Andrade

22.1 Introduction

Rather than managing sustained disease or damage, the field of Regenerative Medicine (RM) is aimed at restoring or establishing normal function by replacing or regenerating human cells, tissues, or organs [1]. As a subdivision of translational research in molecular and cell biology, biomaterial science, and organ and tissue engineering [2], RM holds great promise in addressing the global lack of organ supply, aging-related diseases, and congenital or acquired defects by either actively reconstructing de novo organs [1] and/or tissues or functionally healing previously irreparable tissues or organs by stimulating the body's own repair mechanisms [1].

Under this umbrella, researchers have been working vigorously on the development and bench to bedside translation of a variety of innovative therapeutic products such as: human cell and tissue products, tissue engineered therapeutic products, gene therapy products, and combined products. However, despite the continuous advances in science and technology paving the way in the development of Regenerative Medicine Therapeutics (RMT), to date, only few products have been authorized for marketing in the United States (US) and the European Union (EU).

To better understand how the EU and the US manage the development and manufacture of RM products, details regarding the regulatory process from the first step of classification until market approval will be addressed here.

22.2 EU and US: Different Approaches When it Comes to Medicinal Products

In the EU, the evaluation and regulation of the translation and marketing of RMTs is overseen by the European Medicines Agency (EMA).

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RMTs that are designated as Advanced Therapy Medicinal Products (ATMPs) are pharmaceuticals with a high level of complexity linked to their composition, development, manufacturing, characterization and administration, and represent the forefront of medical research, blurring the lines between medicinal products and medical devices. They are comprised of somatic cell therapy medicinal products, tissue engineered products, gene therapy medicinal products or combined ATMPs (Fig. 22.1). They are derived from living human tissue, which is then manipulated in such a way that it can then be used in a therapeutic setting [3–6].

Somatic cell therapy medicinal products (SCMPs) consist of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions, or structural properties relevant for the intended clinical use have been altered, or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor. SCMPs are presented as having properties for, or are used in or administered to human beings with a view to treating, preventing, or diagnosing a disease through the pharmacological, immunological, or metabolic action of its cells or tissues [7].

Tissue engineered products (TEPs) consist of engineered cells or tissues, and are presented as having properties for, or are used in or administered to human beings with a view to regenerating, repairing, or replacing a human tissue. TEPs may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or non-viable. They may also contain additional substances, such as cellular products, bio-molecules, biomaterials, chemical substances, scaffolds, or matrices [3].

Gene therapy medicinal products (GTMPs) either contain an active substance that consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding, or deleting a genetic sequence. Its therapeutic, prophylactic, or diagnostic effects relate directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence. GTMPs do not include vaccines against infectious diseases [7].

Combined ATMPs contain one or more medical devices as an integral part of the medicine, such as cells embedded in a biodegradable matrix

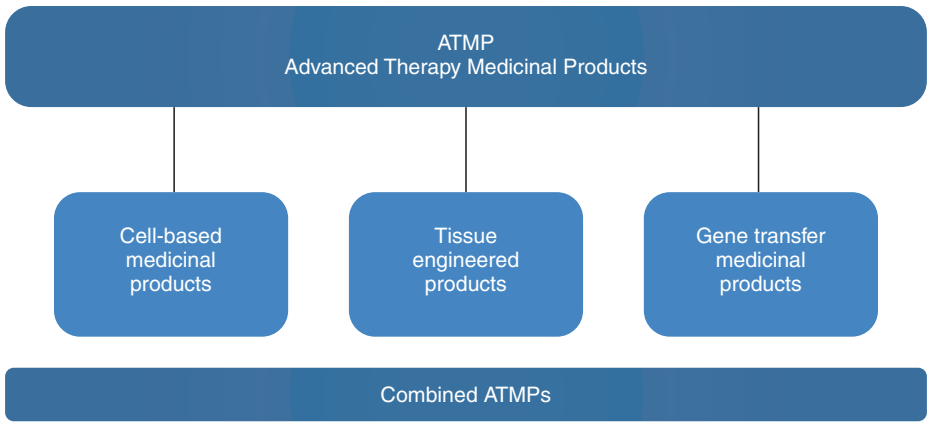

Fig. 22.1 Classification of ATMPs according to EMA. (Adapted from Paul-Ehrlich-Institut Booklet [8])

or scaffold [3]. The translation of different ATMPs into the market requires the obtainment of a marketing authorization (MA). When attempting to translate an ATMP into the market, it is important to note that it is essential to comply with ATMP regulations, as well as EMA's regulatory system.

In the United States, the body responsible for the evaluation and regulation of the translation and marketing of RMT products is the US Food and Drug Administration (FDA). The FDA regulates most RM products under the Food and Drug Safety Act, Title 42, Chapter 6A- Public Health service, Public Health Service Act Section 361 and 351 (PHS Act 361, Title 42 USC Section 264) and Code of Federal Regulations Title 21 Part 1271 (21 CFR 1271) in a risk-based tiered structure [9]. RM products are regulated as human cells, tissues, and cellular and tissue-based products (HCT/Ps) based on the criteria defined in 21 CFR 1271.10 (a) [10], and are defined as "Human cells or tissue intended for implantation, transplantation, infusion, or transfer into a human recipient" (21 CFR 1271.3(d)(1) and Section 361 of the PHS Act). If they meet all of the criteria, they are regulated by the Center for Biologics Evaluation and Research (CBER) solely as 361 products do not require additional regulatory oversight (not subject to pre-market requirements) and follow the 'tissue rules' [11]. Examples of products regulated solely as 361 products include: skin, Dura mater, bone (including demineralized bone), and cartilage [11, 12]. RM products that do not fulfill one or all of the criteria can be regulated as:

1. Drugs and/or Biological Products, such as human somatic cell therapy and gene therapy products (which are also regulated by CBER under Section 351 of the PHS Act and/or the FD&C Act) and require additional regulatory oversight (pre-marketing requirements) [11, 12]. Examples of products falling under this designation include: lymphocyte immune therapy products, gene therapy products, and cultured cartilage cells.
2. Medicinal Devices composed of human tissues (regulated by the Center for Devices and Radiological Health (CDRH), under the FD&C Act and device regulations) and also require additional regulatory oversight (such as preserved umbilical cord vein grafts and human collagen) [11, 12].
3. Combination Products, which combine more than one type of product, are regulated based on the mode of action of the product. Requests for Designations (RFDs) of combination products are handled by the Office of Combination Products (OCP) that determine the responsible office within the FDA [13]. Examples of Combination Products include: encapsulated pancreatic islet cells (regulated as biological products), bone-suture-tendon allografts (regulated as devices), and demineralized bone combined with handling agents (e.g., glycerol or sodium hyaluronate) (regulated as devices) [11, 12].

Most advanced therapy products are regulated as Drugs and/or Biological Products. Recognizing the importance of the regenerative medicine field, and to facilitate the development and approval of RM products, a new program has been established that allows applicants with RM products to file for approval with a Regenerative Medicine Advanced Therapy (RMAT) Designation [14]. Drugs are eligible for RMAT designation (Section 3033 of the Twenty-first Century Cures Act) if:

1. the drug is a regenerative medicine therapy, which is defined as a cell therapy, therapeutic tissue engineering product, human cell and tissue product, or any combination product using such therapies or products, except for those regulated solely under Section 361 of the Public Health Service Act and part 1271 of Title 21, Code of Federal Regulations;
2. the drug is intended to treat, modify, reverse, or cure a serious or life-threatening disease or condition;
3. preliminary clinical evidence indicates that the drug has the potential to address unmet medical needs for such disease or condition [15].

Applicants filing for an RMAT designation must do so in addition to filing an Investigational New Drug application (IND) or as an adjustment to an already existing one [15].

Therefore, addressing regulatory issues concerning cell- and/or tissue-based regenerative medicine products to further develop them into the clinic, begins with the accurate definition and classification of the intended product. It is important to notice that to develop/manufacture a product, the product must be, first, accurately defined and subsequently regulated.

22.3 Classification of RM Products in the EU and the USA

Both EMA and FDA officially classify products when reviewing market or pre-market submissions. However, to select the accurate regulatory submission path and to fully comprehend the extent of regulatory control required to ensure the safety and effectiveness of the product, it is important for manufacturers or stakeholders to identify the classification beforehand [13].

Similarities exist between the EU and the US in the classification of RM or cell- and tissue-based products, mostly about the considerations of: major vs. minor manipulation [16], intended use for its original function, and risk assessment in the classification of the products. However, here is where the similarities end, and this is largely due to the approach in the designation of the products.

In the European Union (EU), cell/ tissue-based products are regulated as ATMP medicines for human use (Article 17 of Regulation (EC) No 1394/2007) (and classified accordingly) [17] when they have been substantially manipulated and/or are not intended to be used for the same essential functions in the recipient as in the donor (not for homologous use). Non-substantially manipulated cells or tissues used for the same essential function are not considered ATMPs.

Minimally manipulated cells and tissues that are not considered medicinal products are regulated under EUCTD (2004/23/EC): donation, testing, procurement, processing, storage, and distribution across the EU. Example: preparation of enriched populations based on immunophenotypic markers such as CD34 or CD133 for haematopoietic transplantation. Examples of procedures that are considered to be of minimal (non-substantial) manipulation include: cryopreservation, cutting, filtering, cell separation, concentration or purification, irradiation, and filtering (i.e., they do not alter the biological characteristics or structural properties relevant for the intended function) [17]. However, in Germany, the Transfusion Act (TFG) defines blood cells and cell preparations from peripheral blood as medicinal products.

Substantially manipulated cells/ not the same essential function(s) are covered by the Regulation 1394/2007/EC and are classified as Advanced Therapy Medicinal Products (ATMPs), which include three categories: (a) somatic cell therapy medicinal products as defined in Part IV of Annex I to Directive 2001/83/EC; (b) gene therapy products as defined in Part IV of Annex I to Directive 2001/83/EC; (c) tissue engineered products as defined in Article 2(1) (b) of Regulation (EC) No. 1394/2007; (d) combined ATMP products under Article (2) (1) (d) of Regulation (EC) No. 1394/2007 [18].

Products in the EU can also be classified according to risk. Cell-based products are considered to be of high risk if the product has been: (a) subjected to a substantial amount of manipulation; (b) used for a function(s) different from its original function); and (c) if the product is combined with another product. All xenogeneic cell-based products are classified as ATMPs by default.

In the US, however, classification, and thus designation of products, is much more complex. RM products can be:

1. HCT/Ps and given the 361 designation are of low risk if they meet all of the criteria under 21 CFR 1271.10(a): (a) it is minimally manipulated; (b) it is intended for homologous use only; (c) it is not combined with another article; and (d) it does not have a systemic effect and is not dependent upon the metabolic activity of living cells for its primary function; or, it has a systemic effect or is dependent upon the metabolic activity of living cells for its primary function, and is for autologous use, for

allogeneic use in a first or second degree blood relative, or for reproductive use [10]. These products are not subject to pre-marketing requirements [19].
2. HCT/Ps that do not meet one or more of that criteria can be classified as either drugs, biologics, and/ or devices under Section 351 of the PHS Act and/or under the Food Drug and Cosmetics Act. These products are of high risk and are subject to pre-marketing requirements [19].
3. Products that contain one or more combination products (as defined in 21 CFR § 3.2(e): products comprised of any combination of a drug and a device; a biological product and a device; a drug and a biological product; or a drug, device, and a biological product) are classified as combination products and based in their classification on their primary mode of action for their primary designation.

On substantially (major) manipulated vs. non-substantially (minimally) manipulated products, generally, both the US and the EU have similar definitions as to what constitutes major/substantial manipulation and/or minor/or non-substantial manipulation. In the EU, substantially manipulated products encompass manipulation throughout the manufacturing process so that the physiological functions, biological characteristics, and/or structural properties of cells and/or tissue(s) have been relevantly modified for their intended function [17]. Some examples include: the enzymatic digestion of tissues and cells that have been artificially expanded in culture, treated with growth factors for differentiation/activation, and/or cells that have been genetically modified. In the US, the FDA substantially manipulated is classified under Title 21 of the Code of Federal Regulations (CFR) Part 1271, specifically 21 CFR 1271.3 (f). Minimally manipulated products are defined as "products that if containing structural tissue, have been processed in a way that does not alter original relevant tissue characteristics related to the tissue's utility for reconstruction, repair, or replacement; and if containing cells or non-structural tissues, have been processed in a way that does not alter relevant biological characteristics of the cells or tissues" [20].

22.3.1 Intended Use or Essential Function (Homologous or Non-Homologous Use)

If no substantial manipulation takes place, products are classified according to the essential function of the cells/tissues. Products are considered of homologous use when the "repair, construction, replacement, or supplementation of a recipient's cells or tissues with an HCT/P that performs the same basic function or functions in the recipient as in the donor" [20] (under the US FDA's regulation Title 21 of the Code of Federal Regulations (CFR) Part 1271, specifically 21 CFR 1271.3(a) (2)/1271.3 (c) criterion and definition of homologous use) [10]; or, "when removed from their original environment in the human body are used to maintain the original function(s) in the same anatomical or histological environment" [17]. An example of homologous use would be donated bone marrow for leukemic patients that have undergone total body irradiation (TBI) [17].

22.3.2 The Product's Claimed Mode of Action (MoA)

The product's claimed mode of action answers questions on whether the product is meant for diagnosis, prevention, or treatment of disease. Does it exert its activity via a pharmacological, immunological, or metabolic action? Alternatively, is the product intended for regeneration, repair, or replacement of cells/tissues? [17].

22.3.3 Other Points Taken Into Consideration When Classifying a Product

1. Their status as living sources: the biologics of the medicinal products for human use [21].
2. The origin of the cells utilized (cell source): autologous or allogenic [16, 21].
3. The application of the anatomical/histological environment [9].

22.4 The EU Legal Framework for ATMPs

A consolidated regulatory framework for the development of ATMPs in Europe came into force in 2008 [22]. The overall framework on ATMPs is provided by Regulation (EC) No 1394/2007, which amended the Medicines Directive 2001/83/EC, and established the Committee for Advanced Therapies (CAT) at the European Medicines Agency (EMA). The EMA is a decentralized agency of the European Union (EU) responsible for the scientific evaluation, supervision, and safety monitoring of medicines in the EU, and the CAT is a multidisciplinary committee, whose primary responsibility is to assess the quality, safety, and efficacy of ATMPs, and to stay up-to-date on the developments in the field. When it comes to regenerative medicine products, the Committee for Medicinal Products for Human Use (CHMP) under the EMA is in charge of evaluation and approval. All legislatures relating to medicinal products, including regenerative medicine products, are data based in the EudraLex. This regulatory framework is designed to ensure the free movement of these medicines within the EU, to facilitate their access to the EU market, and to foster the competitiveness of European pharmaceutical companies in the field, while guaranteeing the highest level of health protection for patients [23].

As this regulatory framework for ATMPs in the EU, because of constant advances in the field of regenerative medicine, is dynamic, complex, and advances rapidly, early interactions with regulatory agencies to ensure collaborative discussions between clinical product developers and regulatory experts are a 'must'. Hence, many regulatory agencies globally encourage and provide opportunities, such as a Scientific Advice Meeting with a national competent authority (e.g., Paul-Ehrlich-Institute, Germany) and/or EMA [6].

The CAT issues scientific recommendations on ATMPs under the ATMP Regulation (Article 17) [17]. Its recommendations are based on definitions laid down in the EU legislative texts:

1. Regulation (EC) No. 1394/2007 on ATMPs provides the definitions of "tissue-engineering product" and combined 'ATMP'.
2. Part IV of Annex I to Directive 2001/83/EC provides the definitions of "gene-therapy medicinal product" and "somatic cell-therapy medicinal product" [17].

At the inception of the development of a product, it is important to consider seeking CAT classification, which is an optional, no-fee procedure. However, achievement of such status holds significant merit in obtaining fee reductions for EMA scientific advice and potential benefits in successfully navigating the clinical trial application process through national authorities in Europe [24].

Developers of ATMPs should also consider if their product may be eligible for the newly introduced Priority Medicines (PRIME), which is a voluntary scheme launched by EMA in 2016 to enhance support for the development of medicines targeting an unmet medical need [6].

In addition to the existing complexity, in Europe certain ATMPs are also considered a Genetically Modified Organism (GMO), i.e., an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination (Directive 2001/18/EC). In the EU, before a clinical trial can commence for ATMPs considered GMOs, besides the ethics committee and competent authority approval, a GMO approval must also be obtained. Nevertheless, the regulatory classification processes and requirements for GMO approval are not sufficiently harmonized between the EU Member States, despite the EU Deliberate Release (2001/18/EC) and the Contained Use (2009/41/EC) Directives, resulting in significant challenges and timeline considerations [6].

The process of ATMP market approval starts with the collection of tissues and cells from donors and their evaluation under the European Union Tissue and Cells Directives (EUTCD), the

EU version of Good Tissue Practice (GTP). Preclinical testing for safety of the product will be performed under Good Laboratory Practice (GLP), similar to the USA [25].

On scientific and technical aspects of drug registration, the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH), as a tripartite initiative between Europe, the US, and Japan, takes place bringing together the regulatory authorities and pharmaceutical industry [26]. To bring new medical product to market, clinical trials are required by regulatory authorities of the originating countries [26]. When the product is adequate for market approval, it will lastly be evaluated by the EMA as a final step to market application. ATMPs will be required to obtain continuous post-market evaluation on the traceability of the donors, products, and patients, as well as the development of risk management systems and pharmacovigilance, especially for follow-up on efficacy and safety. Similar to the situation in the USA, the whole process may take multiple years (Fig. 22.2) [25].

Although the ATMP regulation has been in place for more than 10 years, the number of marketing authorization applications and successful approvals in the EU remains in single figures. Of the 18 marketing authorization applications submitted to EMA since the ATMP regulation came into force in 2009, EMA said nine products have been approved [27]. However, of those nine approved, four have been withdrawn from the market or suspended. For instance, UniQure's Glybera, the first gene therapy authorized in Europe in 2012, was later withdrawn from the market. Similarly, Dendreon's Provenge and TiGenix's tissue-engineered product ChondroCelect, approved in 2009, were also withdrawn. Vericel Denmark's Maci in 2013 was suspended at the recommendation of the Committee for Medicinal Products for Human Use (CHMP)[27].

Other ATMPs remain on the market and include German company CO.DON AG's Spherox, approved in 2017, MolMed S.p.A.'s Zalmoxis, approved in 2016, as well as Chiesi Farmaceutici's Holoclar, GlaxoSmithKline's Strimvelis, and Amgen's Imlygic [27].

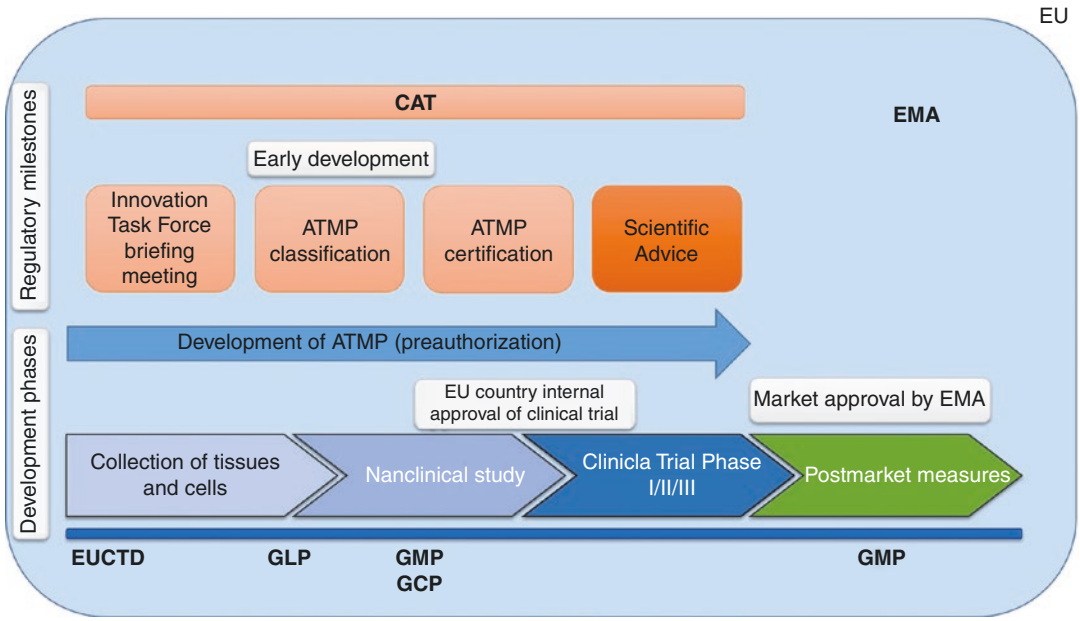

Fig. 22.2 Regulatory pathways and development phases for ATMPs in Europe. (Adapted from Sakai et al., 2017 [23] and Maciulaitis et al., 2012 [25])

22.5 The US Legal Framework

In the ensuing successful pre-clinical investigations (laboratory and animal testing to answer basic questions about safety, devoid of human participants, and mainly conducted under Good Laboratory Practices (GLP) conditions) (21 CFR Part 58) [28–30], with the aim of translation of RM products into the market, sponsors/stakeholders must follow a series of steps under the governance of FDA regulatory requirements (Fig. 22.3) [23].

To select the accurate regulatory pathway and identify its requirements, sponsors/stakeholders must first accurately classify their products. The FDA officially classifies products during the review of the pre-market submission. However, it is the responsibility of the applicant to ensure the correct classification of the product that has been applied [13]. FDA regulation focuses on the:

1. Prevention of inadvertent transmission of infectious diseases such as AIDS and Hepatitis through the use of contaminated tissues.
2. Prevention of contamination and/or damage through improper handling and/or processing.
3. Ensuring that clinical safety and effectiveness is demonstrated, in a risk-based tiered approach [21, 31].

HCT/Ps that meet the requirements (criteria under 21 CFR Part 1271.10(a)):

1. are considered of low risk [19];
2. are regulated under the 361 route;
3. do not need to apply for pre-market approval; and
4. only need to meet the "tissue rules" requirements (21 CFR Part 12714) [11].

HCT/Ps that are more than minimally manipulated, are of non-homologous use, are not combined with other articles (except for water, crystalloids, or sterilizing preserving, or storage agent), do not have a systemic effect and/or are not dependent on the metabolic activity of living cells for their primary function (if they have such an effect, they are intended for autologous use or allogeneic use in close relatives or for reproductive use) (criteria under 21 CFR Part 1271.10(a)), and/or do not qualify for exemptions under 21 CFR 1271.15 [32], are of high risk [19]:

1. are subjected to pre-market review requirements and approval; and
2. are regulated as drugs, devices and/or biological products under 351 of the Public Health Service Act (PHS).

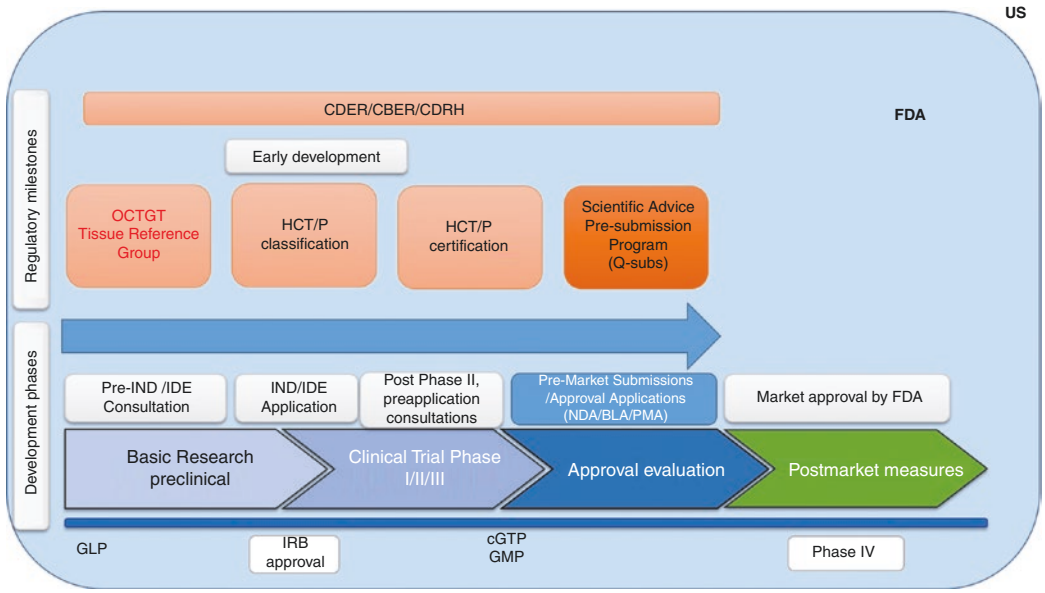

Fig. 22.3 Regulatory pathways and development phases for RM products in the USA. (Adapted from Sakai et al., 2017 [23] and FDA Regulations). CDER: Center for Drug Evaluation and Research, CBER: Center for Biologics Evaluation and Research, CDRH: Center for Devices and Radiological Health, IND: Investigative New Drug, IDE: Investigational Device Exemption, NDA: New Drug Application, BLA: Biologics Licence Application, PMA: Premarket Authorization, OCTGT: Office of Cellular, Tissue and Gene Therapies classification

Examples of products that are regulated as drugs and/or biological products include: gene therapy products, cultured cartilage cells, and unrelated allogeneic hematopoietic cells. Tissue engineered products, however, are more often than not considered to be combination products containing scaffolds, cells, and drugs. Thus, they are regulated according to their primary mode of action (or intended therapeutic effect to be either drugs, devices, and/or biologics) to be determined by the OCP (Office of Combination Products) and forwarded to the relevant centers to lead the review on the product [13].

Pre-market approval applications vary according to the classification of the product. Once the correct classification of a product has been identified, clinical trials can commence under exemption from FD&C Act for new drugs and devices, and PHS ACT for biologics laws, by submitting the applications. Examples of different pre-market approval applications include: Investigational New Drug (IND) (21 CFR 312) for drug or biologics introduction (very common with ATs) or an Investigational Device Exemption (IDE) (21 CFR 812) for device introduction [33]. Applications such as INDs comprise information on the product, data on toxicity and animal studies (possible detrimental side effects), information on the manufacturing process, the planned clinical protocols, data on any prior research on humans, and investigator's information [33, 34]. The FDA also provides pre-investigational new drug (pre-IND) consultation services for researchers and manufacturers and to optimize the IND submission process; after which, formal pre-IND meetings with the Office of Cellular, Tissue, and Gene Therapies (OCTGT) are conducted [35].

Before the commencement of clinical trials, however, the Institutional Review Board (IRB) (21 CFR56) that is responsible for governing the rights of human participants in research programs [33, 36, 37] must review all information related to the IND/IDE application such as: verification of the IND/IDE approval, qualifications of the clinical investigators, suitability of the research site, approval of the Standard Operating Procedures (SOPs), compliance with Good Clinical Practices (GCPs), risk assessment determination, and subsequently, grant approval [33, 36]. Risk assessment determination for the classification of medical devices is initially conducted by the sponsor/stakeholder, and is determined according to the degree of risk a medical device poses according to the following [13, 33]:

- Class I- Low risk products/devices that are subjected to general controls.
- Class II- Moderate Risk products/devices that are subjected general controls and special controls.
- Class III- High risk products/devices that are subjected to general controls and pre-market approval.

Class III- significant risk (SR) designations require an IDE submission and approval, and an IRB approval before clinical investigations [13, 33]. Non-significant risk (NSR) designations do not require IDE submissions [33]. After the submission and approval of the pre-marketing applications, clinical trials can commence.

IND pre-marketing applications for drugs and biologics require three phase clinical trials, and a fourth phase post market approval.

Before applying for final approval, FDA consultations can be held after Phases II and III [23]. With Medical Devices however, single confirmatory feasibility studies conducted under IDE applications that allow for early clinical evaluation of devices to provide proof of principle and initial clinical safety data (with a small number of subjects, first human (FIH) studies), can be sufficient for FDA approval [33].

Post successful Phase III clinical trials, pre-marketing submissions must be handed in to the FDA before marketing [19]. The pre-submissions program allows sponsors or stakeholders to consult the FDA during submission preparations for early feedback before the pre-market submissions in the form of 'Q submissions' or 'Q-subs' [33]. Examples of pre-market submissions include: PMA- Premarket Approval (Class III) submissions and De Novo- new device submissions for Class III medical devices, BLA- biologics license application for biologics [19], and NDA- New Drug Application for drugs [13, 33]. After receiving positive feedback from the FDA, formal applications are submitted.

Following positive review and subsequent approval for marketing, the sponsor/stakeholder can then market the product and post marketing Phase IV data is collected. Recently, the FDA created a new expedited designation for the RM products termed the Regenerative Medicine Advanced Therapy (RMAT) Designation that can be filed with or post IND application [15].

Some of the cell therapy products that have gave gained licensing in the US as biologics include: autologous cultured chondrocytes (Carticel®), autologous cellular immunotherapy (Provenge®), autologous cultured fibroblasts (Laviv®), and hematopoietic progenitor cells, cord blood (HEMACORD®) [23].

22.6 Hurdles in the Development of RM Products

Despite many advances made in science and technology, and large progression in various aspects of the RM field development, many hurdles prohibiting the mainstreaming of such products within a single jurisdiction and on an international scale exist as regards to:

22.6.1 Regulation

A large barrier preventing the mainstreaming and standardization of RM products on a global scale is Regulation. Regulatory barriers can generally be summed up into two themes:

22.6.1.1 Regulatory Clarity

Different countries define RM or Human Cell and Tissue (HCT) products in different contexts [19]. Since accurate product definitions leads to accurate classification, and subsequent application of the correct regulatory requirements, variations in definitions between different jurisdictions create quite a challenge for sponsors/stakeholders aiming to manufacture and/or distribute products in multiple countries, and results in the disruption of a global harmonized development of RM products [16].

In addition, some products that are termed 'borderline products' and defined as "products that might fall between two or more regulated product categories" (ATMPs or blood products and cell transplants) are very difficult to define, and highlight the vague line between what is considered an ATMP and what is not, with important consequence on the developmental pathway the product will take (e.g., lymphocyte immunotherapy) [9].

22.6.1.2 Regulatory Framework and Jurisdiction

The jurisdiction of different regional guidelines vary immensely on product type [16]. For manufacturer/developer to identify product specific requirements, unification and standardization of requirements of is of paramount importance. Thus, variations in guidelines pose a hurdle for global development [16].

22.6.2 Translation: Academia and Industry

Another barrier is the translation of innovative therapeutic science from its source. Academic institutions are vibrant sources for the generation of science leading to novel therapeutics. However, they often face hurdles in the form of lack of regulatory expertise. In academia, the development of RM products is usually science driven rather than product-driven [38]. This generally results in a lack of understanding for basic regulatory requirements of the translation of a product into the market, and a subsequent failure in that regard [16]. In addition, when it comes to funding, it has been observed that for many RM products, especially cell-based and patient-specific treatments, the pharmaceutical industry has limited interest in playing its 'usual' role of financing development and acting as a sponsor in clinical trials. The relative dearth of industrial investment in the RM products lies behind several aspects, including: distribution, economic, as well as cultural issues [39, 40].

22.6.2.1 Distribution Models

Depending on the type of product, decentralization of ATMP manufacturing might be needed,

and to achieve that, it is imperative that the origin, composition, manufacturing process, quality control methods, as well as batch release specifications are aligned between both the regulatory agencies and the ATMP manufacturers. Products that might require a decentralized distribution model are cell-based therapies. When compared to other biologicals they have a shorter shelf life and, therefore, are particularly susceptible to damage during shipping, which influence the final quality of these products. One such example is Holoclar, where patient biopsies must be received by the manufacturer within 24 h following procurement and which has only a 36-h shelf life. Due to the temperamental nature of such products, one suggestion has been the establishment of regional sites or centers of excellence, which could offer a more suitable model for personalized autologous cell products or for rare diseases. Of course, consistency of approach and the necessary standards and guidelines would have to be conserved across these different regional sites and agreed on by the necessary regulatory authorities [40].

Nevertheless, in 2017, using a centralized model, big pharmaceutical companies managed to receive marketing approvals of three gene therapy products: Kymriah, Yescarta, and Luxturna [41, 42].

Economic

1. Intellectual Property. Since ATMP are often not based on a 'simple' cause-effect model, the intellectual property landscape is often more complicated with these products, making it difficult to ring-fence intellectual property, establish freedom to operate, and anticipate the effects of competition [39].
2. Orphan. Many current ATMPs, especially in gene therapy, are applicable for rare or orphan indications. While it is now established that orphan drug status in itself is no bar to profitability, the challenges with developing and finding politically acceptable reimbursement for such products remain [43].
3. Scalability. Even if ATMPs are potentially applicable to a large patient population, their cost and complexity often render it impossible to conduct trials on a large patient population—which is traditionally the specialist expertise that industry brings to the table in translational research. The high cost effort per treatment also yields uncertainties about effective reimbursement [44].
4. Costs. Translational costs related to all aspects of GMP manufacturing, from GMP-grade stating materials to the personnel required to run such facilities, poses a huge burden on the sponsors/stakeholders aiming to market a product [4].

Cultural

1. Complexity. Many ATMPs are manufactured differently from mainstream medicines, requiring investment into new expertise. Cell therapy products often do not have a clearly defined mechanism of action, gene therapy presents unique challenges of long-term systemic effects, and tissue engineering targets a complex interface of material science and biology. The selection, enrichment, or genetic modification of cells and tissues often enhances their sensitivity the effect of which cannot be replicated in vitro [39].
2. Surgical. Many ATMPs are seen to be more closely related to transplantation, an area that does not interface much with established industrial R&D [39].
3. Transferability. Many ATMPs require very specialized, tacit clinical expertise that cannot easily be transferred. Cell populations are necessarily heterogeneous and dynamic, and purification protocols, as they are applied in 'established' biotechnology may actually prove detrimental to the efficacy of the final product [39].

Ethics

A third hurdle that faces RM streamlining surrounds ethical considerations and difference in belief. Ethical hurdles exist on cell sources and ownership, such as: the utilization of embryonic stem cells and/or fetal tissue, which may be allowed in some countries and prohibited in others. In addition to this, controversies exist over whether human cells/tissues can be subjected to

laws regarding property rights [33]. Ethical objections to the implementation of certain procedures such as: therapeutic cloning of cells, genetic engineering of cells, and the mixing of human and animal cells on the production of RM products have been raised [33]. Moreover, a 2008 study indicated that physicians have less confidence in industry-funded clinical trials as opposed to government-sponsored trials [33].

These issues and challenges are not meant to suggest that ATMPs are not attractive for industrial development. Other factors play a role [45] and in fact, the industry sector using ATMP is increasing markedly [46]. However, the above considerations suggest that there are a number of factors that may militate against ATMP development in the private sector and complicate technology transfer from the public sector [39].

22.7 Overcoming Hurdles: the Formation of Collaborative Parties

To overcome challenges resulting from diverse regulatory pathways associated with different countries, and to facilitate the acquisition of marketing approval under multiple jurisdictions, several international collaborations have been established. To that effect, information on science and regulatory convergence related to ATMPs or HCT/Ps manufacturing, and the marketing of products, in several countries can be gained from different alliances groups such as: the EMA-US FDA Parallel Scientific Advice (PSA) [47] (during which each regulatory agency (EMA and FDA) provides the sponsor/ stakeholder with independent advice regarding questions presented) and the Life Sciences Innovation Forum (LISF) (under Asia-Pacific Economic Cooperation (APEC)) both provide meaningful dialogue with stakeholders regarding successful implementation of policies.

Other collaborative clusters that work towards the harmonization of ATMP production include: the US FDA-EMA-Health Canada Advanced Therapy Medicinal Products Cluster [47] and the Regulators Forum (RF): composed of the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) members, the ICH observers, regional harmonization initiatives, and different drug regulatory authorities from various countries (e.g., Brazil and Australia) [47].

On different industries (education, pharma, health care, and regulatory), academic initiatives such as the EuroTech Universities Alliance [48] have been established, and work towards facilitating translation of basic science and technology into the clinic. Groups such as the ATMP Interest Group, founded in 2017 by the European Compliance Academy (ECA), have representatives from the academia, industry, and the regulatory authorities, and aspire to facilitate information exchange between academia, pharma, and regulatory bodies on Good Manufacturing Practice (GMP) and pharmaceutical quality assurance. The ATMP Interest Group in collaboration with the ATMP GMP Open Access Research Alliance (AGORA) group work on the AGORA project [49], which has contributed to the much needed support and training framework for the facilitation of regenerative medicines implementation by establishing a technology transfer network, training programs, and interactive information sources [48].

Other structures, such as the European Society for Blood and Marrow Transplantation (EBMT) [50], have been developed beyond bone marrow transplantation towards modern cell therapies to allow scientists and physicians to share experience and develop cooperative studies. The EBMT together with the International Society for Cellular Therapy (ISCT) have formed the Joint Accreditation Committee ISCT-EBMT (JACIE) [51], which is aimed at promoting high quality patient care through the development of global standards and an internationally recognized system of accreditation. In the US, Foundation for the Accreditation of Cellular Therapy (FACT), together with the JACIE, have formed the JACIE-FACT that is now a unified, leading accreditation agency that insures high quality manufacture of therapeutics.

22.8 Aspects to Consider

Finally, a distinguishing feature of ATMPs deserves to be mentioned again: more than 90% of ATMP development resides in Academia and Small and Medium-sized Enterprises (SMEs). The academic environment has coined the field of ATMP development in many ways. Specific academic features include the close proximity to patients, limited resources in terms of funding, infrastructure and pharmaceutical development, a risk awareness and intentionality shaped sometimes more by disease and suffering than by quality considerations. Academic initiatives have engaged in networks and represent the non-canonical and decentralized developmental pathway of ATMPs [29, 34, 36]. The European Commission has recognized this development and, beyond project-related funding, reached out to and endorsed academic initiatives as a major stakeholder in the field.

22.9 Conclusions

As cell-based medicines often lose magic in the course of clinical development, so does the novelty assigned to ATMPs as the field matures. The development of ATMPs has shown immense success when large pharmaceutical companies have matched centralized models of manufacture and distribution with clinical efficacy (example: Kymriah et al.) [41]. Decentralized concepts, however, will continue to be paradigmatic for many cell-based medicines and to challenge the existing regulatory pathways. The regulatory bodies in the US as in the EU have responded to this demand, sometimes with similar concepts and harmonized incentives, sometimes with a regulatory framework that emerged on extensive stakeholder consultations to allow innovation to reach patients in need [52]. Yet the leading notion continues to be a picture of Europe lagging behind in the global thrive for advanced therapies, which have proven to have both a clinical and commercial potential. As the development of ATMPs cannot be regarded solely under market aspects and a cautious approach must also be considered to ensure patient safety, the EU fortunately continues to establish structures and platforms on a pre-competitive level where stakeholders from all sides are addressed [53]. At the same time, the complexity of ATMPs and the risk inherent in these medicinal products mandate a structured approach in terms of manufacture, application, and risk awareness in terms both clinical and quality risks. The ideal format of specialized, pre-competitive clusters will predominantly be:

1. associated with academic hospitals and centers;
2. defined by processes rather than products;
3. qualified and certified in a way that awaits definition; and
4. seek to find an interaction with industry in a way that respects both the transformative potential of ATMPs and the cautious position of industry that expects the risks inherent in ATMPs to become predictable to a certain extent.

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Translational Challenges in Soft Tissue Regeneration

23

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23.1 Introduction

Soft tissue defects due to trauma, burns, tumor resection, or congenital deformities provide an ongoing reconstructive and regenerative challenge. To cover these defects and promote tissue regeneration, different strategies were developed within the last century and especially during the last few decades. Throughout recent history, there has been a great need to develop new methods and materials which provide soft tissue replacement and promote soft tissue regeneration, especially in plastic surgery.

In this chapter, we would like to first give a comprehensive overview of the epidemiology of each type of soft tissue defects and highlight current successful applications of regenerative medicine and methods of advancing the regeneration of these tissues. Then we discuss current surgical and nonsurgical challenges like hindering scar formation and inducing regeneration, finding biomaterials that are tolerated by or even make use of the immune system, as well as financial, regulatory, and finally ethical boundaries. We conclude with an outlook on necessary future developments for successful translation of advanced current concepts in regenerative medicine to day-to-day routine in plastic surgery.

23.2 Epidemiology of Soft Tissue Defects

Tissue defects happen in all tissues at different rates and with varying consequences for survival and quality of life of the patient.

23.2.1 Skin and Subcutaneous Tissue Defects

Skin and subcutaneous tissue defects are defined as loss of tissues, including epidermis, dermis, and hypodermis [1]. This can arise from trauma, burns, excision of benign or malignant lesions, wounds after surgery, systemic diseases, or vascular problems [2–5]. It was estimated that approximately 11 million people received medical care for burns in 2004 [6]. Surgeons have to frequently deal with wound healing disturbances

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at the donor sites. For example, the incidence of impaired healing after radial forearm free flap (RFFF) harvesting has been reported to be up to 33% [7, 8]. In developed countries, 1–2% of the population will experience a chronic wound during their lifetime, and the incidence is increased to 15–25% in diabetic patients [9, 10]. Injury or illness that cause loss of skin and subcutaneous tissue can lead to substantial physiological imbalance, to significant disability, and ultimately sometimes even to fatality [11].

23.2.2 Muscle, Tendon, and Ligament Tissue Defects

Just below the skin, muscles move us. The loss of skeletal muscle with functional impairment that overextends mammalian regenerative capacity is defined as "volumetric muscle loss" [12–14]. Frequent reasons for the shortfall of muscle tissue are high-energy trauma, combat-related injuries, or surgical treatments (e.g., after compartment syndrome or tumor resection) that lead to an acute tissue loss [15]. Progressive muscle loss can result from metabolic disorders or inherited genetic diseases [16–19]. Up to 10–20% loss of muscle mass can be compensated by the high adaptability and regenerative potential of skeletal muscle. Beyond this threshold functional impairment is inevitable and can lead to severe disability and cosmetic deformities [13, 14, 20, 21], which is why therapeutic options are in urgent demand for these patients.

23.2.3 Nerve Defects

Another reason for impaired muscle function is defects of peripheral nerves, which have a prevalence of about 2.8% [22]. They may occur as a result of trauma or tumor resection and mainly affect younger, male patients [23]. The mean age is 32–35 years old [22, 24, 25]. Nerve injuries are comparatively rarer than other soft tissue defects, but cost more. A nerve injury may result in loss of sensory function, motor function, or both. For example, the nerve defect of the hand represents a serious impairment of the patients' quality of life with intensive and long-term rehabilitation, and impaired employability. This results in high follow-up costs for the health system [26, 27].

23.2.4 Fat Tissue Defects

Local loss of fat tissue can be due to traumatic deformities, burns, post-irradiation defects, and breast post-mastectomy, as well as congenital deformities such as Poland syndrome, Romberg syndromes, or pectus excavatum [28]. While most of these causes are fortunately rare, aging leads to changes in metabolism of preadipocytes, so in older age, relative loss of subcutaneous peripheral fat is a common problem [29]. In 2009, fat grafting represented 5.9% of all nonsurgical aesthetic procedures [30].

23.3 Successful Application of Regenerative Treatments

Already today regenerative therapy concepts are successfully used in several applications of plastic and aesthetic surgery.

23.3.1 Breast Reconstruction

Autologous fat transplantation is widely used in breast cosmetic surgery or reconstruction: treatment of micromastia, correction of post-augmentation deformity, treatment of tuberous breasts and Poland's syndrome, surgical repair of post-lumpectomy deformity and post-mastectomy deformity, treatment of deficits or tissue damage caused by conservative or surgical reconstruction (Fig. 23.1) and nipple reconstruction [31–36].

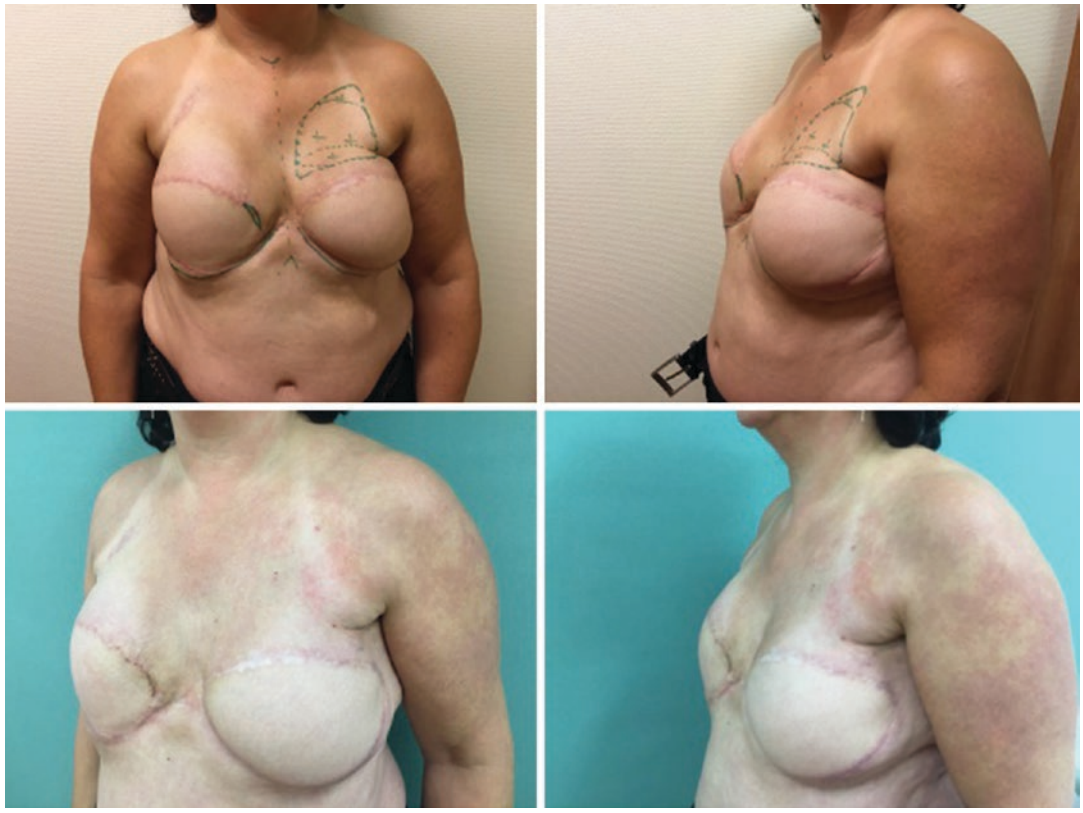

Fig. 23.1 54-year-old female patient, status after right- and left-sided breast cancer, necessary mastectomy of both breasts followed by bilateral (both sides) reconstruction with pedicled transverse rectus abdominis myocutaneous (TRAM) flap. Pictures above: preoperative planning of autologous fat transplantation. Pictures below: postoperative result after 3 months

23.3.2 Breast Augmentation and Asymmetry

Initially, fat grafting was considered to be a promising technique for breast augmentation and correction of breast asymmetry because of advantages such as easy availability of donor tissue, absence of a scar, and short recovery time. Moreover, because the procedure could be performed in an outpatient setting and avoid the complications of prostheses, it gained significant initial popularity [37]; however, the value of this technique in augmenting breasts and filling breast defects became controversial because the results are not always sustainable. Zocchi and Zuliani [38] and Khouri et al. [39] developed a new surgical technique to improve graft survival. They combined external skin expansion with BRAVA® and autologous fat grafting (AFG) and gained a volume persistence at 1 year of up to 70% in an early study (average 55%) and up to 82% in later work [38, 39].

23.3.3 Scars and Burns

Patients with retractile and painful scars compromising the normal daily activity and/or mobility of the joint involved can take advantage of lipofilling treatment. In fact, fat transplantation can

be used not only to fill atrophic scars, but also to reduce scar contracture as a regenerative alternative to other surgical techniques [40].

A burn injury is a devastating trauma with systemic consequences. Autologous fat grafting with adipose-derived stem cells can be effectively used to correct postburn scars with minimal risk of hypertrophic scarring [41–43].

23.3.4 Rejuvenation

Autologous fat grafting has an important role in facial rejuvenation: filling of the nasolabial folds and the lips, facial contouring, and facial tissue augmentation [44–46]. Guyuron and Majzoub [47] also developed a core fat grafting technique for lip augmentation and correction of malar and buccal deficiency. Furthermore, autologous fat transplantation is often used to correct the profile of the nose [48].

The appearance of the hands is a telltale sign of a person's true age [49]. Because fat not only serves as a filler, but also has the regenerative potential to improve the quality of soft tissue and skin on the dorsal side of the hands, fat grafting is an attractive procedure for hand rejuvenation [50].

23.3.5 Gluteal Augmentation

Gluteal augmentation is often performed by means of intramuscular implants, but lipofilling has begun to gain popularity in recent times. Fat grafting will play an important role in gluteal augmentation and may replace implant-based gluteal augmentation if the patient has a great enough amount of fat as a donor material [51–53].

23.4 Challenges Associated with Regeneration

23.4.1 Scar Formation vs. Regeneration

Wound healing is a complex, interactive, cellular, and biochemical process involving inflammation, blood clotting, cellular proliferation, and migration of various cell types, resulting in closing of the wound [54]. The typical wound of mammals like us heals by scarring. Scarring is a natural way of quickly filling large voids in tissue, with a haphazard arrangement of connective tissue elements [55]. Scarring is much faster than regeneration and therefore probably favored by evolution, as in the jungle an open wound strongly increased the chances of fatal infection. On the negative side, scarring is characterized by lack of appendages such as hair follicles and sweat glands. Due to its distinct function and appearance of the original intact tissue, the formed scar can result in devastating functional, cosmetic, and psychological problems, including limited movement, restricted growth, difficulty in sweating, and temperature equilibrium. This can reduce the quality of life of the individuals [56, 57]. Furthermore, scar formation has been frequently cited as a physical and chemical barrier for tissue regeneration [58, 59], thus preventing final return of the defect site to its original state. Modern medicine now allows us to take longer time until wound closure has to be achieved, and therefore waiting for regeneration has become an option. Wounds in the early embryo can heal without scarring, raising the hope that the genetic program for regeneration may also be present in adults [60].

23.4.1.1 Skin and Subcutaneous Tissue Defects

A complete regeneration of functional skin and subcutaneous layers would have to include all the skin appendages (hair follicles, sweat glands, and sensory organs) and layers (epidermis, dermis, and hypodermis), and a preformed functional vascular and neuronal network [61]. It therefore requires the coordination of multiple cell types to regenerate epidermis, dermis, and hypodermis.

Substantial evidence shows that scar formation can indeed be cancelled and regeneration achieved by appropriate modification of the wound healing process. Specifically blocking of the normal wound contraction process seems promising and understanding how this regenerative mechanism of scar-free healing is activated has become a major quest [62].

23.4.1.2 Muscle, Tendon, and Ligament Tissue Defects

Scar formation can severely impede motion and thereby aggravate the consequences of muscle tissue loss, e.g., after burns. Different from tissue regeneration, neo-angiogenesis and fibroblast proliferation are deemed counterproductive and are antagonized, e.g., by injection of 5-fluorouracil and bleomycin to reduce scar formation (Fig. 23.2) [63, 64]. Scar improvement can also be achieved by (non-ablative and ablative fractional) laser therapy with release of contracture and functional improvements after 6–12 months treatment [65, 66].

Regeneration with regression of scar tissue and functional recovery can furthermore be optimized with fat grafting [67]. The proclaimed mechanism is that subdermal tissue is filled and thereby tension is reduced. Another option for consolidation of scar tissue is by treating the wound with Cx43 analogon [68]. Cx43 is important for gap junctional communication between cells and therefore for healing processes [68, 69]. Treatment of scars with the gel formulation of the Cx43 analogon aCT1 has been shown to improve scar scores by 47% and improve pigmentation, thickness, surface roughness, and mechanical suppleness [68].

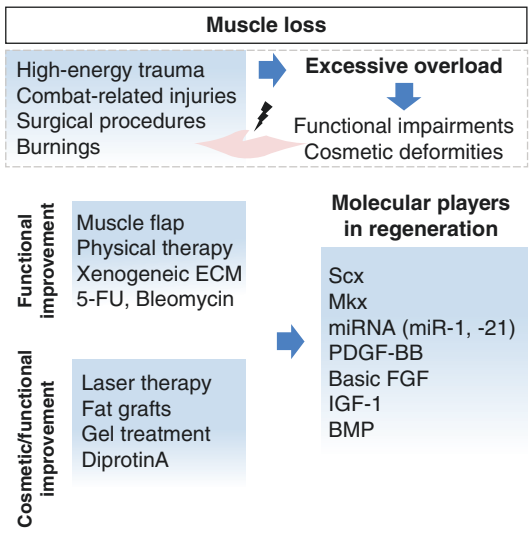

Fig. 23.2 Current methods for muscle and tendon regeneration

Novel insights into the molecular mechanisms of tendon regeneration reveal that in neonates, tendons heal via a new-formed tendon, while in adults, they heal with a fibrovascular scar, leading to impaired function. Locally, the scleraxis (Scx)-lineage cells seem to be the key players in newborns, while they are lacking in adults [70]. Transcription factors like Scx and Mohawk (Mkx) are key regulators of collagen maturation, so the ECM seems to play an important role in the process (Fig. 23.2). Cells with a high alpha-SMA expression form a permanent scar leading to non-regenerative tendon healing. Healing with scaring can be reduced with an inhibitor of dipeptidyl peptidase-4, Diprotin A, since a subpopulation of fibroblasts, which expresses dipeptidyl peptidase-4, promotes fibrosis and stroma formation [71]. A different approach for scarless healing is the use of miRNAs (including miR-1 and miR-21), which partly led to promising results [72–74].

23.4.1.3 Nerve Defects

After nerve injury, different molecular processes run in the proximal and distal stump. The proximal stump begins to swell, but experiences only minimal damage via retrograde degradation [75, 76]. Migrating macrophages and monocytes remove resulting myelin and axon debris. Schwann cells proliferate to form bands of Bungner, and produce neurotrophic factors like interleukin-6 and extracellular matrix (ECM) to enhance axon growth. Regeneration begins at the proximal stump directed towards the distal stump. New axonal sprouts origin from the nodes of Ranvier. In this progress Schwann cells play a central role as they remyelinate the regenerating nerve. Functional reinnervation is possible when the axons reach their synaptic target [76, 77].

At the distal stump Wallerian degeneration begins as a result of protease activity and separation from the metabolic resources of the nerve cell bodies due to the injury. Myelomonocytic cells destroy myelin and initiate mitosis in Schwann cells [76]. The cytoskeleton begins to break down, followed by dissolution of the cell membrane. Wallerian degeneration remains one of the major biological hurdles faced in rapid and complete functional reinnervation and recovery

[78]. Furthermore, the functional outcome of nerve repair is also affected by age-related proliferation potential of regenerating structures [76].

Thus, also in nerves, depression of scarring and promotion of regeneration seem to matter, and consequently proliferation of Schwann cells and delay of Wallerian degeneration are main focusses of research in nerve regeneration [79].

23.4.2 Replacement vs. Regeneration

The big advantage of replacement vs. regeneration is again that it can be performed much faster, thereby decreasing the probability of complications like infection. Also again, this comes at the cost of an imperfect outcome. Thus, if it would be possible to accelerate regeneration, it may be the better option for a variety of cases.

23.4.2.1 Skin and Subcutaneous Tissue Defects

Different therapeutic strategies are applied to close the wound as efficiently as possible (Table 23.1). This starts with gluing or stitching for smaller wounds, a huge number of dressing materials (including films, hydrogels, hydrocolloids, foams, alginates, etc.), and skin grafting with autografts and flap transfer for bigger wounds [80–83]. This tissue harvesting, however, can cause complications on donor sites such as scarring, infection, loss of motion, unhealed wounds, and even amputation of extremities [84, 85]. In patients with chronic wounds or extensive burns, this treatment option is further impeded because of expected poor healing of the donor site and/or limited donor sites [86, 87]. These problems have led to an increased focus on regenerative medicine in this area to develop replacement tissues and biologically compatible constructs to improve wound coverage and attempt to restore skin and subcutaneous tissues without causing novel defects.

Replacement by allografts or biomaterials have always been the temporary coverage of choice when donor autografts are limited. They have been shown to prevent evaporative water loss, reduce infection, and promote autologous epidermal growth within the defect area [88]. Cadaveric allograft and inner amnion have gained popularity on temporary wound coverage, from which commercial products were made available in the past few years [11, 89, 90]. However, disadvantages include the potential transmission of infectious pathogens and some cultural and religious considerations for allografts; difficulty in handling and fast degradation, in addition to lack of adherence in full-thickness defects for application of amnion [91]. Replacement made of biomaterials is another choice of temporary coverage due to good biocompatibility, less risk of infection transmission, and much lower cost [88]. Silver-impregnated temporary dressings (Anticoat, Aquacel Ag, Biobrane) have been shown to reduce the likelihood of infection and decrease wound healing time and pain [88]. However, the temporary replacement of the skin and subcutaneous tissue defects is not sufficient for tissue regeneration.

23.4.2.2 Muscle, Tendon, and Ligament Tissue Defects

Current therapeutic principles are based on physical therapy or muscle flaps [12]. Functional free muscle flaps can lead to at least decent functional results; they cause however substantial donor site morbidity [92]. Other nonsurgical solutions like bracing focus on the area below the knee. Modern approaches use xenogeneic extracellular matrix made from the small intestine submucosa and autologous tissue to restore functional muscle and simultaneously generate a biological niche for recovery [93]. Since a devitalized scaffold without myogenic cells is not able to promote muscle fiber regeneration, cellular matrices are necessary [74, 94].

Extracellular matrix of tendon consists of collagen type I with a complex interlaced structure [95], while ligaments consist of fibroblasts and collagen type I and III. Natural regeneration of tendon and ligaments is so far limited in adults [70]. Defects heal with a fibrovascular scar with harmed function due to the absence of tenogenic cells. Replacement of tendon and ligament tissues with allograft is quite popular in the clinic. Problems include immune response, the risk of infection, and delayed wound healing [96–98].

Table 23.1 Current methods of advancing skin tissue regeneration

	Description of each soft tissue			
Strategies	Skin and subcutaneous tissue defect	Muscle, tendon, and ligament defect	Nerve defect	Fat defect
Surgical technique	Tissue transfer (FTSGs, STSGs, flaps)	Autologous muscle/tendon transfer	Autologous nerve graft transplantation	Fat tissue transplantation
	Tissue expansion (skin expansion, DermaClose RC)	Autologous muscle/tendon transplantation	Nerve transfer	
	Vacuum assisted closure system	Reconstruction of ligaments with tendons	Autologous conduits (veins, arteries, soft tissues, muscle-vein-combined conduits)	Fat tissue aspiration and reimplantation by injection
	Transverse tibial bone transport	Tendon or tissue allograft		
Scaffold-based therapy	Acellular dermal matrix (AlloDerm Regenerative Tissue Matrix)	Muscle-derived matrix	Decellularized nonneural tissues, decellularized allogenic/xenogenic neural/nonneural tissues	Natural polymers (collagen, silk, fibrin, gelatin, hyaluronan, adipose-derived ECM, decellularized human placenta, and matrigel)
	Cellular dermal matrix (Apligraf, Dermagraft)	Small intestine submucosa, porcine bladder acellular matrix		
	Other tissue derived matrix (DHACM, PSIS)		Biodegradable synthetic polymers (aliphatic polyesters, polyurethanes, piezoelectric polymers and some electrically conducting polymers, PGA, PLC, etc.)	Synthetic polymers (PLA, PGA, PEG, PLGA)
	Biomaterials (Collagen-GAG sponge, Integra Dermal Regeneration Template, gelatin, PEG, fibrin)	Biomaterials (polyurethane-based porous scaffold, fibinogen/Laminin-111,PLLA,PCL,PLGA,CEB)	Naturally derived polymers (collagen, laminin, fibrin, chitosan, polysaccharides, silk fibroin, keratin, biodegradable synthetic polymers)	
Drug-based therapy	Growth factors (PDGF, EGF, IGF, VEGF, FGF, TGF), Deferoxamine	Growth factors (BMP, myostatin, TGF-b, HIF-PHDs, PDGF-BB)	Growth factors (NGF,BDNF,NT-3,GDNF,CNTF,FGFs,VEGF)	Growth factors (VEGF,bFGF)
Cell-based therapy	Cultured cell therapy (fibroblasts, keratinocytes, bone marrow-derived MSCs, ADMSCs, cultured epidermal cell sheet)	Fibrin microthreads with adult human stem cells (MSCs)	Neural stem cells, embryonic stem cells, Schwann cells, bone marrow stromal cells, MSCs	ADMSCs, adipose-derived stromal cells
	No cultured cell therapy (ReCell system)	Autologous multipotent stromal cells in a vicryl mesh tube	Gliogenic secondary neurospheres derived from iPSc	
Others	Platelet-rich plasma, Emacure system, cold atmospheric plasma	Platelet-rich plasma	Electrical stimulation, genetic engineering of autologous cells	

23.4.2.3 Nerve Defects

Nerve defects are typically classified based on the classification by Seddon (1943) [99] and Sunderland (1951) [100]. This allows selection of the required treatment (Fig. 23.3) and allows an estimated prognosis for recovery [101]. Current strategies for nerve defects are mainly based on microsurgical connection of the two sides by autologous nerve graft or approaches of tubulization [102, 103]. Despite the advantages of existing approaches, complete recovery is inauspiciously infrequent, misdirection and development of debilitating neuropathic pain unfortunately common [101].

Currently, autologous nerve grafts or nerve allografts have been widely used for replacing the defects [104]. Nerve transfer is another way of replacing the proximal part of the involved nerve.

23.4.2.4 Fat Tissue Defects

Historically, the primary method was to cover subcutaneous defects of fat tissue according to the philosophy "replace like with like" via fat transfer. The first surgeons who used fat grafts were Neuber in 1893 [105] for unilateral facial atrophy, followed by Czerny [106], who performed one of the first breast enlargements with autologous fat, which he had isolated from a lipoma, and Lexer [107] for soft tissue filling after zygomatic bone fractures. In the following years, autologous fat grafts were used in several other fields [108].

The liposuction technique, introduced by Fisher in 1974 [109], accelerated the development of the lipofilling technique, which started in 1986 when Illouz and Pflug [110] and Chajchir and Benzaquen [111] published their works about reinjection of liposuctional fat tissue**;** however, in the following years the use of fat as an implant material was not favored, because the method of harvesting was standard liposuction [112–115]. Standard liposuction caused significant damage to the fat cells by rupturing cell membranes and subsequently causing cell death. Since then, autologous fat transfer has been improved. Coleman [116] described a new way to harvest fat with atraumatic low-vacuum technique to increase intact and viable lipocytes in transplanted fat for transfer. His technique remains the gold standard for liposuction and lipofilling, but it has undergone some technical modifications [117]. Nowadays, autologous fat transplantation is one of the most popular procedures performed by plastic surgeons.

Despite fat grafting being a well-established method, the long-term survival rate of autologous fat grafting remains unpredictable. Peer [118, 119] studied the long-term survival of autologous fat grafts and showed in 1950 that, of the autologous transplanted fat grafts, more than 50% of their weight and volume was reduced after 1 year; several other studies in the following years confirmed this [120]. Some authors described 30–70% reduction in graft volume within a year [120]. Additional, although rare, severe complications could be seen in the literature related to autologous fat grafting including vision loss, stroke, and even death [121]. That is why recent research up to this point has included attempts to further improve autologous fat transfer and numerous natural, synthetic, and hybrid materials have been used to act as adipose surrogates.

Every step in fat transplantation—harvesting, processing, and transplantation—is important, but the viability of the harvested fat cells is crucial [122]. The chances of survival are higher if the fat graft is manipulated less and reinjected quickly [123].

Typical donor site complications include swelling, hematoma formation, paresthesia, or donor site pain. In a few cases, infection, hypertrophic scarring, contour irregularities, and damage to the underlying structures occur, due to intraperitoneal or intramuscular penetration of the cannula [32, 124–128]. Multiple complications were reported in the literature concerning the recipient region, including edema, bruising, bleeding, dysesthesia, infection, fat necrosis, less than expected beneficial outcome, microcyst, microcalcifications, fat embolism, and severe complications including stroke, vision loss, systemic infection, sepsis, or death [121, 129, 130].

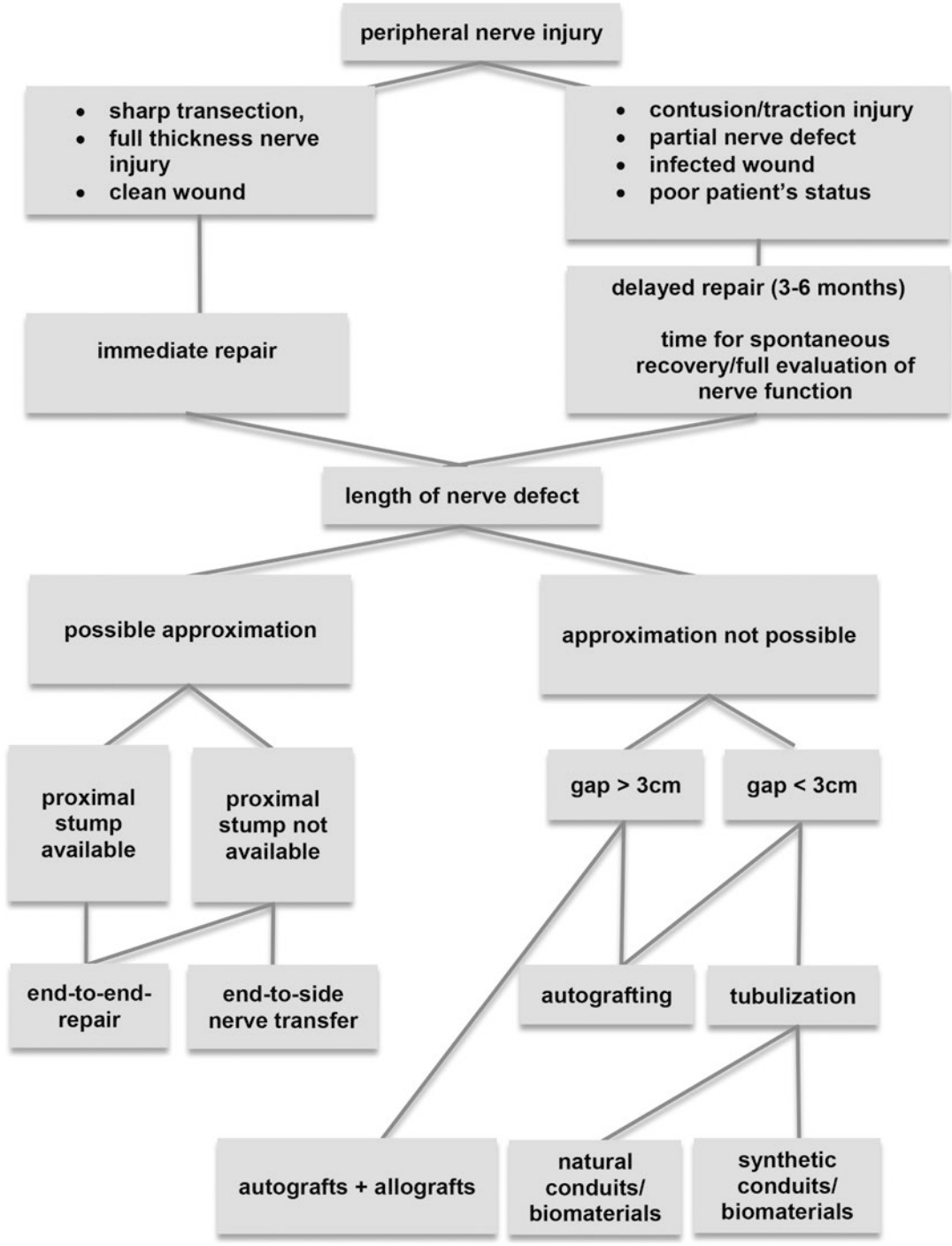

Fig. 23.3 Surgical algorithm of peripheral nerve repair (modified based on[150])

23.5 Advancing Surgery for Soft Tissue Regeneration

23.5.1 Surgical Techniques

23.5.1.1 Skin and Subcutaneous Tissue Defects

Vacuum-assisted closure (VAC) after debridement is a popular technique in wound care which speeds up wound healing by improving tissue perfusion, suctioning the exudates and thus increasing the granulation tissue regeneration [131]. Skin graft transplantation is needed thereafter to completely close the defect. The application of transosseous-osteosynthesis with the Ilizarov technique, which is expected to improve local microcirculation, showed great effect in promoting wound healing in diabetic foot ulcer [132]. Tissue expansion is commonly used for skin and subcutaneous tissue defects after excision of tumor lesions or severe scar [133, 134]. In addition, a continuous external tissue expansion system (DermaClose RC) has been reported to be an effective technique for achieving definitive large wound closure, potentially reducing the donor-site morbidities needed for larger reconstruction measures [135].

23.5.1.2 Muscle, Tendon, and Ligament Tissue Defects

Autologous muscle or tendon transfer is commonly performed in the clinical situation, when there is muscle loss following trauma, tumor resection, or nerve injury, which impairs the irreplaceable motor function [136, 137]. Latissimus dorsi muscle transfer showed safe and efficient restoration of elbow flexion after injuries [136]. Tendon transfer of the flexor carpi ulnaris is considered as a good option to restore the hand extension function following high radial nerve injuries[138]. When no adjacent muscle is available because of high-level nerve injuries, autologous muscle transplantation together with neurorrhaphy is typically applied [139, 140]. It can also be used for muscle weakness after facial palsy or for pelvic floor reconstruction [141, 142].

In addition to tendon transfer, autologous tendon grafting can facilitate restoring tendon length and strength when there is a tendon defect at the level of the hand or wrist [143]. Palmaris longus tendon is one of the most commonly used grafts in hand surgery [144, 145]. Tendon allografts (Achilles tendon) processed from cadavers is another good option, which avoids injuries in the donor area [146].Tendon allografts are commonly applied for both tendon reconstruction and ligament revision [146, 147].

After establishment in mouse models, restoration of vastus medialis muscle in patients could be performed with the use of a multilayered scaffold made of extracellular matrix derived from porcine submucosa [15, 20]. Abdominal musculoskeletal wall defects were restored with a porcine small intestinal submucosa-extracellular matrix that was sutured at the defect corners and subcuticularly closed with a Vicryl suture [93].

23.5.1.3 Nerve Defects

If a direct tension-free end-to-end or end-to-side neurorrhaphy is not possible, the interposition of a graft between the nerve stumps is required to bridge the gap and support axonal regrowth [78]. To ensure a tension-free repair it is advised to choose a graft that is 10% to 20% longer than the existing nerve gap [76, 148]. Implantation of an autologous nerve graft, which is a functionally less important nerve segment from another site of the body, remains the most reliable repair technique [76, 148]. Autografts are ideal nerve conduits for longer gaps (>3 cm), critical nerves and proximal injuries [78, 149]. They provide a permissive and stimulating scaffold, including Schwann cell basal laminae, adhesion molecules, and neurotrophic factors and they constitute a supportive structure for the ingrowing axons [78, 150, 151].

Only approximately 25% of the axon will, however, successfully regenerate through the graft's two coaptation sites with an estimated loss of 50% of axons at each of them [78]. Single grafts describe a segment of a donor nerve of similar diameter [78, 152]. Cable grafts join nerve gaps with large diameter by using multiple lengths of a smaller diameter (sensory)

donor nerve. They are reversed in orientation [152]. Trunk grafts mix motor and sensory grafts and are integrated as a large donor nerve of an entire segment of a very proximal nerve injury. Trunk grafts showed no convincing results due to poor vascularity and internal fibrosis [78, 152]. Interfascicular grafts close the gap between groups of fascicles in the damaged nerve [78, 153].

Nerve transfer is another surgical technique. It involves isolating nerves with less important roles or branches of a nerve that perform redundant functions, and "transferring" them to restore the function of a more crucial nerve which has been severely damaged. Typically, the functioning nerve that is close to the target muscle or sensory area is transferred or "plugged in" to the injured nerve that no longer functions (Fig. 23.4).

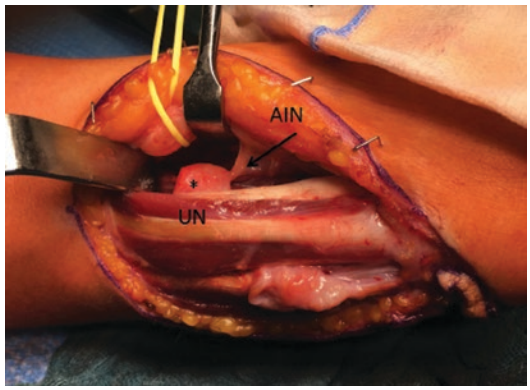

Fig. 23.4 Illustration of selective nerve transfer of the ulnar nerve (UN) to the anterior interosseus nerve (AIN) for neuroma treatment of the distal forearm. The challenge of this nerve repair is the correction of the size mismatch resulting from the end-to-end adaptation performed nerve transfer to avoid following misdirecting of sprouting axons compromising the sensory and motor recovery.

23.5.1.4 Fat Tissue Defects

It is widely accepted that less traumatic methods of fat harvesting result in increased graft survival of the transplanted fat [154]. It has been shown that a number of possible harvesting options allow the collection of regenerative adipose tissue-derived stem cells [155–157]. However, there is an ongoing discussion about which methods increase intact and viable adipocytes. The main techniques are vacuum aspiration, syringe aspiration, and surgical excision. Similarly to adipocytes, lipoaspirate contains collagen fibers, blood, and debris. Those elements can cause inflammation in the recipient site, and thus fat processing is advisable. Various processing techniques such as centrifugation, sedimentation, washing with physiologic solution, and gauze filtration have been proposed (Fig. 23.5) [50, 158–166]. The most common technique is centrifugation as described by Coleman [50].

Principles of fat reimplantation are based on optimal recipient site vascularity for increased fat survival [167]. Through a skin incision, which is sized corresponding to the diameter of the cannula, the fat graft is inserted into the area of the anatomical region affected. On the one hand, small-gauge cannulas reduce the risks of bleeding and hematoma formation, but on the other hand they cause poor graft oxygen diffusion [167]. It is thus suggested to use cannula with similar hole sizes for aspiration and utilize minimal amounts of suction force to avoid mechanical damage. Because revascularization starts at the periphery, ischemic time is longer in the center of the graft; therefore, fat reinjection in multiple small-volume sessions is preferred rather than one single injection [168]. Fat grafts are

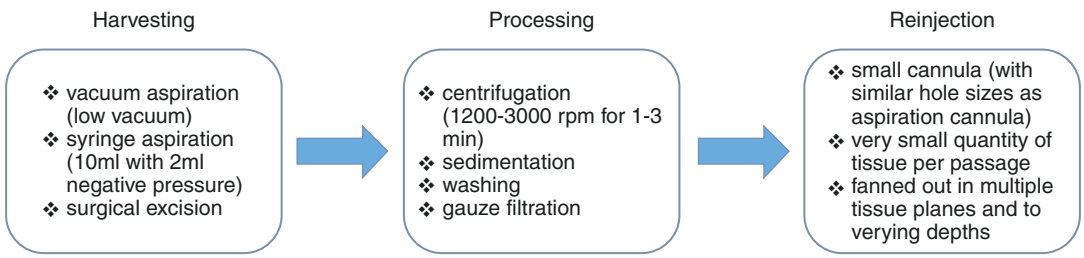

Fig. 23.5 Common procedures of fat/ADMSCs harvesting and reimplantation

therefore distributed in small aliquots and fanned out in multiple tissue planes and to varying depths in the soft tissue to create a three-dimensional network and to avoid excessive interstitial pressure at the recipient site [167].

23.6 Scaffold-Based Treatments

23.6.1 Skin and Subcutaneous Tissue Defects

Tissue-derived matrix has been widely applied for treating skin and subcutaneous defects (Table 23.1). It can be processed either from cadaveric allografts of skin tissue, placenta tissue (Dehydrated Human Amnion/Chorion Membrane, DHACM), or from porcine small intestinal submucosa (PSIS) [169]. Those scaffolds provide nearly perfect extracellular matrix architectures for three-dimensional cell growth and rebuilding of multilayer tissue structures within scaffolds after implantation, promoting tissue regeneration [170]. Biomaterials including natural and synthetic polymers have also been employed to fabricate skin substitutes (Table 23.1). Gelatin-sulfonated silk composite scaffolds have been produced based on 3D printing and showed favorable properties for skin regeneration by stimulating epidermal growth and dermal neovascularization [171]. Gold nanoparticles (GNPs) whose surface has been functionalized with PEG show accelerated cell migration, successful scaffold colonization, and regeneration [172]. Collagen-GAG scaffolds have been cocultured with autologous fibroblasts and keratinocytes or used directly with uncultured keratinocytes or stem cells obtained at the point of care in the operation room [173].

23.6.2 Muscle, Tendon, and Ligament Tissue Defects

ECM scaffolds can fill the defect and restore morphology temporarily [21]. The in vivo microenvironment needs to facilitate remodeling of the neo-tissue [174]. Functional muscular impairment can therefore be addressed by a muscle-derived matrix, which is filled with bone marrow-derived mesenchymal stem cells (MSCs) after implantation and growth of the layer. This enriched matrix gains more blood vessels and regenerates more myofibers than "conventional" extracellular matrix [21, 175]. Comparable to muscle-derived matrix, small intestinal submucosa-extracellular matrix can lead to contractile sheets of skeletal muscle with comparable contractile force [93]. One obstacle in muscle regeneration is the musculotendinous junction. This can be partly restored in the absence of implanted cells by extracellular matrix-based platforms and has been shown to withstand half of the force of the contralateral site after complete resection in a mammalian model [174].

For in vitro muscle tissue engineering, rat myoblasts have been preconditioned on a porcine bladder acellular matrix in a bioreactor and then been implanted in nude mice at a muscle-defect to restore muscular tissue [176]. The newly formed muscle cells show better adherence to 3D polyurethane-based porous scaffolds with low stiffness and larger roughness values [177]. Another hydrogel with the composition of fibrinogen and Laminin-111 (LM-111), combined with electromechanical stimulation, delivered a promising scaffold for myoblast cultures [178]. Laminin seems to play a crucial role for muscle injury since a novel synthesized laminin-mimetic bioactive peptide (LM/E-PA) was shown to stimulate activation of satellite cells and lead to myofibrillar regeneration in rat models reducing the time necessary for functional recovery [179].

Ligaments and tendons often heal with scar formation and low vascularization [180, 181]. For ACL-replacement, ligamentous regeneration has recently gained attention. PLLA (Poly L-lactic acid) nanofibers with a shell of electrospun PCL nanofibers (with bFGF and platelet-derived growth factor (PDGF)) helped hMSCs to proliferate in vitro with subsequent upregulation of collagen I and III and multiple ligament markers, forming fibers similar to the natural ligamentous structure [182].

Tendon regeneration is similarly challenging because of the long time for rehabilitation and lack of regeneration to native composition and structure. It may therefore need additional cell/molecular elements for treatment approaches [183, 184]. One promising report used PLGA (polylactic-co-glycolic acid) fibers for release of bFGF (Basic fibroblast growth factor) which increased collagen production in tendon regeneration. In the rat model, promising in vitro and in vivo results with a collagen-BDDGE-elastin (CBE)-based device for tendon tissue engineering were reported [95, 180, 185].

23.6.3 Nerve Defects

One major drawback for current nerve graft techniques is the requirement of a secondary donor site and subsequently injury, the limited supply of donor nerves, and a mismatch between the donor nerve and the recipient site [186–188]. The limits have encouraged collective development of alternatives to autologous nerve grafts mainly through tubulization. Tubulization means bridging the gap between the nerve stumps by using nonnervous tubes [189–192]. Good results of this method were reported for bridging nerve gaps less than 3 cm long (Fig. 23.6) [193]. Natural biomaterials for such neural scaffolds fall into two categories:(1) autologous nonneural tissues and allogeneic/xenogeneic neural/nonneural tissues that have been decellularized [194]; (2) naturally derived polymers, including extracellular matrix (ECM) molecules (collagen, laminin, fibrin, fibronectin, and hyaluronan), polysaccharides(chitosan, alginate, agarose), and proteins (silk fibroin, keratin) [195]. Used tubes include hollow veins, arterial and soft tissues (muscle, tendon) grafts [78, 196]. Veins alone and in combination with intraluminal muscle inlays have a high tendency to collapse. Another strategy for avoiding collapse is filling the vein lumen with small pieces of nerve tissue [197]. Muscle–vein-combined conduits have been used in the clinical practice filling gaps up to 6 cm with good results in to 85% of the cases [104, 189]. An effective nervous tissue construct seems to require a combination of a scaffold, cells, and signaling factors [78, 149]. Nerve conduits should be porous to provide sufficient diffusion of oxygen and metabolites for supporting Schwann cells proliferation, as well, be low-antigenic, biocompatible, biodegradable, conductive and resistant to infections and fibroblast infiltration [76, 78, 198, 199].

The idea of employing muscle fibers for axonal regeneration is based on the similarities between the muscle basal lamina and the endoneurial tube [200, 201]. Both fresh and denatured muscle conduits led to reported successful nerve repair. A comparison of nerve regeneration through nerve and muscle grafts has been reported for the rat sciatic nerve [202]. Results indicate the suitability of either acellular muscle or nerve grafts for nerve repair compared with conventional fresh nerve grafts [203–208]. The advantage of autologous conduits is that they are almost cost free (apart from the increased operation time) and prepared according to reconstructive needs after consideration of nerve size and length defect [209]. Despite all advances in microsurgery, satisfactory results of motor recovery from nerve injury are still reported in less than 40% of cases [210].

Current methods of advancing nerve regeneration in Tissue Engineered Nerve Grafts (TENGs) have emerged as a potential alternative to autologous nerve grafts. To overcome the limits of autografts, various artificial and biologically based nerve conduits have been developed varying in the levels of success. TENGs can be categorized into biological and artificial nerve grafts [188]. Biologically nondegradable inert silicone elastomer was the principal material used in the beginning. More recently, different classes of biodegradable synthetic polymers including aliphatic polyesters, poly(phosphoesters), polyurethanes, piezoelectric polymers, and some electrically conducting polymers have served as a scaffold in neural tissue engineering [188]. Today, commercially available products are made of polyglycolic acid (PGA) and poly(D,L-lactide-co-ε-caprolactone) (PLC) (Neurotube® and Neurolac®) (Fig. 23.7) [211, 212].

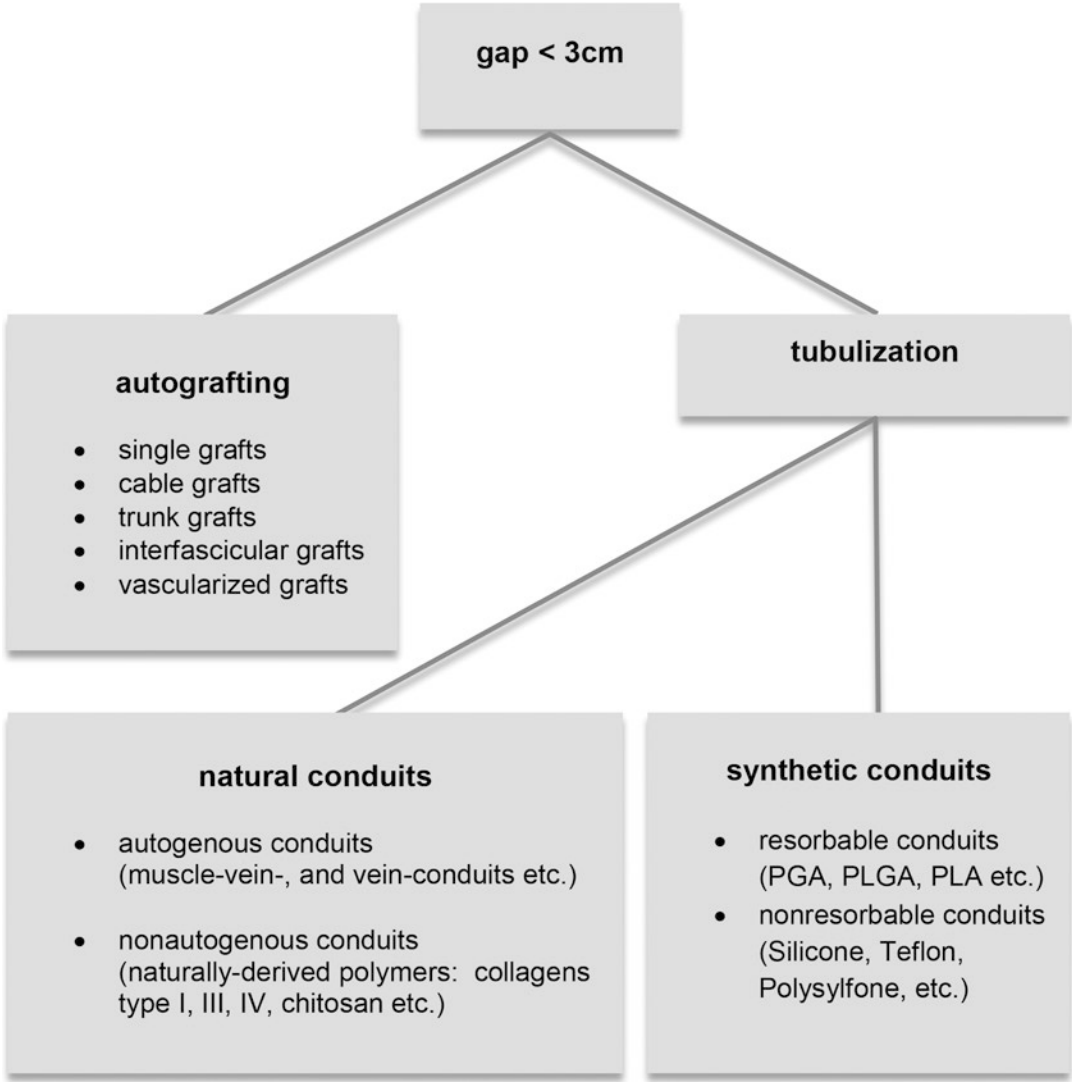

Fig. 23.6 Systematic overview for autografting techniques, natural and synthetic conduits if the nerve gap is <3 cm.

The majority of approved commercially available products are made of Type I collagen (Neurotube®, NeuroGen®, NeuroFlex®, NeuroMax®, NeuroWrap®, NeuroMend®), or chitosan-based nerve grafts (Reaxon®) [211–213]. In addition to natural and synthetic polymers, ceramic, carbon, and metallic-based materials have been investigated (Fig. 23.7) [214–220].

The selection is decisively influenced by a high priority of avoiding unwanted inflammation-inducing properties of the biomaterial, especially regarding long-term stability [221, 222]. To meet these requirements, biomaterials are usually modified or blended with each other [188].

Recently, nanoscale fabrication technologies have made it possible to synthesize neural scaffolds with submicron architecture closely resembling the architecture of natural ECM [223]. They provide a greater surface-area-to-volume ratio, which enhances cell attachment, differentiation, and growth when compared to microscale scaffolds [224]. Nanotechnological fabrication techniques as electrospinning, phase separation, self-assembly, and computer-aided design-based

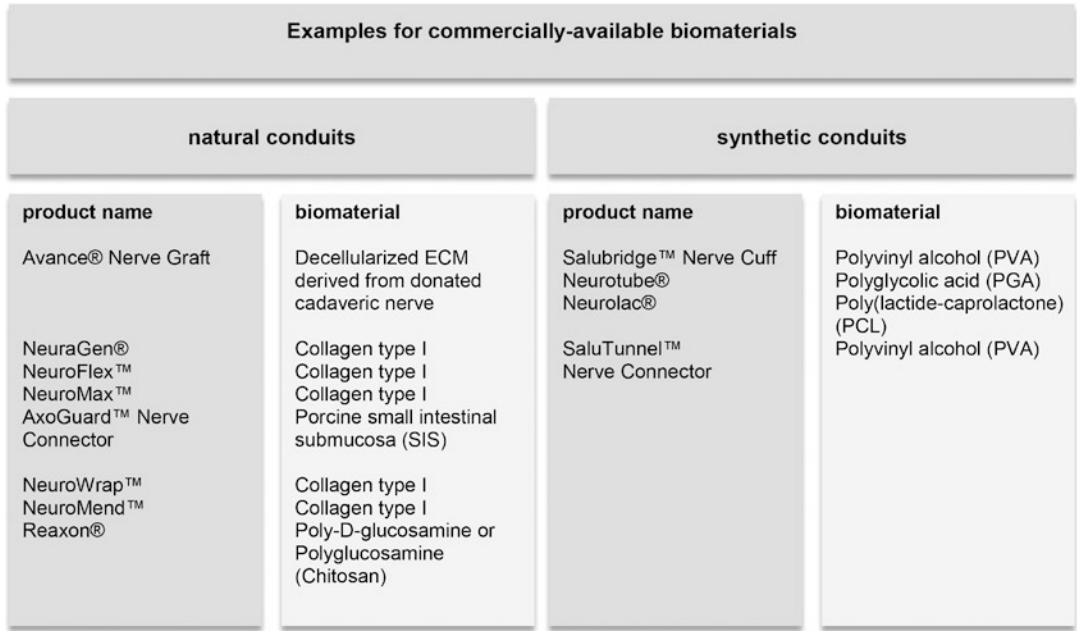

Fig. 23.7 Examples of commercially available natural and synthetic conduits (modified based on [150]) which successfully bridge gaps <3 cm. Within the German Health System exists a code for reimbursement of CE-certified nerve conduits (Operations and Procedures Key (OPS), Codes: 5-085.40, 5-058.41, 5-058.42, 5-058.43, 5-058.4x).

fabrication techniques improved regeneration regarding neurite length and linear orientation in vitro and in vivo [225–234].

23.6.4 Fat Tissue Defects

For the formation of large-volume three-dimensional fat tissues, scaffolds are used. These scaffolds stabilize the growing tissue and should enable stronger proliferation of adipocytes and endothelial cells [235]. Therefore, various natural and synthetic scaffolds have been tested in vitro and in vivo. Natural polymers that have been explored include collagen, silk, fibrin, gelatin, hyaluronan, adipose-derived ECM, decellularized human placenta, and matrigel. Matrigel is both angiogenic and adipogenic and improves the adipose graft longevity and volume maintenance when mixed with adipocytes [236, 237]. Synthetic materials have also been widely tested in adipose tissue engineering [238]. Polymers such as polyactic acid (PLA), polyglycolic acid (PGA), polyethylene glycol (PEG), and the copolymer poly lactic-co-glycolic acid (PLGA) have been extensively used for soft tissue applications. The chemical-physical properties of PLA and PGA showed potential in supporting tissue regeneration in in vitro and in vivo studies as 3D scaffolds or grafts for adipose tissue engineering [239–241].The long-term availability of PGA meshes in vivo supported evident adipogenesis and vascularization [242].

23.7 Drug-Based Therapy

23.7.1 Skin and Subcutaneous Tissue Defects

Exogenous administration of growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), vascular endothelial growth factor(VEGF), fibroblast growth factor (FGF), and transforming growth factor (TGF), has potential benefits in wound healing and tissue regeneration [243]. They can affect chemotaxis

and stimulate mitosis of quiescent cells, angiogenesis, and synthesis and degradation of the extracellular matrix (ECM) [244]. EGF stimulates epidermal and mesenchymal regeneration and cell motility through interaction with the EGF receptor on epidermal cells and fibroblasts [245]. IGF-1 plays an important role in stimulating collagen synthesis in fibroblasts, the proliferation of fibroblasts and keratinocytes, and angiogenesis [246]. PDGF can regulate the maturation of blood vessels and re-epithelialization and also stimulate proliferation of fibroblasts, thus increase ECM production [247]. Because each growth factor has specific functions and is present at different stages of wound healing and tissue regeneration, recently researchers suggested a cocktail of growth factors to be a more promising treatment [248]. The low-molecular-weight protamine (LMWP) conjugated with EGF, IGF-1, and PDGF-A via genetic modification accelerated wound re-epithelialization significantly, accompanied by the formation of healthy granulation tissue within 9 days (Fig. 23.2) [243].

Deferoxamine (DFO), an FDA-approved iron-chelating agent, currently corrects impaired HIF-1a-mediated transactivation in diabetes by preventing iron-catalyzed reactive oxygen stress. Duscher et al. reported that transdermal delivery of DFO was found to prevent diabetic ulcer formation and improve wound healing in preexisting ulcers by decreasing oxidative stress [249].

23.7.2 Muscle, Tendon, and Ligament Tissue Defects

Pathogenesis of sarcopenia as one of the most frequent muscular diseases involves different molecular pathways, out of which BMP and myostatin pathways seem to be most promising [250]. Medication with human recombinant BMP-2/7 and anti-myostatin can help to reduce sarcopenic symptoms [251]. Cachexia is addressed with anamorelin, a ghrelin agonist and selective androgen receptor modulator as well as anti-cytokines/myokines [252]. Another factor involved in muscle healing seems to be TGF-β. Increased TGF-β1 levels, which could be detected after the use of nonsteroidal anti-inflammatory drugs, helped to regenerate muscle tissue [253–255].

Ameliorating tendon repair is especially of interest in rotator cuff tear reparation. Here, HIF prolyl 4-hydroxylase (PHDs)-inhibitors have been shown to improve enthesis mechanics in a rat model [256]. For further tendon reparation, tendon multipotent stem cells (tendon-derived stem cells, TDSCs) may be the key to biological drug delivery therapies (BDDT). Blood-derived TDSCs can be applied intratendinous and intraligamentous with fibrin scaffolds to repair tendon tears [257, 258]. As another factor of tendon regeneration, platelet-derived growth factor (PDGF-BB) can increase flexor tendon fibroblast proliferation and matrix synthesis in vitro, yet without effecting improvements in biomechanical properties [259, 260].

23.7.3 Nerve Defects

Neural cells in the distal nerve stump secrete endogenous growth factors and support axon regeneration. This stimulus declines over time. To maintain the supportive action additional of exogenous growth factors, Gu et al. [188] classified the existing growth factor into two classes: (1) neurotrophins: NGF, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3); (2) growth factors with neurotrophic actions: glial cell line-derived neurotrophic factor (GDNF), ciliary neurotrophic factor (CNTF) and fibroblast growth factors (FGFs) [261, 262].

For continuous release of growth factors from TENGs, different delivery systems were compared [236]. Classical delivering strategies are adsorption of growth factors to the surface, the bulk of a scaffold, incorporation of growth factors into the scaffold materials during the scaffold fabrication, entrapment of growth factor-loaded microspheres into a scaffold, covalent immobilization of factors onto the scaffold, and installation of an osmotic minipump or injection device. Different microsphere designs allow effective technologies to encapsulate

growth factors in the delayed nerve repair [263–271]. The combination of cellular components and growth factors within TENGs seems to allow a prolonged release from their cell environment. Another approach for prolonged release of growth factors is immobilization or high-affinity binding of growth factors to cells or scaffold biomaterials [272, 273]. Diverse techniques immobilize neural growth factors onto neural scaffolds. Among various newly developed procedures, cross-linking is commonly used for immobilization. Used cross-linkers are glutaraldehyde, carbodiimide, and genipin [274–277]. Furthermore, photochemical reactions, coaxial electrospinning, and differential adsorption achieved immobilization of neural growth factors [278–280].

VEGF has neurotrophic activity to stimulate axonal outgrowth and to enhance survival and proliferation of Schwann cells. It also improves intraneural angiogenesis by promoting endothelial sprouting during peripheral nerve regeneration [281–284]. Long-term observation demonstrated that VEGF significantly increased vascular and axonal regeneration and enhanced target muscle reinnervation [285]. Bio-printing was used to incorporate VEGF-releasing fibrin gel and neural stem cells into a collagen hydrogel scaffold [286]. Manipulation of nitric oxide supply within TENGs could contribute to new capillaries and regenerating axons [287].

23.7.4 Fat Tissue Defects

Tissue engineering strategies are being investigated to develop methods for generating adipose tissue for regenerative and aesthetic medicine. A current concept is to harvest fat cells from a patient, amplify them in a laboratory, and then seed adipose tissue-derived stem cells (ADSC) or adipose progenitor cells onto a scaffold that supports cell proliferation. The cell-covered scaffold could be implanted into soft tissue defects of the patient for adipogenesis. After guiding tissue regeneration, the biodegradable polymer scaffolds will then decompose, leaving the newly formed tissue [288].

Hereby, the key factors in tissue engineering might be the seed cell, scaffold, and the microenvironment [289, 290].To support and accelerate tissue growth, a synthetic or biological matrix is used in most tissue engineering procedures in combination with a pool of growth factors to enable or support angiogenic induction.

Meanwhile, a new strategy to improve the vascularization of implanted fat grafts and the survival of transplanted fat is incorporation of vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) gene-transfected adipose stem cells [291]. Circulating endothelial progenitor cells (EPC) are considered an important factor for stimulation of tissue vascularization [292]. Thus, mixing EPCs with fat grafts may also potentially enhance vascularization and increase long-term survival of autologous fat grafts.

23.8 Cell-Based Therapy

23.8.1 Skin and Subcutaneous Tissue Defects

Nearly every cell type that is present in the skin has been isolated and added to wounds to improve healing. Fibroblasts and keratinocytes have been used for augmenting healing of wounds [293]. Armenio et al. [294] have achieved very promising results with sealing diabetic foot ulcers and providing a moist environment for fibroblast growth and neoangiogenesis of the neo-dermis by combining autologous fibroblasts grafts and V.A.C. Transplantation of keratinocytes and dermal fibroblasts cultured on PLGA microspheres were presented as a potential alternative for the treatment of skin wounds [295]. For ulcers, bilayered cellular constructs containing both fibroblasts and keratinocytes have been shown to promote healing of chronic wounds [293]. Cutaneous wounds demonstrated successful closure after treatment with bone marrow-derived MSCs impregnated fibrin polymer spray [296]. Adipose-derived MSCs being cultured within human fibroblast(HS27)-derived conditioned medium(F-CM) facilitated type I

collagen synthesis for wound healing and skin regeneration [297]. For burn wound treatment, autologous non-cultured cell therapy using the ReCell system has been proposed [298]. In this, the cells from the dermal-epidermal junction of the skin are harvested, typically producing a complete cell population, including keratinocytes, melanocytes, Langerhans cells, and fibroblasts, which can be directly applied intraoperatively.

23.8.2 Muscle, Tendon, and Ligament Tissue Defects

For regeneration of muscle tissues, implanted cells alone show only low survival in vivo and minor formed neo-muscle [299]. This can be improved by surrounding the cells with microthread bundles, e.g., fibrin microthreads with adult human stem cells [300]. Stem cells also drive regeneration in tendon defects; however, they may not be sufficient on their own [301]. For xenograft experiments in rats, human MSCs have been used in vivo for repair of patellar tendon [253, 302]. Typically, they are treated with growth factors like GDF5 and GDF7 for tenogenic differentiation[303–305]. For regeneration in a preformed shape, autologous multipotent stromal cells in a vicryl mesh tube have been shown to fill defects (e.g., in the Achilles tendon) with good results and completely regenerated tendon [306]. There seem to be differences in performance of MSCs from different sources for different fields of application [307].

23.8.3 Nerve Defects

For augmentation of nerve tissue regeneration, neural stem cells, embryonic stem cells, Schwann cells, and bone marrow stromal cells (BMSCs) have been the most studied types of cells [262]. Schwann cells within TENGs have shown positive effects in experimental studies [308–311]. Their clinical use is however limited as autologous Schwann cells are difficult to obtain in large number, and allogenic Schwann cells are involved in immunological rejections. Thus, stem cells from different sources (e.g., bone, fat, amniotic fluid) have become a promising alternative as they can be easily harvested through the aspiration of bone marrow and expanded in a large scale in vitro culture [312–314]. Diverse animal models containing either undifferentiated or differentiated MSCs have bridged peripheral nerve gaps of different lengths [315–329].

Furthermore, gliogenic secondary neurospheres derived from induced pluripotent stem (iPS) cells showed promising results, when added to a PLC-based NGC and implantation across a sciatic nerve gap in mice [330].

23.8.4 Fat Tissue Defects

Adipose tissue-derived stem cells induce neovascularization, and release high levels of angiogenic growth factors like epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β), and insulin-like growth factor (IGF) or brain-derived neurotrophic factor (BDNF) [331–337]. Consequently, the implantation of adult stem cells could induce angiogenesis and/or vascularization during the de novo adipogenic process, and may develop fully vascularized fat tissue. Kato et al. indicate that adipose tissue of nonvascularized grafts is completely remodeled within three months [338]. In a further study based on this, Kentaro et al. [339] showed that the majority of host-derived cells detected during remodeling of the grafted fat were macrophages and that at 12 weeks mature adipocytes were largely derived from adipose-derived stem/stromal cells of grafts. This underlines the importance of adipose stem/stromal cells for adipogenesis.

A relatively recent development is the use of genetic engineering to instruct autologous cells to produce their own biological drugs. Current methods allow overexpression or downregulation of any known gene in any known cell type. For example, nucleofection of fibroblasts with plasmids containing VEGF and bFGF has been shown to improve vascularization and wound

healing in vitro and in vivo [340–342]. Through RNA interference (RNAi) particular genes can be silenced using so-called short interfering RNAs (siRNAs) and microRNAs (miRNAs), which regulate gene expression at the posttranscriptional level in cells [343]. This methodology has been successfully used to modify the Ras/Raf/ERK pathway and induce an increase in neurite outgrowth in vitro and in vivo as well as increases of Schwann cell migration and peripheral nerve regeneration [344–347]. Another approach is the use of gene-modified stem cells for neural tissue engineering. Examples are genetically engineered BMSCs expressing nerve growth factor (NGF) via an adenoviral vector [348]. Nanocarrier systems, including liposomes, nanoparticles, dendrimers, and carbon nanotubes, could be future siRNA delivery vectors to overcome current problems with biodegradation, renal clearance, unselectivity, and immunology [349, 350].

23.9 Other Treatments

23.9.1 Blood-Based Treatments for Skin Tissue Regeneration and Tendon Healing

Blood cells are the first to enter a new wound and play an important role in the healing process. Consequently, blood-based therapeutic approaches like platelet-rich plasma (PRP) or the cell-free EmaCure technique use these autologous source of active growth factors, such as PDGF, platelet-derived angiogenesis factor (PDAF), platelet-derived epidermal growth factor (PDEGF), TGF-b, platelet factor-2 (PF-4), IGF1, FGF, and EGF, which are important in the regulation of the healing process [351–355]. Their subcutaneous infiltration at the wound boundaries were found to enhance wound re-epithelization and contraction within 3 weeks, without chronic effects or formation of exuberant tissue granulation and with minimum scarring [356]. In addition, local injection of PRP could also improve tendon healing in acute tendon injury and tendon-bone interface regeneration for ligament restoration [357, 358]

23.9.2 Electrical Stimulation for Nerve Tissue Regeneration and Muscle Reinnervation

Electrical stimulation (ES) enhances neurite extension on the substrates that are based on electrically conducting polymers [359]. A Canadian group showed that brief direct nerve stimulation after nerve injury improved the amount and accuracy of motor and sensory reinnervation [360–364]. ES prevents degenerative changes like axotomy during the delays caused by slow axonal regrowth leading to an improved functional outcome [78]. The time course of electrical stimulation is important, and a rapid onset of electrical stimulation may accelerate axonal regrowth across the nerve gap [365]. Distal electrical stimulation addresses the muscles directly and is one method to maintain muscle architecture, function, and responsiveness [78]. Animal studies with an implantable electrical stimulator on limb and facial muscle improved morphology and functional capacity of the reinnervated stimulated muscles [78, 366, 367].

23.10 Unsolved Questions

23.10.1 Functional Tissue Regeneration

Lack of organogenesis is stated as one big shortcoming of the current models. Epidermal skin substitutes are effective in providing rapid and temporary external coverage of wounds, but lack the underlying connective tissues that provides the elasticity and mechanical stability of regenerated skin [368]. The 3D structure of the multilayers is difficult to achieve even with the use of dermal scaffolds. Bioprinting provides the capability of producing an organized structure in the biomimetic skin with fibroblasts and keratinocytes [368, 369]. Melanocytes and stem cells can also be incorporated within the printed cells [370]. However, recapitulating more complex skin functions is still challenging as the current bioprinting resolution is limited to few hundreds of micrometers [371]. It is not only the topogra-

phy, but also the structure and function of the skin layers which would determine if the regenerated tissue can be considered as skin and used for regenerative purposes. Furthermore, the conformation of the sweat glands, hair follicles, nerve endings, and blood capillaries needs a stringent signaling cascade [372]. Disruption of the latter would lead to the loss of skin architecture, which is the case during scar formation [373].

Vital signal molecules (HGF) lack after demolition of the basal lamina, which makes fibrin microthreads loaded with HGF a plausible solution. Three-dimensional microscale assay systems with these HGF-loaded, cross-linked fibrin microthreads have been demonstrated as a platform for "axially aligned tissues" [374].

The long-term results of autologous fat grafts are often disappointing because of the unpredictable partial reduction in graft volume of up to 70%, and therefore unpredictable success rates. To the present day, there has not been agreement among physicians concerning the specific techniques of fat graft harvesting, fat processing, and injection [30, 120, 375]. The Coleman technique should be considered the standard method for harvesting and processing; however, one of the problems observed in this method is an increase of apoptotic death rate for mature adult adipocytes because of damage caused during the aspiration and centrifugation steps. Thus, the centrifugation phase, as recommended by Coleman, has been demonstrated to decrease the number of fat cells [50, 376–378].

Therefore, an important challenge is to optimize the individual steps of the autologous fat transplantation so that during the harvesting, fat processing, and injection of fat grafts as many intact and viable adipocytes could be preserved as possible.

23.10.2 Vascularization in the Process of Regeneration

The absence of immediate blood supply is one main reason for the failure of the integration of bioengineered soft tissue constructs [379]. For example, revascularization of skin substitutes occurs by ingrowth of bed vessels into the graft, which might take up to 3 weeks and significantly limits the capacity to obtain wound closure in a short period of time [380]. Their inability of fast vascularization results in cell death and ultimate sloughing away from the host [381]. For bioengineered scaffolds with biomaterial and cells, the only cells up to a distance of approximately 200 μm have access to sufficient nutrients by diffusion [382]. Insufficient vascularization can lead to nutrient deficiencies and hypoxia deeper in the scaffolds, which could result in non-uniform cell differentiation and integration, and thus decreased tissue functionality [383].

Development of a network for blood supply is one major issue during the integration of newly formed tissue. Encouraging, after an acellular biologic scaffold/ECM is implanted at the site of injury, neovascularization and perivascular stem cell mobilization could be detected in human muscle defects [384]. Different approaches for vascularization are conceivable: One way is through angiogenesis growth factors like Basic FGF, which seems to accelerate neoangiogenesis in an early stage of healing, but diminishes flexibility of the new tendon [385, 386]. Another possibility is the coculture with endothelial cells [387]. In addition, integration of vascular networks into bioengineered scaffolds by microfluidic systems or bioprinting is expected to provide solutions in the near future [388–391]. Maybe the combination of several of these approaches will lead to the vascularization of the designed tissues [386].

23.10.3 Immune System Problems

For matrix derived from tissues, both allografts and xenografts are often rejected because of host immune response arising from antigens present in the donor tissue [392]. For polymeric biomaterials, immunological compatibility remains a problem since limited biocompatibility causes site morbidity and chronic inflammation [253]. One reason could be that polymeric

biomaterials seem more often to undergo disintegration because of multinucleated giant cell-induction. Therefore, decellularization techniques for the enrichment of native tissue seem to be more promising for soft tissue regeneration [393]. Embryonic and adult stem cells seem to have less immunogenicity [394]. Therefore, cells isolated from cord blood and autologous stem cells would be preferred for clinical application. The induced pluripotent stem cell (iPSCs) has the wide ability for differentiation, but there are still safety concerns for the use of iPSCs in patients requiring extensive studies in the future [395].

In addition, the response of the immune system to tissue injury is related not only to tissue repair but also to tissue regeneration. The process of inflammation may preclude the ability of a structure to regenerate [396]. Study of immunomodulation by scaffolds might provide new ideas for strategies to enhance skin tissue regeneration.

23.10.4 Problems with Biomaterials

The scaffolds created with natural polymers (e.g., collagen, fibrin, hyaluronic acid) are usually associated with poor mechanical stiffness and rapid degradability [397]. Synthetic polymers (e.g., poly-ethylene glycol (PEG), poly(lactic acid) (PLA), polyglycolide (PGA), poly(lactic-co-glycolic acid) (PLGA), polycaprolactone (PCL)) provide an artificial alternative which have flexible mechanical properties [398, 399]. However, the use of synthetic scaffolds can be associated with side effects such as inhibition of cell migration and cell-to-cell communication, which can lead to loss of the cell phenotype [400]. For 3D bioprinted biomaterials, the main technical difficulties are associated with nozzle blockage and shearing stresses on the cells [372]. Therefore, reliably printing large areas of skin is not yet possible.

The mechanical and surface properties of the scaffold will also affect the cell behavior in terms of adhesion, proliferation, migration, and differentiation [401]. If stem cells are seeded onto scaffolds, they may differentiate into different types of cells based on the scaffold properties [402, 403]. Therefore, better understanding of cell-scaffold interaction and development of a carrier scaffold that stimulates the niche environment for ongoing remodeling processes would be valuable.

As first steps in this direction, mechanical properties of hydrogels can be engineered with microfluidic techniques, generating gradients of tissue properties and with electrospinning it is possible to form tunable fiber arrays. Furthermore, mechanical stimulation can increase the proliferation rate of tendon stem cells, modulate collagen synthesis, and stimulate matrix turnover and remodeling [301, 404, 405]. Since the musculotendinous border is partially mineralized, calcified nanofibrous electrospun PLGA scaffolds could depict an opportunity for toughening the attachment between tendon and bone [406].

After establishing and integrating biomaterials, insufficient mechanical properties often tend to cause reinjuries. For ligament repair, low-intensity pulsed ultrasound has been proposed as intervention for the strengthening of the implanted biomaterials. This effect is thought to act through increasing interleukin 1β and consequently angiogenesis and protein synthesis [306, 407, 408]. Fibrous matrices can also be augmented by mineral deposits in the tendon-bone connection which may serve as barrier against reinjury [406].

23.10.5 The Time Challenge

Especially in nerve reconstruction, the main challenge remains the race of axon regeneration at velocity of an 1 mm/day versus the fast-progressing muscle atrophy. Even if nerve reconstruction is successful, there is no satisfying reconstruction outcome if the muscle is conversed irreversible to fatty and fibrotic tissue or the neighboring joints are already stiffened [409]. Consensual recommendations are that nerve injuries should be repaired early and within 3 months if there is no sign of reinnervation [410]. If reinnervation occurs uncontrolledly, branching of

growing axons at the lesion site and misdirection of axons and target organ reinnervation errors are common. These complications require sensory reeducation in the following rehabilitation process [76, 411, 412]. Obstacles are compatibility with the surrounding tissues and regenerative environment likewise the protection of regenerating nerve fibers from scar invasion.

23.11 Back from the Lab to the Real World

Despite the plethora of positive in vitro and in vivo results, still only a fraction of these promising approaches is successfully clinically translated. In part this may be due to differences between the scientific model and the real patient. In part it may be completely independent of science and due to financial and regulatory reasons and due to the structure of the respective health care system.

Getting marketing authorization for drugs or medical products is a highly regulated process in most countries, which needs expert knowledge in several fields and takes years to decades. The whole process and especially clinical testing is extremely expensive. Given the relatively high risk, private investors like pharmaceutical companies will usually only invest, if there is a chance of a high return of this investment. Fortunately, recently there has been a rise in federal funding, so hopefully more of these early stage projects will be able to jump the early financial "valley of death." Even the best drug or medical device has to be manufactured, distributed, and controlled which needs further investments. This is mainly interesting for manufacturers and distributors, if they have strong protection of their product like patent protection. Otherwise, once it becomes obvious, that the approach works, it can be copied at a fraction of the original cost, leaving the first manufacturer with non-competitive prizes. If the science is published, before strong IP-protection is secured, this can therefore stop promising approaches, even before they even can reach the clinical stage.

Another problem lies in the structure of reimbursement in the health care system. While regenerative therapies are usually beneficial for patients, as they provide a long-lasting solution for the problem, this is not necessarily beneficial for all stakeholders in the health care framework. Apart from this, a socially organized system may not be able to afford the optimal solution for each problem for each member, so negotiations for prioritization are necessary.

Taken together, the translation of regenerative approaches for clinical use is a field with huge opportunities and great challenges.

If we will be eventually able to beat all the scientific, technical, regulatory, and financial challenges of regenerative medicine, this may even result in a completely new realm of philosophical, psychological, and ethical challenges around the concepts of immortality and eternal youth.

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24 Bone Tissue Engineering Challenges in Craniofacial Reconstructive Surgeries

Seyed Mohammad Zargar and Nima Jamshidi

24.1 An Overview of Craniofacial Defects

The craniofacial region has always been one of the most noticeable regions of the human body for physicians, surgeons, and engineers. This region consists of a wide variety of soft tissues as well as diverse bones, including frontal, occipital, parietal, and temporal bones (which form the cranium); the two jaws that are named maxilla and mandible; and other kinds like zygomatic, nasal, sphenoid, and ethmoid bones. It contains six various cavities that are cranial, orbital, nasal, oral or buccal, and middle and inner ear ones. Hence, not only is it directly pertinent to some critical functions including breathing, speaking, eating, seeing, hearing, etc., but it also plays an essential role in social relationships thus it is indubitably important to scrutinize different types of the defects and deformities of this region and discover the reasons why these appear.

Craniofacial deformities can be categorized into congenital, traumatic, and cancerous ones. According to The International Statistical Classification of Diseases and Related Health Problems (ICD-10) [1], congenital malformations and deformations are divided into several groups. The first one is craniosynostosis, that is, when some of the cranial sutures ossify before the brain has matured suitably. It brings about some alterations in the pattern of skull growth. For instance, scaphocephaly and oxycephaly can be mentioned. The second division is craniofacial dysostosis, which is defined as a disorder of bone development, such as Crouzon and Treacher Collins syndromes, as well as hypertelorism. Other deformities are classified into the third division. Examples are macrocephaly, platybasia, plagiocephaly, and saddle nose syndrome.

Cancer may lead to the devastation of both soft and hard tissues in the craniofacial region. Yet, traumatic injuries result in a great number of diverse craniofacial defects, including lacerations, blunt traumas, and burns. Vehicle accidents, chemicals, heat, electricity, assaults, and falls are some examples of such injuries [2]. Regarding the importance of the roles which the craniofacial region plays as well as the prevalence of its deformities, pondering over finding appropriate therapies has always been an interest of technicians, such as physicians, surgeons, and engineers. Although there have been grandiose notions for repairing the deformities of the craniofacial region, some have become pragmatic after passing standard tests. Some main methods of treatment are discussed in the next section.

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24.1.1 Available Therapies

Repairing the defects and deformities of the craniofacial region has always been a concern for specialists due to destructive impact thereof. No record is found from early Greek physicians, such as Hippocrates and Galen, with regard to craniofacial reconstruction. However, dating back to the Incan Empire, there is some evidence in which some valuable metals and gourds are reported to be utilized in order to repair craniofacial defects. The application of grafts, harvested tissues, in reconstruction surgeries was first published by Meekeren in 1668. He utilized canine bone to reconstruct a defect in the cranium of a Russian man. Afterward, in the late nineteenth century, further experiments resulted in more progress in craniofacial reconstruction surgeries. Moreover, the special circumstances provided by warfare in the twentieth century brought about a leap in reconstruction surgeries. The specialists, at that time, sought applicable metals and plastics to use for larger defects [3]. The advancement in reconstruction surgery methods applying distinctive grafts experienced an incremental trend with the passage of time. In this section, the main purpose is to discuss these methods and various grafts.

Bone defects can be of different sizes. They can be either as small as periodontal defects (in millimeters) or large, which are mainly traumatic or caused by surgical incisions or cranioplasty. The vivid similarity among most of the defects is the complicated 3D structure thereof. The main expectation of reconstruction surgeries is the restoration of the functionality that has been restrained as well as the appearance that need to be aesthetically reconstructed due to the severe dependency of social relationships on it [4]. All of the process must be pursued in a way that controls the morbidity of both donor and recipient sites [5].

There are three significant criteria to amend the functionality of any graft, namely osteoconductivity, osteoinductivity, and osteogenesis. Osteoconductive grafts are those whose surfaces permit the formation of new bone cells along themselves. However, osteoinductivity causes the supply of the factors that are required for bone-forming cells to be recruited to the graft site and differentiate. In addition, osteogenic grafts benefit from bone-forming cells that can be induced or inducible. Thus, they can commence with the bone formation as soon as they are transplanted [4]. An ideal graft is the one which meets all the aforementioned criteria. Provided that the graft lacks osteoconductivity, the incorporation process of it into the recipient site declines intensely. Besides, if the graft is not osteoconductive, it does not tend to recruit bone-forming cells, such as osteoblasts and osteoprogenitors, and the stem cells cannot be differentiated due to scarcity of the needed factors. The circumstances exacerbate when the graft does not contain induced and inducible cells or in other words when the graft is not osteogenic.

Degradability is one of the vital factors depending on the purpose for which the graft is implanted. The condition that the graft is to stimulate bone formation and has to degrade as fast as the new bone tissue forms. Otherwise, degradability is absolutely a demerit for the grafts intending to contour the normal appearance or have mechanical functions. In this case, calvarial and cortical bones can be applied [6].

The ideal graft is the one which is not only osteoconductive, osteoinductive, and osteogenic but also suitably porous with interconnected pores. Porosity provides larger contact surface between the graft and recipient site and the cells thereof. So the osteoblasts can penetrate into the graft and form new bone structure and, on the other hand, osteoclasts can have a wider connection with the graft's surface so that resorption is facilitated. These are key to the incorporation of the graft into the recipient site. Besides, blood vessels are also required for nutrition delivery to the cells and their recruitment.

In reconstruction surgeries, the applied grafts can be classified into groups based on their source. Autografts are those which are harvested from the patient's own body. Spongy and cortical bones, bone marrows, and vascularized bones are examples of tissues that can be autografted. Autografts benefit from osteogenic cells which

do not activate the immune system. However, surgeons encounter a higher level of morbidity at the donor site as well as a restrained amount of harvestable tissues [7]. Grafts that are harvested from a person who is genetically identical to the patient are called isografts. The merits and demerits of isografts are roughly the same as autografts due to the similarities in genetics.

The next group of grafts, allografts, are those that are harvested from another individual with different genetics from the patient. Allografts are mostly harvested from a cadaver and used as augmentation for autografts. Before being applied, these grafts are generally decellularized since there is a huge risk of transplant rejection by the immune system. Allografts are also treated in preoperational procedures for decreasing the chance of disease transfer from the cadaver to the patient [5, 6].The last category of grafts is pertinent to xenografts that are defined as those harvested from animals. They can be bovine or porcine, or only collagen from such animals [8].

Grafts can be applied for various purposes such as filling a defect, mechanical functions, or triggering bone formation. There might be no need for its incorporation into the recipient site in some cases whereas large defects' reconstruction requires an incorporated graft. In other words, the graft has to be remodeled. The graft should allow bone cells to proliferate on its surface, that is, in contact with the recipient site. Afterward, the graft ought to begin its degradation process in order to permit the new bone cells to form the former normal tissue gradually. Deficiency of blood vessels restrains the remodeling process enormously [4, 9–11]. Thus, provided that the surgeon faces a huge loss of both soft and hard tissue, it is recommended to apply vascularized grafts since they can supply sufficient blood for the remodeling process. In the case of only hard tissue defects, nonvascularized grafts might also be applicable due to the ability of the recipient site to supply blood.

The incorporation quality depends mainly on the applied graft, recipient tissue, and the interface between graft-tissue and physiological capacities. One of the most influential factors of the applied graft is its degree of porosity. A more porous graft has wider contact with the recipient tissue. In osteoconductive grafts, the large surface allows much more bone cells to migrate and proliferate on it as well as facilitates the degradation process by permitting more osteoclasts to be in contact with the graft. In addition, being porous results in more blood vessels' invasion into the graft so the matrix will be demineralized and its proteins will be released, such as bone morphogenic protein (BMP), which provides osteoinductivity [4, 5].

Cortical bones are not porous in comparison with trabecular bones. Blood vessels and recruited cells can merely attach to the outer surface of such bones. Therefore, the integration process is prolonged and often incomplete. The application of vascularized grafts, even when they contain cortical bones, will accelerate integration [6, 9].

Another main challenge is for the graft to remain fixed in its place, as any strain may result in the failure of the remodeling process. There is a broad range of fixators and alloplastic materials, such as titanium reconstruction plate, that not only keep the graft strongly in its place but also assist the contouring procedure. For instance, according to Kim and Donoff (1992), titanium reconstruction plates that were applied in a lateral mandible reconstructive surgery showed acceptably low failure rate [4, 5, 12].

Vascularity and viability are the most important criteria for an appropriate graft bed or recipient site. The applied graft is needs access to the viable bleeding bed. Redundant reaming or high temperature throughout surgery might cause necrosis in recipient site's cells. On the other hand, other factors such as prior radiotherapy might jeopardize the success rate of reconstructive surgery due to its impact on vascularity and fibrosis of the recipient tissue. Thus, vascularized grafts are highly preferred in the cases that have experienced radiotherapy before reconstructive surgery. In these cases, anastomosis of the blood vessels should also be done in order to increase the success rate. In addition, prior surgeries and chemotherapy may become hazardous, in particular, in cases where tissue has been radiated simultaneously [4, 5].

A broad range of diverse grafts has been applied in reconstruction surgeries. In some cases, the hard tissue has been harvested whereas others have tried both hard and soft tissue that was concurrently harvested as vascularized pedicles. For instance, in an experimental study, calvarium bone grafts were used for 222 patients with mainly posttraumatic or congenital deformities [13]. Other grafts that have been used were harvested from various parts of the body such as acromion and spine of scapula [14], rib [15], radius [16], iliac crest [17, 18], tibial plateau [19, 20], fibula [21, 22]. Some studies reported the usage of myocutaneous free flaps that supply muscle mass, epithelial tissue, and blood such as trapezius and pectoralis major myocutaneous flaps [14, 23]. In some cases, even the resected tissue was applied as a graft [24].

24.1.2 Tissue Engineering Approach

Reconstructive surgeries with the application of grafts, including autografts, allografts, etc., are the best existing treatments for deformities in the maxillofacial region. However, there are some inevitable complications in these surgeries such as the probability of donor site morbidity, restraints in harvesting suitable tissue regarding the quality and the quantity thereof, and the vivid drawbacks of alloplastic materials. To address these complications, biochemical and biomaterial engineering are tried to be combined with cell transplantation studies in order to achieve a fabricated tissue or organ that not only reconstructs the defect but also does not trigger immune response when transplanted. This field of research is named Tissue Engineering and defined as "a new approach applying the principles of biology and engineering to the development of functional substitutes for damaged tissue" by Langer and Vacanti in 1993 [25–27].

Tissue engineering consists of three main strategies that are based on the materials utilized for treatment. Isolated cells or substitutes thereof can be used for improving the functionality of the tissue. There are some substances that can result in tissue formation induction. This strategy relied on the application of these materials. Engineered constructs, called scaffolds, can be utilized and implanted into a defect and lead to the reconstruction of the lost or deformed tissue [28].

24.1.3 Scaffolds

Three-dimensional structures, scaffolds are the main part of tissue-engineered constructs. Tissue engineering scaffold should benefit from some criteria such as biocompatibility in order to have an appropriate function when implanted. For preventing immune rejection, scaffolds must be biocompatible. In some cases, scaffolds are designed for a temporary function. So, they should degrade when their mission is completed. These scaffolds are supposed to be biocompatible in both implantation and degradation time. In other words, the products of their degradation must be nontoxic and safe, as well as themselves. Also, the degradation rate is important and has to be measured and well adjusted.

Since scaffolds are implanted to function as the extracellular matrix of the tissue, they should have suitable mechanical properties. In fact, the preferred scaffolds are those which mimic the native tissue properties. Similar to grafts, scaffolds should be adequately porous and penetrable. The size of pores and their interconnectivity are really important for cell and blood vessel invasion. The cells' diameter is the determinant of pore size. Suitable surface properties are vital as well in order to achieve cell attachment [29].

When a scaffold is to be designed, the first and foremost step is the selection of suitable materials. The materials should be biocompatible and biodegradable and have suitable mechanical and surface properties. A broad range of materials have been introduced with the potential of being used as scaffolds. They can be classified into four groups, namely, polymers, ceramics, metals, and composites [29–31].

24.1.3.1 Polymers

Polymers can be a golden choice for tissue engineering scaffolds due to their high ability to be designed in a way to address the needs. For this,

their composition and structure can be altered [1, 32]. Polymers are divided into two groups of natural and synthetic ones, and each has its pros and cons. Natural polymers are derived from either plants or animal sources similar to the nature of human body, so they are less likely to be rejected by the immune system. Due to their origin, they have variations that may cause an inaccuracy in their engineering and functionality. Their major demerit to some extent are their weak mechanical properties [29, 33].

Collagen can be named as one of the major natural polymers that have been used as tissue engineering scaffolds. Although 28 different types of collagen are known, the collagen type 1 is found the most in human body tissues like bones, tendons and ligaments [34, 35]. Collagen is used because it is not only profuse but biocompatible. In addition, it has the ability to be highly porous and easily processed. Also, it is a hydrophilic and absorbable material having low antigenicity [36, 37]. In addition, some other natural polymers have been used as scaffolds and have shown good performance such as chitosan and hyaluronic acid [38, 39].

Synthetic polymers are aimed to meet the deficiencies in natural ones. They do not have variations and their degradation is always the same, on any patient. This similarity is due to chemical hydrolysis of synthetic polymers rather than an enzymatic one [29]. Some synthetic polymers that have been used for tissue engineering scaffolds are poly lactic acid, polyglycolic acid, and polycaprolactone [40–44].

In a study, the scaffolds made of poly-DL-lactic-co-glycolic acid (PLGA) by solvent-casting particulate-leaching technique were used for repairing defects in porcine mandible. The scaffolds were accompanied by porcine mesenchymal stem cells derived from ilium. The results were satisfactory and PLGA scaffolds could cause bone regeneration at the implantation site [40]. PLGA scaffolds were used in a rabbit mandible with pore sizes of 100–250 μm and resulted in adequate bone formation [41].

Polycaprolactone was used in a dog's mandible in order to reconstruct the mandibular defect [42]. It was also implanted into the anterior mandible of a 71-year-old woman and the results illustrated new bone formation and higher bone volume in comparison with controls [43]. In another study, polylactic acid was used as a scaffold for a defect of critical size. The results were satisfactory, and the PLA scaffold could play a role in bone formation due to its proper mechanical properties and suitably low degradation rate [44].

24.1.3.2 Ceramics

Ceramics can be used widely as bone tissue engineering scaffolds due to their great biocompatibility and bioactivity [29]. Ceramics are highly osteoconductive [45] and osteoinductive [46–48]. Unlike the polymers that are mostly ductile, ceramics are stiff and brittle materials. They are usually used in combination with polymers [29] in order to obtain better characteristics.

There are a broad range of ceramics that have been used as tissue engineering scaffolds, such as hydroxyapatite [49, 50], bioglass [51], titanium oxide [52], and zirconia [53]. For instance, among ceramics, bioactive glasses are not only osteoconductive and bioactive [49, 54–57] but are also able to deliver cells [58]. Furthermore, their degradation can be controlled [59–61]. Bioactive glasses or bioglasses can be fabricated porously with suitable shape and pore size by replication technique [62–64]. In a study, a new sintering method was tested and bioglasses could achieve appropriate mechanical strength as well [51].

Hydroxyapatite (HAp) can be another example of widely used ceramics. Hydroxyapatite is used not only for tissue engineering scaffolds but also as a coating for implants and fillers. All of these applications are due to its high biocompatibility, even with soft tissues, low degradation rate, and proper osteoinductivity and osteoconductivity [28]. HAp is chemically kind of similar to the inorganic component of the bone matrix, so it can form strong chemical bonds with the recipient tissue [65]. HAp does not benefit from suitable mechanical strength. In a study, it has been shown that if HAp is used in nanoscale, the mechanical strength thereof rises [28].

Generally, the materials have to be selected properly in order to achieve suitable characteristics. However, there are some methods of fabrication that confer specific features to the scaffold such as porosity. These methods consist of firing powder and firing slurry [66]. Replication technique is an example [51]. The scaffolds that are fabricated via replication technique are suitably porous. For having a scaffold with desired characteristics, not only is it vital to choose a proper material but also by the use of some techniques, the characteristics of the chosen material can be manipulated as desired .

24.1.4 Cells

Embryonic stem cells (ESCs) are pluripotent stem cells which can be found in the inner cell mass inside the blastocyst. These stem cells have the potential to recreate every organ of the human body. For this, ESCs have to be divided into the groups of cells with the potential to work more specifically, called multipotent stem cells. Mesenchymal stem cells (MSCs) are derivatives of ESCs and play an important role in forming the craniofacial structure by differentiating to various forming cells, such as chondroblasts, osteoblasts, etc. [67]. Mesenchymal stem cells can be easily harvested, isolated, and proliferated. In addition, in freezing, they do not lose their osteogenic potential [5]. MSCs might be the best choice for tissue engineering approach.

Generally, stem cells' division is slightly different from other mature cells. Stem cells need to be constantly available inside the human body. Whenever they differentiate, they produce a cell with characteristics identical to their own. These identical stem cells remain inside various tissues and will be recruited and used whenever required [67]. So, although there are some available sources of MSCs inside the human body, the presence of these cells in a scaffold seems to be beneficial. For instance, for stem cell recruitment, there is a need for blood supply in the defect site while in some cases, in particular in large defects, blood supply shortage is clear.

MSCs have been embedded in various kinds of scaffolds, such as adipose [68–73]. In a study, MSCs that were derived from adipose tissue were embedded in apatite-coated PLGA scaffolds and implanted into a large defect in calvarium. The results were satisfactory and MSCs could induce bone formation [73]. In some studies, MSCs were seeded in an injectable hydrogel, such as the composite of oligo (poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles [74] or sodium alginate hydrogels [75]. Radiation therapy affects reconstruction adversely. It not only endangers vascularization but also makes an incremental change in apoptosis of the embedded cells [76, 77].

In order to induce chondro- or osteogenesis by stem cells that are embedded in a biocompatible and biodegradable scaffold, growth factors need to be added [67].

24.1.5 Growth Factors

There are various growth factors used concomitantly with scaffolds and cells in order to induce tissue formation. Some of them are discussed here, including platelet-derived, insulin-like transforming growth factors, as well as bone morphogenic protein and platelet-rich plasma.

Platelet-derived growth factor (PDGF) is known to affect bone formation by amending the proliferation of both osteoblasts and osteoclasts [78, 79]. It improved bone formation when an absorbable scaffold containing it was implanted into a rat calvarial defect [80], and new attachment and bone defect filling were observed when implanted in monkeys [81].

Insulin-like growth factor (IGF) is one of the growth factors that seem to be effective on general growth of body skeleton [82]. Its systemic application for a critical-size defect in rats that were under radiation prior to reconstruction improved bone formation [83]. IGF has been mostly applied in combination with PDGF [84–86], and in a study, it was illustrated that these growth factors can lead to a dose-dependent improvement in bone formation when applied concomitantly [87].

Transforming growth factor beta (TGFβ) is one of the most prevalent and multipurpose cytokines that generally have an influence on various tissue formations [88]. There are 30 proteins included in the TGFβ superfamily, such as activin, bone morphogenic proteins, and TGFβs themselves [82]. These growth factors have been applied in various studies, albeit with somewhat vague results. Yet, it has been shown that the effectiveness of TGFβs is largely dependent on their carrier and its degradation pace [89]. For instance, $TGF\beta_1$ could not affect bone formation in the rabbit calvarial defects when it was administered freely; however, it vividly resulted in bone regeneration when embedded in a gelatin capsule [90].

Bone morphogenic protein (BMP), as is clear from its name, plays a role in the morphogenesis of bones, specifically three types: BMP2, BMP4, and BMP7. These three types have been shown to induce dose-dependent ectopic and orthotopic bone formation [91, 92]. Recombinant human bone morphogenic type 2 has been used for an elderly female patient in a polycaprolactone carrier and shown to induce de novo bone formation [43]. Some other studies, in particular animal studies, have tested the BMP [93–97].

Platelet-rich plasma (PRP) is a source of platelets produced by blood centrifuging. It benefits from a high amount of thrombocytes containing several prepacked growth factors, such as PDGF, TGFβ, IGF, and VEGF (vascular endothelial growth factor) [82, 97, 98]. In some studies, the application of PRP has led to more bone volume and bone regeneration [42, 43].

24.2 Conclusions

The craniofacial region of the human body plays many vital roles in our lives; therefore, the defects in this site have to be considered as those of critical nature to be reconstructed. Implanting autologous grafts was a gold standard due to not triggering immune rejection, etc., yet, nowadays, the tissue engineering approach seems to be much better since it does not result in donor site morbidity nor does it have the problem of lack of suitable source in terms of quality and quantity. Tissue engineering approach is to implant or inject a biomaterial called scaffold that benefits from some criteria such as biocompatibility, biodegradability, porosity, etc., in combination with cells and some inducing factors in order to enhance the capability of the body to reconstruct the defect. Although a lot of work and experimental studies have been carried out in this regard, there is a broad range of studies that remain and hoped to be done for finding the best cure for these kinds of problems.

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Translational Challenges: Lymph Node Tissue Engineering

25

Philipp Neßbach and Matthias M. Aitzetmüller

25.1 Introduction

Chronic lymphedema results from a progressive pathological condition of the lymphatic system. It is a combination of an accumulation of protein-rich fluid, inflammation, fibrosis, and hypertrophy of adipose tissue. This often leads to a clinical picture of disfigurement and decreased mobility and function. The dysfunction of lymphatic transport can be described as a lack of removal of net fluid efflux from the capillaries. An accumulation in the interstitium and following pressure on the skin is the result. This interstitial fluid is up to 90% reabsorbed by venous capillaries. In healthy patients, the remaining 10% are removed by lymphatic vessels to blood as lymph [1]. Macromolecules like proteins are first degraded by macrophages and normally afterwards also removed by lymphatic vessels from the interstitium [2]. Chronic lymphedema results from an imbalance in lymph transport.

Lymphedema is classified into primary and secondary causes. Among primary, all congenital causes are summarized. Furthermore, secondary lymphedema comprises all causes which are acquired by disruption of lymph transport. Congenital hereditary lymphedema as well as Milroy disease occur in the first 2 years of life. Milroy disease is an autosomal-dominant pattern which mostly affects lower extremities and causes intestinal lymphangiectasia and cholestasis [3]. It is known to be connected genetically to a mutation in the VEGFR-3 tyrosine kinase signaling pathway [4, 5]. Familial lymphedema praecox, also known as Meige disease, occurs during puberty. Another autosomal-dominant pattern which is associated with the loss of hearing, cerebrovascular malformations, vertebral defects, and distichiasis [6]. Lymphedema tarda is the primary form which occurs latest in life, that is, after the age of 35 [1].

The most prominent etiology of secondary lymphedema is filariasis secondary which is caused by a nematode *Wuchereria bancrofti* and affects more than 90 million people worldwide [7]. In industrial countries, female breast cancer and its treatment play an important role in the development of chronic lymphedema. According to the American Cancer Society, there are two million breast cancer survivors of which 20% suffer from chronic lymphedema [8]. In Germany there are

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approximately 1.2 million patients suffering from lymphedema [9]. Mostly upper extremities are affected by lymphedema with rates from 24% to 49% after mastectomy [10–14] and 4–28% after lumpectomy [15, 16]. Both main treatments for breast cancer patients, radiation and dissection of lymph nodes in the axillary region, increase the risk of chronic lymphedema [15, 17–20]. After introducing sentinel lymph node biopsy to detect breast cancer spread, a significant decrease in lymphedema cases had been shown in comparison to the traditional dissection of lymph nodes [21–25]. Various known factors such as obesity, infection, and trauma increase the risk of lymphedema for women after breast cancer therapy [11, 16, 26, 27].

25.2 Clinical Presentation and Diagnosis

Lymphedema is characterized by an accumulation of subcutaneous interstitial fluid and adipose tissue. Inflammation occurs simultaneously with the accumulation of fluid and the decreased removal of lymph fluid. This also leads to more fat deposition and lipogenesis which results in an increased activation of fibrosis and proliferation of connective tissue [28–30]. Subcutaneous tissue in patients becomes harder and fibrosis develops. Furthermore, hypertrophy of adipose tissue occurs. This first results in a swelling of the diseased site and is often characterized as fibrotic and soft, and turns into a hardened state over time. The International Society of Lymphology defined the clinical classification for lymphedema using the following terminology [31]:

- Stage I: Represents an early accumulation of fluid relatively high in protein content (e.g., in comparison with "venous" edema) and subsides with limb elevation.
- Stage II: Signifies that limb elevation no longer reduces tissue swelling, and pitting is manifest. Later in Stage II, pitting is less evident as tissue fibrosis supervenes.
- Stage III: Encompasses lymphostatic elephantiasis where pitting is absent and trophic skin changes such as acanthosis, fat deposits, and warty overgrowths occur.

Even though a swelling by itself does not induce serious symptoms, cellulitis may occur due to proliferation of microbes in the accumulated interstitial fluid. A common effect is the progression of lymphedema by lymphangitis, which results in lymphatic vessels destruction. Papillomatosis, hyperkeratosis, and skin breakdown are additional changes in skin quality that regularly occur [32]. Lymphangiosarcoma, Kaposi sarcoma, and lymphoma represent cutaneous malignant tumors which are rare complications of chronic lymphedema [33]. A subsection of women who developed a significant lymphedema following radical mastectomy and who were diagnosed with ensuing lymphangiosarcoma are suffering from Stewart Treves syndrome. Around 200 patients are reported up to now with a mean survival time of 19 months [34–38].

Patient history and clinical presentation are the basis for diagnosis of lymphedema in later stages (Table 25.1).

Common causes of limb edema are challenging to differentiate from early stages of lymphedema. Cardiac failure, protein-losing conditions, local etiologies including lipedema, vein thrombosis, myxedema, chronic venous insufficiency, and idiopathic edema are systemic causes for differential diagno-

Table 25.1 Symptoms and factors for lymphedema diagnosis

Associated symptoms
Chronic skin breakdown
Recurrent cellulitis
Clinical signs
Soft, pitting edema (early stage)
Cellulitis
Fibrosis and induration (late stage)
Hyperkeratosis
Papillomatosis
Peau d'orange skin changes
Positive Stemmer sign (lower extremity lymphedema)
Reported risk factors
Familial history of congenital lymphedema
History of infection
History of malignancy
History of radiation therapy
History of trauma
Obesity
Prior surgical procedures, particularly nodal dissection
Travel to geographical region with endemic filariasis

sis of lymphedema [1]. Positive Stemmer sign as well as peau d'orange changes in the skin and cutaneous and subcutaneous fibrosis are indicators for lymphedema during physical examination [39, 40]. Circumferential and volumetric measurements are commonly used for lymphedema documentation as well as comparison between a patient's healthy and diseased limbs. Tonometry, perometry, and bioelectric impedance analysis are noninvasive, advanced methods for clinical examination [41–44]. Analysis in body composition typically make use of bioimpedance. This technology allows a direct measurement and is able to differentiate between edema and limb volume [45, 46]. Bioelectric impedance is a reliable and reproducible technique which has shown the capacity to indicate lymphedema in women following breast cancer treatment [47–49].

Magnetic resonance imaging, computed tomography, and lymphoscintigraphy are used if clinical examination is not capable of a secure diagnosis. Lymphoscintigraphy makes use of a radiolabeled marker which is intradermally injected and stains lymphatic vessels [50, 51]. Delayed transport of the radiolabeled marker, backflow, dermal diffusion, and meager visualization of lymph nodes and vessels are common anomalies for lymphedema [52]. Furthermore, computed tomography is a 100% specific and 97% sensitive technique to confirm lymphedema diagnosis [53]. Magnetic resonance imaging is more cost intensive but able to show a more detailed lymphatic structure. In addition it was shown to have similar sensitivity and specificity for lymphedema diagnosis, without exposure to radiation [54].

Radiologic examination techniques for lymphedema diagnosis are only necessary in cases in which patient history and physical examination are insufficient. For most patients, there is no need for lymphoscintigraphy, magnetic resonance imaging, or computed tomography, and clinical diagnosis guides the treatment algorithm.

25.3 Traditional Treatment

The most prevalent conservative treatment is complete decongestive therapy (CDT), which has become internationally recognized. More than 90% of lymphedema patients are treated with CDT. This therapy is supported by its four mainstays: manual lymphatic drainage, compression treatment, exercise, and skin care. It needs to be provided by specialized physiotherapists. In addition, CDT is a symptomatic treatment, which makes it necessary to perform CDT lifelong. The biggest disadvantages are the lack of disease elimination and the high expenditure of time. The use of special compression devices that mimic manual lymphatic drainage is rare. Due to the danger of centralized edema, this procedure is effective only in a small proportion of patients and must be supervised by physicians [55].

Since several decades various surgical procedures have existed; however, none has been established as a gold standard for lymphedema treatment. For a small group of patients, who are suffering from a special combination of lymphedema and lipedema, liposuction offers a long-term symptomatic improvement. However, tissue removal is a very stressful procedure, which is rarely performed today [56].

The goal of reconstructive surgery is to correct the cause of the disease by restoring lymphatic drainage. Reconstructive procedures are only offered in a very few specialized hospitals. Established operating procedures are bypasses between lymphatic vessels (lymphatic grafting) and connections between lymphatic vessels and veins (lymphovenous anastomosis). Both procedures use elaborate microsurgery with relatively high treatment risks and long surgical times [57–59]. Alternatively, an autologous transplantation of lymph nodes (microvascular lymph node transplantation), lymph node bundle with surrounding fatty tissue, in the sense of a free flap, is taken from a healthy site and microsurgically transplanted into the diseased site. Thereby blood vessels are connected to each other by complex microsurgery, resulting in higher risks for the patient and frequent problems at the donor site, such as secondary lymphedema. Among the existing surgical procedures, microvascular lymph node transplantation is the most promising [60–63]. A graphical overview of treatment options is provided in Fig. 25.1.

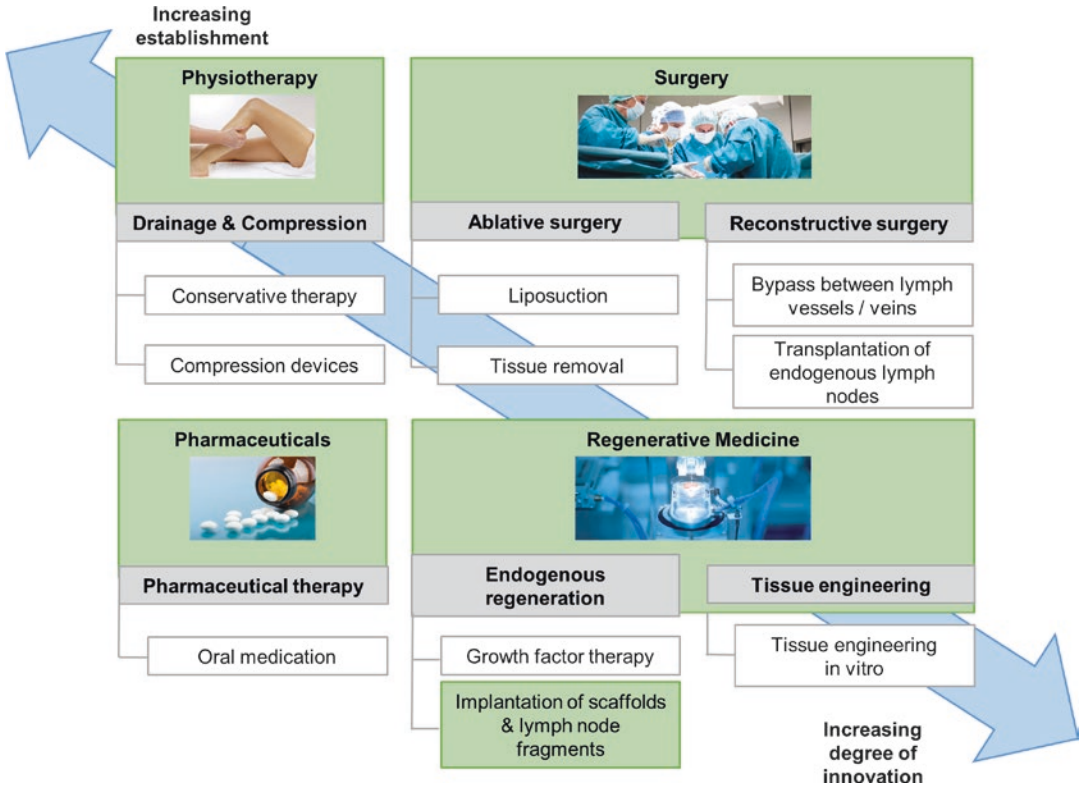

Fig. 25.1 Traditional and regenerative treatments for chronic lymphedema

25.4 Regenerative Treatment: Lymph Node Tissue Engineering

Tissue engineering and its area of clinical application, regenerative medicine, aim to regenerate the body's own tissue and organs by introducing the use of three-dimensional scaffolds, different cell types, and growth factors. The scaffold serves as temporary three-dimensional support structure for mechanical stabilization, organization, proliferation, and differentiation of the involved cells. Scaffolds take over the function of the extracellular matrix and are subject to correspondingly high demands. An ideal scaffold should have an interconnecting pore structure that allows the migration of cells and their supply of nutrients and oxygen [64, 65]. These properties were previously difficult to implement by using conventional fabrication methods for scaffolds. In traditional techniques (textile technologies, plastic processing), the results are not reproducible (pore size, mechanical properties, filament thickness), or cytotoxic or carcinogenic solvents are used. These residues have a negative influence on the biocompatibility of the scaffold. Through the introduction of additive manufacturing techniques in tissue engineering, many obstacles were overcome. The mechanical properties of the scaffolds could thus be controlled down to small levels and play an important role in the field of tissue engineering and especially in lymph node regeneration [66, 67]. A suitable scaffold should have the same biomechanical properties as the surrounding tissue at the time point of implantation. The degradation of the biodegradable polymer must fulfill requirements such as maintaining rigidity and the three-dimensional structure as well as allowing surrounding cells to migrate and proliferate [64, 66].

The high potential for regeneration of lymphatic tissue is characterized by numerous studies

by Jaffe et al. and known since the 1920s [68]. In a rat model, they were able to show, that autologous transplanted lymph nodes regenerate within 6 days. These lymph nodes were previously transplanted avascular into muscular abdominal wall. Several years later, in the 1980s and 1990s the group Papst et al. introduced the concept of transplanting autologous lymph node fragments [69–71]. Lymph node fragments were transplanted in different anatomical regions (inguinal, mesentery of ileum, and omentum majus) of minipigs. Inguinal transplanted fragments showed good regeneration, whereas the omentum majus fragments did not show regenerative characteristics. The group Papst et al. [69] assumed that subcutaneously transplanted lymph node fragments are more capable of connecting to afferent lymphatic vessels than the fragments of omentum majus.

After several decades and with increasing progress in regenerative medicine, approaches of artificial lymph nodes were introduced [72]. The complex three-dimensional structure with cortex and the composition of various cell types is a big challenge for researchers [73]. First artificial lymph nodes were made using collagen sponges and stroma and dendritic cells [74]. These artificial lymph nodes were transplanted subcapsular into kidneys of immunodeficient mice. A secondary immune response was shown, starting from the artificial lymph node after antigen exposure. B and T cells migrated from the artificial lymph nodes to the spleen and bone marrow and became antibody-secreting plasma cells [74, 75]. Another approach is using polyurethane scaffold which is seeded with murine T zone fibroblastic reticular cells. A connection was discovered between interstitial flow velocity and the secretion of CCL21. At low flow rates, gene expression of CCL21 was downregulated [76]. CCL21/CCL19 and its receptor CCR7 have an influence on the migration of antigen-presenting cells and T lymphocytes from the periphery to the lymph nodes [77–79]. The mentioned artificial approaches combine the time-consuming cell isolation and culturing steps for the production of artificial lymphatic tissue.

A further development of the already referred methods is the introduction of the idea to combine autologous lymph node fragments and biodegradable scaffolds. There are two promising ideas combining lymph node fragments and scaffolds. On the one hand, Hadamitzky et al. [80], who used aligned nanofibrillar collagen scaffolds and, on the other hand, Kwak et al. [81, 82] who used polycaprolactone (PCL) tubes.

Hadamitzky et al. [80] performed a porcine model of secondary lymphedema in minipigs. Here, 10 aligned nanofibrillar collagen scaffolds (10–12 cm each) were implanted into the groin of a previously operated and irradiated minipig. Three months after implantation the scaffolds were analyzed histologically, the lymphatic function was determined by bioimpedance ratio, and the lymphatic regeneration was quantified by computed tomography. It was found that aligned nanofibrillar collagen scaffolds provide mechanical support for directed lymphangiogenesis. The density of lymphatic vessels increased as well as the lymphatic function improved.

The whole concept of Kwak et al. [81] is provided in Fig. 25.2. A lymphedema patient provides a lymph node from a healthy donor site. This removed lymph node is then fragmented and fixed inside the PCL scaffolds by using fibrin glue (bioartificial lymph node). Several of these bioartificial lymph nodes are then implanted close to an artery into the diseased region. In the following months, the bioartificial lymph nodes induce growth factor release and regeneration of the lymphatic system.

In previous studies, Kwak et al. used tubular, porous PCL scaffold in immunodeficient mice in combination with human lymph node fragments. After 8 and 16 weeks, samples were taken and histological and immunohistochemical staining were performed. It was shown that the combination of lymph node fragments and PCL scaffold result in higher vascularization as well as presence of lymphatic endothelial cells [82]. These cells are responsible and essential for building lymphatic vessels and regenerate lymphatic function.

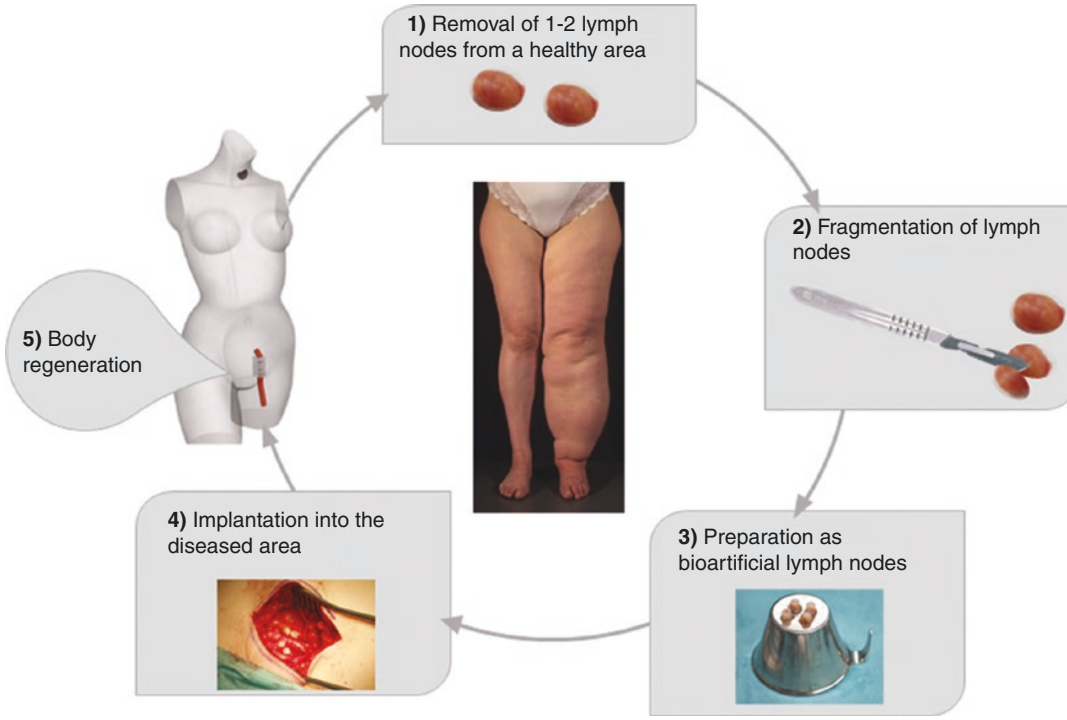

Fig. 25.2 Method for bioartificial lymph nodes according to Kwak et al. [81]

25.5 Conclusions

Lymphedema patients often go through a long ordeal. They consult a number of physicians according to their cancer disease and following lymphedema symptoms. A well-documented clinical history and physical examination typically are sufficient to provide clear diagnosis of chronic lymphedema. In obscure cases, additional lymphoscintigraphy and/or magnetic resonance imaging lead to a diagnosis.

Even after diagnosis, the suffering of patients with chronic lymphedema is high. The existing options for treatment often do not contribute to a significant improvement in the quality of life. Complete decongestive therapy is effective, but is only symptomatic and needs to be continued lifelong [55, 83]. Microvascular lymph node transplantation is the most promising traditional treatment [60, 84]. The removal of a lymph node bundle with surrounding fatty tissue from a healthy region contains higher risks for inducing another lymphedema in the donor site [60, 84]. In order to avoid these risks, in the future, novel translational treatment approaches harnessing the possibilities of regenerative medicine are warranted.

The most innovative regenerative medicine paradigms are related to cell processing and culturing, which typically leads to a harsh regulatory situation which is time- and resources consuming. The approaches that combine autologous lymph node fragments and biodegradable scaffolds therefore are particularly promising. They do not require problematic regulatory processes because they are considered medical devices (no cell processing). Additionally, they promote true regeneration of the lymphatic system instead of treating the disease only symptomatically.

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26 Implementing Tissue Engineering and Regenerative Medicine Solutions in Silicone Implants

Jae Chul Lee and Chan Yeong Heo

26.1 Introduction

This chapter introduces the concepts of tissue engineering and regenerative medicine, presenting some of the previous work in this area. However, because biointegration, as applied to the interface between a silicone implant and a biological tissue, is a concept that is still in development, this chapter is in fact a mix of review articles and white papers to help disseminate ideas in the field of biointegration of tissues and silicone implants. It is important to note that this chapter does not describe the use of scaffolds, biomaterials, biomolecules, cells, and tissue-engineered constructs, which are single-use, soft, biodegradable materials, and these will not be considered as "silicone implants" for the purpose of this discussion. This chapter presents the following: (1) a discussion on the need to improve the long-term integration of biological tissue and silicone implants; (2) an introduction to the problems of interfacing biological tissue and silicone implants; (3) a description of methods currently available to modify implants; and (4) a description of novel ideas drawn from tissue engineering, regenerative medicine, and biomedical engineering that may be applicable and useful to the implementation of interfacing biological tissue and silicone implants. These methods should be applicable to implants that are intended to be in place for an extended period of time, and, in most cases, indefinitely. There is a need to improve surgical silicone implants integration with surrounding tissues.

Nowadays, an incredible variety of devices using disparate materials (from plastics to metals) have been developed by researchers, constructed by the medical device industry, and implanted by surgeons. However, it is important to evaluate whether current medical implants and technology require substantial improvements, and, if so, how by understanding the shortcomings of these current devices. Existing literature about the failure rates and economic impact of all currently available medical implants is difficult to find. Nonetheless, it is possible to find specialized reviews by implant type, and the economic costs of implant failure have been evaluated for certain types of implants [1]. Moreover, some patients require multiple device replacements, and it is expected that the number

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of medical procedures with more sophisticated implants and longer periods of implantation will increase in the near future due to an plastic surgery patients and the general population with several medical complications [2]. By any measure, the above figures are staggering, indicating a clear necessity for the improvement of the process of long-term integration of tissue and silicone implants.

26.2 Problems Associated with Medical Implants

Medical devices and surgical implants have seen a drastic technological improvement in the past few decades, widely extending their applicability and usability. In the field of cosmetic and reconstructive surgery, silicone implants are widely used [3, 4]. As such, there has been an increased interest recently to find solutions that better provide long-term integration with surrounding tissues and organs. A possible way to better biointegrate silicone implants and biological tissue may be to implement methods drawn from the field of tissue engineering and regenerative medicine to the areas in contact with medical implants, in addition to the normal approach of functionalizing or modifying surgical implants. Although silicone implants are approved for clinical use, local complications, such as capsular contracture, implant rupture, and gel bleeding, have not yet been fully resolved [5, 6]. One of the most serious complications is capsular contracture, which has been reported to occur in up to 30% of patients after the insertion of silicone implants [7, 8]. When a silicone implant resides in the body for a prolonged period, excessive fibrous connective tissue, which is mainly composed of collagen and fibroblasts, accumulates around it, isolating the implant from the local tissue environment [9, 10]. Subsequently, a contractile force originating from the collagen and myofibroblasts, which are differentiated from fibroblasts, causes capsular contracture around the silicone implant. In serious cases, this can require secondary surgery, posing a great deal of inconvenience to patients [7, 11].

26.3 Suppression of Cysteinyl Leukotriene to Reduce Capsular Contracture Around Silicone Implants

This pathological phenomenon usually occurs due to prolonged inflammation, resulting in chronic inflammation from acute inflammation [12]. In such a scenario, the period of acute inflammatory response can also be prolonged from days to weeks [13]. Once the silicone implant is inserted, acute inflammation initiates with infiltration of polymorphonuclear leukocytes (PMNs) from the blood vessels to the implant site [13]. Then, these PMNs secrete cysteinyl leukotrienes (CysLTs), which are potent inflammatory lipid mediators [14] and also involved in the recruitment and survival of PMNs via autocrine and paracrine signaling [15, 16]. The persistent presence of the silicone implant leads to chronic inflammation, in which CysLTs stimulate the migration and proliferation of fibroblasts [17, 18]. During this stage, fibroblasts differentiate into myofibroblasts and synthesize collagen, which is mediated by transforming the growth factor (TGF)-b secreted by the fibroblasts themselves [19–21], leading to the formation of capsules by fibrosis. Eventually, both the smooth muscle actin (i.e., a-SMA), which is expressed on the myofibroblasts, and tension, which is caused by collagen fibrils, cause capsular contracture, i.e., a contractile force around the silicone implant [9, 22, 23]. Therefore, inhibiting CysLT production may prevent the formation of capsular contractures. Furthermore, decreasing the number of CysLTs would reduce the recruitment and proliferation of fibroblasts, thereby decreasing the amount of myofibroblasts and collagen during the chronic inflammation stage. Montelukast is a potential therapeutic agent for the inhibition of CysLT production [24, 25]. Montelukast, a specific leukotriene receptor antagonist, selectively blocks the production of leukotriene D4 (LTD4) by binding to the type 1 CysLT receptor (CysLT1) located on the outer plasma membrane of PMNs [22–24]. This drug has been reported to inhibit the accumulation of fibroblasts/myofibroblasts as well as to inhibit

the deposition of collagen [26, 27]. It is already being used clinically to treat asthma or other allergic diseases caused by the overproduction of CysLT [28–31]. Therefore, to minimize or prevent capsular contracture, we recommend using a local, sustained release of montelukast around the silicone implant. We hypothesize that the formation of fibrous capsule, which occurs during the chronic inflammation stage, can be reduced by inhibiting the production of CysLT even during the acute stage of inflammation. Periodically, early suppression of CysLT should decrease the amount of PMNs, thereby reducing the amount of CysLTs, which are secreted by the PMNs themselves. Decreasing the amount of CysLTs would result in minimized recruitment and proliferation of fibroblasts during the chronic stage of inflammation [17, 32], leading to a reduction in TGF-b secretion, a decrease in myofibroblast differentiation, and a reduction in collagen synthesis, ultimately resulting in diminished capsular contracture.

26.4 Problems Associated with Silicone Implants and Biointegration

The main reason for the failure of implants is the lack of integration between the silicone implant and the tissue around it. Here, we define "biointegration" as the recurrent, long-term mechanical, biological, and physical adjustment of the interface between the surgical implant (silicone) and the surrounding biological tissue. An alternative, but not equal, term is "bio-functionalization"; however, this expression is better used to describe some of the methodologies for improving implant performance. The term bio-functionalization is also encountered in biomaterial technology to refer to the development of coatings of implant materials or the characterization and evaluation of silicone implants [33–37]. This idea behind biological integration has been proposed by tissue engineers or biomedical engineers to describe the short-term "integration" of biodegradable, soft scaffolds of varied sorts with tissues [38, 39]. This generally implies some type of cell and tissue penetration of the implant surface, requiring direct functional and structural connection between the tissue and the silicone implant.

26.5 Overview of Solid Implant Types

Solid implant materials commonly used in facial reconstruction include silicone (polydimethylsiloxane), Silastic (solid silicone elastomer; Michigan Medical Corporation, Santa Barbara, CA), GoreTex (expanded polytetrafluorethylene; W. L. Gore & Associates Inc., Flagstaff, AZ), MedPor (high-density porous polyethylene; Porex Industries, Fairburn, GA), and Mersilene (nonresorbable polyester fiber; Ethicon, Somerville, NJ). Other less common implant options include Supramid (polyamide nylon mesh; Ethicon), PMMA (polymethylmethacrylate), and Permacol (porcine dermal collagen; Tissue Science Laboratories PLC, Aldershot, United Kingdom) (Table 26.1).

Midface augmentation is generally used to address volumetric deficiencies of the soft tissue and skeletal framework, such as malar hypoplasia, submalar soft-tissue insufficiency, or a combination of the two [40]. Nasal implants are commonly used for dorsal augmentation, as well as for the correction of structural deficiencies and contour irregularities in primary and secondary rhinoplasties. Mandibular augmentation is most frequently used for defects caused by microgenia or a poorly defined mandibular angle border [40]. Poor projection of the pogonion and soft-tissue inadequacy of prejowl sulcus can be corrected with the use of chin, extended chin, chin jowl, or prejowl implants. The mandibular angle region can also be augmented to more sharply define its borders or provide lateral enhancement using posterior mandibular angle implants [41].

26.6 Solid Implant Complications

Several studies over the years have investigated the postoperative outcomes following facial reconstruction using different types of solid

Table 26.1 Solid implant materials commonly used in facial reconstruction

Material	Description	Advantages	Disadvantages	Common uses
Silicone [41, 45, 50]	Polydimethylsiloxane; smooth, nonporous	Greater degree of augmentation, lower early risk of infection, easily carved, easily removed, low cost	Chronic inflammatory process leading to capsule formation, mobility of implant (leading to seroma, chronic peri-implantitis, extrusion), bone resorption, increased risk of late infection	Forehead, malar, chin, premaxillary, nasal augmentation
GoreTex (ePTFE) [41, 45, 46, 51]	Fibrillated polymer with small pores (10–30 μm); sheets or premolded into desired shapes	Simplicity of operation, lack of capsule formation, minimal displacement/extrusion (fibrous tissue ingrowth), easily carved, good biocompatibility, easier to remove than larger pored materials	Limited amount of augmentation, slightly higher chance of infection (smaller pores decrease access of immunomodulators; hydrophobic so does not absorb antibiotic solution), higher cost, hardens in contact with blood	Forehead, mandible, orbital floor, zygoma, chin, malar region, nasal dorsum, nasolabial fold, lip implants
MedPor (HDPE) [41, 45, 46, 50, 51]	Similar to PTFE but larger pores (100–300 μm)	Lower infection risk long term (larger pores allow macrophage entry), minimal displacement/extrusion (fibrous tissue ingrowth), less bone resorption, easier dissection (in avascular subperiosteal plane), stability under subsequent trauma		

ePTFE expanded polytetrafluorethylene, *HDPE* high-density porous polyethylene. Notes: An overview of the three most common solid implant materials—silicone, GoreTex (W. L. Gore & Associates Inc., Flagstaff, AZ), and MedPor (Porex Industries, Fairburn, GA). A general description of the material is provided along with the advantages and disadvantages seen with use of each material as well as the most common uses for each implant type

implants. Each type has advantages and disadvantages. Reported complications include infection, extrusion of the implant, malpositioning, irregular contouring of the overlying skin/tissue, pain or patient discomfort, and hypoesthesia. The risks and complications of implants can be categorized according to the material and/or the location of the implant. Tables 26.2 and 26.3 highlight the most common complications.

Infection in the setting of alloplastic implantation has always been an issue, as these materials lack the vascular ingrowth seen with autologous grafts (and thus have a decreased ability to fight infection, if present), and additionally serve as a surface for bacterial colonization and biofilm formation. When it comes to implantation in the head and neck, it remains controversial as to whether porous materials (including MedPor, GoreTex, and Mersilene) have a greater risk of infection compared with their smooth counterparts (silicone). Several studies have evaluated the infection rates of GoreTex, MedPor, and silicone, with inconsistent results. Bacterial biofilm formation, which has been a well-known and well-studied problem in orthopedic surgery and ophthalmology, has recently gained much interest in the field of facial plastic surgery. Biofilms are relevant because they contribute to confounding scenarios, such as chronic or subacute infection, chronic pain, and delayed infection. In contrast to procedures performed in orthopedic surgery, however, facial plastic procedures are generally clean-contaminated, rather than sterile, and there is a question of whether these implants might be seeded with bacteria at the time of implantation, increasing the risk of infection and biofilm formation. In 2013, Desai et al. [42] published a case report documenting the presence

Table 26.2 Complications seen with different implant materials

Material	Common complications
Silicone [41, 44, 45]	Late onset infection
	Extrusion
	Migration
	Seroma formation
	Chronic peri-implantitis
MedPor [44, 46, 48, 50, 51]	Early-onset infection
	Extrusion
	Fistula formation
	Hypoesthesia
	Palpable or visible implant
	Difficult removal
GoreTex [45, 46, 51, 54]	Infection
	Extrusion
	Hardening of edges on contact with blood
	Palpable or visible implant
	Over augmentation
	Seroma
Mersilene mesh [41, 51]	Increased operative time (secondary to formation of implant in OR)
	Decreased solidity
	Smaller augmentation
	Migration
	Difficult removal

OR operating room

Notes: Most common complications seen with use of different implant materials. Infection and extrusion are often seen with use of silicone, MedPor (Porex Industries, Fairburn, GA), and GoreTex (W. L. Gore & Associates Inc., Flagstaff, AZ), but less common with Mersilene mesh (Ethicon, Somerville, NJ). The porous materials (MedPor, GoreTex, and Mersilene) are more difficult to remove secondary to ingrowth of fibrous tissue. Silicone is easier to remove because of its smooth surface, but this also predisposes it to migration

Table 26.3 Complications seen with different areas of reconstruction

Location	Most common complications
Midface [40, 44, 47]	Inadequate correction or overcorrection
	Malpositioning
	Migration
	Infection
	Extrusion
	Hypesthesia/hypoesthesia
	Facial nerve injury
Orbit [47]	Diplopia
	Enophthalmos
	Infection
	Extrusion
	Migration
	Lacrimal duct obstruction
	Hematoma formation
	Erosion into maxillary sinus
	Lower eyelid deformity
Nose [43, 51, 55, 57, 59, 60]	Aesthetic failure
	Migration Extrusion
Mandible/chin [40, 41, 44, 48, 60, 62]	Inadequate correction or overcorrection
	Asymmetry
	Malpositioning
	Bone resorption
	Infection
	Extrusion
	Hypesthesia/hypoesthesia

Notes: The most common complications seen in different areas of reconstruction within the head and neck. Infection, extrusion, and migration can be seen with all implants, regardless of location. Patients with midface and mandibular/chin implants are more often predisposed to hypesthesia/hypoesthesia given the close proximity of the infraorbital nerve, the inferior alveolar nerve to the implant pocket. Chin implants are at highest risk for bone resorption given the pressure exerted on the implant by the overlying mentalis muscle. Aesthetic failure is commonly seen in areas with thin, taut overlying soft tissue, such as the nose

of biofilm on a nasal GoreTex implant that was removed secondary to chronic infection. This was the first documented account of biofilm formation on nasal implants. This year, a study published by Walker and Toriumi [43] looked more closely at the presence of biofilms on explanted alloplastic facial implants. Four of the seven implants in the study were silicone, and the remaining three were MedPor. Six of the seven were nasal dorsal implants, and the seventh was a midface silicone implant. Only two of the seven implants were free of biofilm formation. For the other two silicone implants, one was found to have focal areas of moderate-to-severe biofilm formation and the others had focal areas with diffuse moderate-to-severe formation. Evaluation of the MedPor implants revealed one with severe biofilm formation on the entire implant and the other two with areas with mild and severe biofilm formation. The authors concluded that both

silicone and MedPor can promote and harbor biofilm formation, but biofilms appear to grow more readily and more densely on implants with rougher surfaces, such as MedPor [43]. However, this does not directly imply that MedPor is more susceptible to infection.

26.6.1 Silicone

Smooth implants, such as silicone, illicit a mild inflammatory reaction at the time of implantation and gradually develop a fibrous capsule around them, causing some authors to be concerned for possible infection and rejection in the near future. The rate of infection with silicone implants hovers around 3.9% with an extrusion rate of 2.9% [44]. Serna et al. [41] hypothesized that infection from silicone implants may be secondary to limited delivery of immunocompetent cells to the implant pocket due to the thick capsule. These implants also experience frequent shifts in position, given the smooth surface and lack of soft-tissue ingrowth, as well as higher rates of bone resorption compared with their softer, porous counterparts. If the implant pocket is too large, the mobile implant can induce persistent seroma or cause chronic peri-implantitis, both of which increase the tendency for extrusion [41, 45]. However, these implants are easily removed (because of the fibrous capsule), have lower costs, and are easy to use [41].

26.6.2 MedPor

MedPor is a high-density porous polyethylene material with a fairly large pore size (100–300 μm) that allows for significant fibrous tissue ingrowth over time [46]. In a literature review by Ferreira et al. published in 2016, a broad spectrum of complications was noted for MedPor implants in facial reconstruction. These complications included diplopia, hypoesthesia, enophthalmos, limited eye movement, hematoma, infection, swelling, pain, displacement of implant, fistula formation, palpability of the implant, scar formation, seroma, and dystopia. It is important to note that their review included the use of solid implants for orbital floor repair following blowout fractures [47]. Ridwan-Pramana et al. [48] looked at the outcomes from 69 MedPor facial implants and found an overall complication rate of 31.8%. This was significantly higher than the rates documented in other studies (which ranged from 10.3 to 26.3%). This is in part because the author included "patient dissatisfaction with appearance" as a complication, thus increasing the overall complication rate by 10%. This was the highest reported complication (10.1%), followed by infection (7.2%), enophthalmos (4.3%), limitation ocular movement (2.9%), diplopia, fistula, dizziness, and pain. The infection rate of MedPor varies from 0.9 to 12.5%, and much of this variation is attributable to the implant location [44, 48, 49]. Given the ingrowth of soft tissue seen with these implants, authors additionally report a more intense fibrotic reaction, and implant removal tends to be very difficult [50, 51]. In augmentation procedures using MedPor, overcorrection must be avoided as this can promote extrusion of material, especially when the skin is under tension and the soft-tissue mantle is damaged. However, if extrusion occurs, the surgeon can remove a part of the implant while leaving the remainder of the implant in situ [51]. Serna et al. also noted that MedPor has scant flexibility, requiring a submergence in warm saline to be able to bend the implant, as well as a larger incision to place it in the soft-tissue pouch without buckling or folding. They recommended fixation of the implant in place with osteosynthetic material, but this increases surgery time, cost of the procedure, and surgical complexity [41].

26.6.3 GoreTex

GoreTex is an expanded polytetrafluoroethylene material with medium-sized pores (10–30 μm), permitting limited fibrous tissue ingrowth [46]. A meta-analysis by Peled et al. showed that the overall complication rate was lower when using GoreTex compared with MedPor or silicone

[52]. However, this does not mean that it is without its own complications, which include infection, extrusion, migration, shrinkage, and scarring [46]. Berghaus [51] revealed that polytetrafluorethylene (a nonexpanded form of GoreTex) failed to show the same biocompatibility and stability in both experimental and clinical models compared with MedPor, and was more susceptible to infection. The average rate of infection noted for mixed facial GoreTex implants is around 2.2% [44]. The expanded form of the material (GoreTex) seems to have better biocompatibility, but when used by Bracaglia et al. [53] to fill smaller defects in secondary rhinoplasty, they found high rates of infection and rejection (10.6%). Yang et al. also reported that the edges of the implant can harden when they come in contact with blood, leading to a palpable implant within the subcutaneous tissue. As a result, gaps form between the bone and the implant, resulting in increased augmentation and a possible formation of hematoma and seroma [54]. In addition, GoreTex tends to be more expensive compared with MedPor, silicone, and Mersilene.

26.6.4 Mersilene Mesh

Mersilene mesh is a woven polyester fiber net material with a pore size of approximately 120 μm, and is used mainly for volumetric correction in the region of the nose and chin. Ingrown connective tissues hold the mesh in place, but can make its removal difficult. The material generally has good outcomes with low complication rates [51]. Serna et al. [41] looked at the postoperative outcomes with several different implant materials used in chin implantation and found that Mersilene mesh has reasonably low infection and resorption rates (2.3%). However, there is increased operating time secondary to production of prosthesis on the spot. Additionally, they showed that the material lacks solidity of other materials, achieves a smaller degree of augmentation, and has slightly higher rates of displacement when compared with GoreTex and MedPor.

26.7 Complications Seen in Particular Implant Locations

26.7.1 Midface

Complications of midface implantation include inadequate correction or overcorrection, malposition, implant migration, infection, extrusion, nerve hypesthesia/anesthesia, and facial nerve injury [40]. For malar implants, the average infection rate is 2.4% and displacement rate is 2.3%. [44] In terms of orbital and periorbital implants, Rubin and Yaremchuk [44] noted high rates of extrusion with smooth implants, such as silicone (3.1%), as well as high overall rates of infection and extrusion, averaging 2.1% and 1.6%, respectively. de Moraes Ferreira et al. [47] found that the most common complication in orbital implantation was diplopia (3.85%), followed by enophthalmos (1.44%) and infection (0.41%). There were many accounts of late complications with orbital implants (especially silicone and GoreTex), as late as 21 years postoperatively. These included infections, extrusion, migration with hematoma formation, migration with lacrimal duct obstruction, erosion into the maxillary sinus, and lower eyelid deformity [44].

26.7.2 Nasal

The anatomy of the nose puts it at high risk of postoperative complications following implant placement. Its prominent position on the face makes it receptive to microtrauma and major blows. The lower two-fifths of the nose is also highly mobile, the soft-tissue coverage is very thin, and there is continuous strain by the host tissue on the implant [55]. When it comes to nasal reconstruction, several different alloplastic materials have been used [56]. GoreTex gained popularity in the 1990s, but before this, silicone, Supramid, Proplast (Vitek, Inc., Houston, TX), Silastic, and Mersilene mesh have been popular options. However, these materials were associated with high rates of

complications, including infection, migration, resorption, extrusion, and difficulty with removal. Today, GoreTex, MedPor, and Silicone are most widely used. The main complications of these materials include aesthetic failure, dislocation/movement of the implant, infection (most commonly with *Escherichia coli*, *Proteus*, and *Staphylococcus aureus*), and extrusion (given the thin nasal skin, mucosal coverage of the implant, its dual-surface exposure and increased risk of subsequent nasal trauma) [43, 44, 57, 58]. Several studies have shown increased complication rates with secondary (revision) rhinoplasty and alloplastic graft use. Jin et al. [59] found a complication rate of 4.6% in revision cases, but only 1.9% in the primary rhinoplasty. Similarly, Godin et al. [60] looked at 309 rhinoplasties augmented with GoreTex implants and found an overall infection rate of 3.2%. However, there was a significant difference between infectious complications in the primary surgical procedures (1.2%) and revision surgical procedures (5.4%).

26.7.3 Mandibular

According to Vuyk [61], the ideal material for chin implantation should be natural in consistency with the ability to be molded well to the mandible that can easily be shaped and secured, but resistant to possible future trauma, and have minimal morbidity. Mandibular implant complications are fairly analogous to those of midface implants, and include inadequate correction or overcorrection, asymmetry, malpositioning, bone resorption, infection, extrusion, and nerve hypesthesia/anesthesia [40]. Several studies have focused on the complications of solid chin implants in particular, and were in agreement that one of the most common complications was dysesthesia secondary to manipulation of the mental nerves. This is seen in 20–30% of patients, more often with anatomical implants and larger implants; most patients resolve their symptoms over a period of several months [41]. Another complication is malpositioning due to incorrect design of the implanted pouch. This is seen more often with an intraoral approach, since a larger tunnel must be dissected to insert the prosthesis. In a study by Zide et al. [62] analyzing 100 cases of complications following chin implantation, they found that almost all problems occurred in implants placed transorally. They asserted that a disruption of the origin of the mentalis muscle from the mandibular symphysis with this approach led to problems with implant positioning and ultimate location as well as the appearance of the labiomental sulcus. However, Gross et al. [63] conducted a similar study looking solely at Mersilene chin implants and found no difference with respect to the complication rates between the transoral and submental approaches. Regarding infection, the average rate with chin implantation was 1.4% [44], with the highest percentage of infections occurring in mandibular angle implants (27%) [48]. One of the most concerning complications of chin implantation is bone resorption. Morera Serna et al. [5] noted that this occurs to some degree with almost every implant, and seems to be secondary to the continuous pressure exerted by the mentalis muscle on the prosthesis, and in turn, by the prosthesis on the external bony cortex. Theoretically, the greater the degree of retrogenia and larger the prosthesis, the greater the activity of the muscle and the greater the bone resorption. Luckily, this bone resorption results in very limited aesthetic impact, and little impact on dental stability. Controversy still remains, however, on whether resorption continues/increases over time or is only seen during the first few months following implantation. A study by Godin et al. in 2003 [64] looked at GoreTex implants and found no signs of resorption with any implant. They found that 2 of the 324 implants became infected and were ultimately removed after failing multiple courses of antibiotics (one was placed transorally, the other submentally). Other complications included patient dissatisfaction with appearance (4/324) secondary to implant location.

26.8 Principles and Techniques for Avoidance of Postoperative Complications

26.8.1 Patient Selection

Appropriate patient selection is a very important part of any preoperative workup and evaluation before facial reconstruction with solid implants. Identifying ideal candidates for allogenic implantation is necessary, and recognizing those with high risk of postoperative complications (e.g., diabetics, immunosuppressed, smokers, patients with a history of facial radiation, those with preexisting septal perforation in the case of rhinoplasty) [56] is important to be able to determine whether the benefits outweigh the risks.

26.8.2 Choosing the Proper Procedure

With respect to midface reconstruction, preoperative examination is crucial in each patient to determine the ideal position of their malar prominence. This will help surgeons delineate whether repositioning of the soft tissue or implant augmentation is required. The soft tissues are then evaluated to determine the type of implant needed: malar, submalar, or combined malar–submalar [40]. In chin augmentation, it is important to recognize that micrognathia, retrognathia, and vertical lower facial height inadequacy are not corrected using implants, but rather masked with these reconstructive techniques. Patient's maxillomandibular occlusion and facial proportions must be evaluated, and surgeons must then present all reconstructive options to patients, including implantation as well as more invasive (but comprehensively corrective) orthognathic and orthodontic procedures. Implantation can also be used as an adjunct to orthognathic procedures [40]. Additionally, genioplasty must also be within the surgeon's armamentarium when orthognathic surgery is not warranted and implants are not ideal for the selected patient.

26.9 Infection

There is no consensus among surgeons regarding the use of antibiotics or intraoperative techniques for the prevention of infection in facial implantation. There are only a few studies that focus on this issue, and most of them are retrospective reviews with inconsistent results. There are no formal controlled trials to confirm the efficacy of these techniques, thus it is difficult to develop evidence-based guidelines. However, there are several common practices employed by surgeons to help reduce postoperative infection:

1. Preoperative and/or intraoperative systemic antibiotics
2. Preoperative soak or impregnation of implant with antibiotics
3. Meticulous aseptic technique
4. Irrigation of the implant pocket with antibiotic solution
5. Watertight intraoral mucosal closure
6. Postoperative oral antibiotics

Brandt and Moore [40], in their review of facial implants, suggest a protocol for reducing infection and implant extrusion, based on existing literature. They advocate meticulous attention to implant sterilization preoperatively, as well as maintenance of wound sterility as best as possible (given that many of these procedures are clean contaminated). Their patients receive perioperative antibiotics, and the implants are submerged in an antibiotic bath (50,000 U/I bacitracin saline solution) before placement. Intraoperatively, the implant pocket is irrigated with an antibiotic solution, and patients with intraoral incisions perform postoperative 0.12% chlorhexidine gluconate mouth rinses to reduce risk of postoperative infection. Moreover, Morera Serna et al. [41], in their review of chin implants, advocate the use of careful aseptic technique during implant handling and intraoperative steps, the soaking of implants in antibiotic solution before use, and the airtight closure of intraoral mucosal incisions to prevent saliva entry into the implant pocket. Similarly, Niamtu [64] published a

technical note on cheek and midface implantation based on his operative experience. He suggests an intraoperative irrigation of the implant pocket (using 300 mg clindamycin in 30 mL sterile water) as well as postoperative antibiotics and a steroid taper. His overall infection rate was 1.5%. In the realm of augmentation rhinoplasty, Dong et al. [65] emphasized the importance of sterile technique, limited air exposure of implant and contact with other materials, soaking of implant in dexamethasone/gentamicin solution before placement, and the use of postoperative oral antibiotics. Their overall infection rate was 1%.

26.10 Intraoperative Antibiotics

Intraoperative antibiotics are almost unanimously given in head and neck surgical procedures, including facial reconstruction with implants. This is a known important component of infection prevention and a part of routine evidence-based practice. In the study by Ridwan-Pramana et al. [48] looking at the complication rates of MedPor implants, all patients received pre- and/or intraoperative antibiotics; however, the implants were not soaked in antibiotics before implantation, and they did not disclose whether patients received a course of postoperative oral antibiotics. The overall infection rate was 7.2%, which is on the higher end of the range documented in the literature. Some authors also suggest a short course of intraoperative and postoperative intravenous (IV) and oral corticosteroids to minimize the body's inflammatory response to the implant occurring in the immediate postoperative period.

26.11 Preoperative Antibiotic Soak or Impregnation

The use of alloplastic material preimplanted with antibiotics has been described in orthopedic surgery for years. Several different techniques have been noted in the literature attempting to infiltrate selected antibiotics into porous alloplastic implants during head and neck reconstruction, including just dipping the implant into the antibiotic, completely immersing the implant in the antibiotic, and, finally, applying negative pressure to create suction and infiltrate the implant with the antibiotic. In 2009, Keefe and Keefe [66] published an in vitro study looking at the efficacy of the above techniques in preventing *Staphylococcus* growth on GoreTex and MedPor implants. They found that the negative pressure technique was most effective, with a statistically significant advantage over the other two techniques. This information is beneficial, but there is still a paucity of objective evidence in the literature suggesting that these intraoperative techniques are effective for preventing postoperative infection in vivo. In a prospective study by Niechajev [57] looking at complications following MedPor implantation, all implants were impregnated with cloxacillin using a suction infiltration technique before implantation. The author also reports the use of densely placed sutures to create a watertight seal, preventing contamination of the implant pocket with microorganisms, as well as a 7-day postoperative course of flucloxacillin. Their overall infection rate was 2.8%; all cases were nasal reconstructions. Several other authors are proponents of preoperative antibiotic soak or impregnation, especially during rhinoplasty [61, 65–71]. Although the rates of infection are relatively low in these studies, none of them are formally controlled trials, and there is little objective evidence that the above techniques are effective. Furthermore, in 2003, Godin et al. [72] investigated the complications following GoreTex chin implantation and found no difference in the infection rate between preoperative antibiotic soaking (gentamicin or bacitracin) and no preoperative antibiotic soaking. They did not discuss the use of perioperative systemic antibiotics or postoperative oral antibiotic prophylaxis.

26.12 Postoperative Antibiotic Use

Regarding postoperative treatment with antibiotics, some authors are strong proponents of a short (5–7 days) prophylactic course, while others express it is unnecessary when it comes to changing the postoperative infection rate. A study by Villarreal et al. in 2002 [71] looking at the complications following orbital floor reconstruction with MedPor implants found no difference in the infection rates among patients who received amoxicillin–clavulanate, those who received clindamycin, and those who received no antibiotics (p = 0.958) postoperatively. All of their patients received a pre- or intraoperative dose of IV antibiotics. Their overall infection rate was 12.5%. Another study by Shadfar et al. [56] evaluating the complications following MedPor implants for nasal reconstruction highlighted the use of perioperative and postoperative antibiotics (7-day course). They did not use preoperative antibiotic soaking for their implants, and their overall infection rate was 2.9%.

26.13 Implant Shaping

Pre- or intraoperative shaping of the implant is also an important step in the prevention of complications. Yang et al. described a modified carving technique for GoreTex to produce better curvature and a flush fit in the implant pocket. This technique involves the creation of multiple V-grooves on the posterior side of the implant. The center V-groove functions as an anchor for immobilization and positioning adjustment, and the margins of the implant taper imperceptibly into the bone [16]. Another modified shaping technique was described by Wong [73] for forehead implantation using a silicone. The author created corrugated edges and central perforations to permit smoother contour and fixation of the implant with minimal capsule contraction.

26.14 Surgical Approach

In terms of surgical approach for implant placement, both external and intraoral techniques have shown to be efficacious [40]. Several studies have looked at the risk of postoperative complications between these two approaches, and the results are inconsistent. Zide et al. [62] found that most of their complications following chin implantation occurred in cases of intraoral approach. They postulated that the increased complication rate was due to the release of attachments of the mentalis muscle as well as increased risk of wound contamination because of breach of the intraoral mucosal barrier. However, Gross et al. [64] and Godin et al. [72] looked at similar outcomes following chin implant placement and found that the risk of postoperative complication was equivocal between these two approaches. Regardless, it is accepted that intraoral incisions must be closed meticulously to ensure a watertight seal, preventing the leak of saliva into the implant pocket. Aynehchi et al. [74] published a study in 2012 advocating a vertical intraoral approach for chin augmentation. The authors argued that this approach preserved the attachment of mentalis muscle (reducing the risk of implant movement and malpositioning) while achieving augmentation results and complication rates equivalent to other approaches. However, Vuyk [61] favored a submental approach over an intraoral approach for silicone chin implant placement, as he endorsed lower infection rates with this approach and avoided suture line irritation. The submental approach does have a risk of small scar formation which is avoided with intraoral approach.

26.15 Intraoperative Techniques and Implant Placement

26.15.1 Chin Implantation

Irrespective of the surgical approach, a tightly dissected tissue pocket is critical for implant fixation. It is still debated whether this pocket

should be subperiosteal or supraperiosteal [74]. Additionally, some authors (e.g., Godin et al. [72]) will leave the midline mandibular periosteum down, but elevate the subperiosteal tunnels to accommodate for the implant arms. A study by Vuyk [61] looking at silicone chin implants supported a subperiosteal insertion. The author emphasized caution when making the implant pocket, the right size, to prevent shifting (if too big) and buckling (if too small). He noted that the pocket should be kept below the labial mental sulcus, and effort should be taken to maintain fibrous attachments of soft tissue to the lower border of the mandible through the majority of the pocket to prevent inferior displacement of the implant toward the neck. Yang et al. [54] also supported subperiosteal insertion for the same reasons. In submalar implantation, an even more limited subperiosteal dissection is performed to avoid upward displacement of the implant during wound contracture. Fixation of the implant with suture or screws also helps prevent migration [40].

26.15.2 Mandibular Implantation

Brandt and Moore [40] detailed their intraoperative technique for placing mandibular implants, and endorsed the use of a tight subperiosteal pocket with rigid fixation of the implant using screws (or suture). However, one must ensure that the implant is not buckled within the tight pocket before fixation. In the prevention of mental nerve hypesthesia, knowledge of the anatomy is crucial, with the placement of the fixation screws above the mandibular foramen or along the posterior aspect of the ramus to avoid damage to the inferior alveolar nerve. Looking at lateral mandibular angle implants, the fixation is highly important, as these implants have a tendency for rotational, vertical, and horizontal displacements. Yaremchuk [50], on the other hand, favored a wide subperiosteal exposure of the area to be augmented, as this would enable accurate positioning of the implant and a tension-free closure. They felt that screw fixation was adequate in preventing movement of the implant. Additionally, it obliterates any gaps between the implant and the recipient bed. Elimination of movement is thought to hasten fibrous incorporation, minimize capsule formation, and decrease the risk of bone resorption. The combination of wide exposure and screw fixation also allows for in-place contouring of implant at the recipient site, eliminating the perceptible border between implant and recipient bed.

26.15.3 Midfacial Implantation

In terms of midfacial implantation, Brandt and Moore highlighted their intraoperative technique, noting the creation of a subperiosteal pocket over the region for implantation, which typically extends to the inferior orbital rim superiorly and masseteric tendon laterally. The authors noted that a dissection over the zygomatic arch and malar eminence should remain in the subperiosteal plane, with particular stress on proceeding with caution to avoid injury to buccal and frontal branches of the facial nerve. Additionally, knowledge of the anatomy is crucial as care must be taken to avoid infraorbital nerve injury [40].

26.15.4 Nasal Implantation

With the endonasal technique, success depends on a precise size of the implant pocket [57]. Dong et al. [65] highlighted the importance of decreasing the surface tension on the prosthesis and stabilizing the position of the prosthesis in GoreTex rhinoplasty. They provided the following guideline for prosthesis placement: After creating the prosthesis, there should be no sharp angles, especially in the tip region. If the cross-sectional area of the tip of the prosthesis is smaller than 4×4 mm^2, increased pressure on the skin may induce ischemia and ulceration; therefore, in such cases, they recommend the placement of autogenous cartilage to cover the tip of the prosthesis. The width of the columellar portion of the prosthesis must be trimmed to less than half the width of the columella to be free of tension in its desired location and prevent distortion of the

implant in the dorsal tunnel. In terms of preventing migration, which can cause pressure on the local tissue, the authors support using an external rhinoplasty approach with a v-shaped incision, allowing broad exposure for implant placement. A silicone sizer is used to determine the appropriate size for augmentation, which prevents frequent placement of the implant into the pocket and subsequent distortion. The pocket must be dissected 1 cm wider than the prosthesis, as too small a pocket size may lead to bending or distortion, increasing surface pressure on the adjacent tissue (Table 26.4).

Table 26.4 Principles for avoidance of postoperative complications with solid facial implants

Patient selection	Identification of poor implant candidates [56]
	Diabetes
	Immunosuppression
	Smokers
	Preexisting septal perforations
	Thorough preoperative examination [40]
	Repositioning versus augmentation
	Corrective versus masking procedures
	Type of implant needed
Infection [40, 41, 64, 72]	Pre- and/or intraoperative systemic antibiotics [48]
	Preoperative soak or impregnation of implant with antibiotics [57, 61, 64–70]
	Meticulous aseptic technique with implant handling, patient preparation, field sterility
	Irrigation of implant pocket with antibiotic solution
	Watertight intraoral mucosal closure
	Postoperative oral antibiotics [56, 71]
Implant shaping	GoreTex: Multiple V-grooves for anchoring, tapering of margins [54]
	Silicone: Corrugated edges and central perforations to permit smoother contour and better fixation [73]
Surgical approach [40, 60–63, 74]	Intraoral versus extraoral
Intraoperative technique	Chin implantation [40, 59, 61, 62, 74]
	Subperiosteal dissection
	Appropriate tissue pocket size
	Location of pocket
	Fixation of implant
	Mandibular implantation [40, 50]
	Tight subperiosteal pocket versus wide exposure
	Fixation of implant
	Location of screw placement
	Midface implantation [40]
	Subperiosteal pocket
	Location (extend to infraorbital rim and masseteric tendon)
	Subperiosteal dissection over zygomatic arch and malar eminence
	Nasal implantation [57, 64]
	Precise implant pocket size
	Open V-approach
	Use of silicone sizer
	Use of cartilage graft over tip of prosthesis

Notes: Common principles and techniques for the avoidance of complications associated with solid facial implants. Careful patient selection and proper choice of operative procedure are both important preoperative considerations to ensure implant success. Additionally, techniques for infection control, including preoperative or intraoperative systemic antibiotics, sterile technique, soaking, or impregnation of the implant with antibiotic, irrigation of implant pocket with antibiotic, meticulous closure, and postoperative oral antibiotics are used by many surgeons to reduce complication risk. Preoperative implant carving, proper selection of surgical approach, and use of certain intraoperative techniques (specific to implant location) can also help reduce complications seen with solid implants

26.16 Conclusions

Approaches from regenerative medicine with respect to medical implants are somewhat novel concepts. Such approaches and methods are a new area in regenerative medicine as well. The objective of this chapter was to inform readers about the possibilities of learning from and adopting from concepts that are still in their nascent stages of development in tissue engineering and regenerative medicine to achieve total and permanent integration between silicone

implants and surrounding biological tissues. Allogenic implants have become an increasingly popular alternative to autologous grafts in facial reconstruction. These materials have shown to be advantageous with respect to availability, donor-site morbidity, operation time, and cost. However, there are risks and complications associated with the use of these implants, many of which depend on the type of material used and the location of implantation. These include the use of meticulous aseptic technique, pre-/intra-/postoperative antibiotic administration, soaking or impregnating the implant in antibiotics, careful patient selection, and procedure selection, as well as certain surgical approaches and intraoperative techniques based on implant type and location. Larger, controlled trials are still needed to determine the efficacy of these techniques in reducing complication rates with the use of solid facial implants. It is possible that in the future, implants will be designed as a single unit with proactive delivery systems that simultaneously provide long-term access to the tissues. Artificial implants will continue to increase in complexity and number, while their failure rates will remain basically unchanged unless existing and novel methods are developed to better integrate them with the human body.

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Part V

Regenerative Plastic Surgery

Microdeformational Wound Therapy

27

Douglas Helm and Dennis P. Orgill

27.1 Introduction

Chronic and complex wounds are increasing healthcare burdens both domestically and internationally. Simple wounds heal without significant intervention; these are a commonly planned surgical incisions or linear lacerations from minor trauma. The cascade of wound healing from the inflammatory phase, proliferative phase, and maturation phase all occurs in sequence to reconstitute the skin and soft tissue barrier.

The elegant natural mechanisms of wound healing can be interrupted or delayed by a variety of intrinsic and extrinsic factors. Insufficient blood flow to the wound, which frequently occurs because of peripheral arterial disease, interrupts transport of inflammatory cells, oxygen, and nutrients and finally new progenitor cells. Venous insufficiency, where blood outflow is blocked, also reduces blood flow to the wound and also causes extravasation of red blood cells. Poor glycemic control from diabetes has several proposed mechanisms that inhibit wound proliferation further delaying wound healing. In addition, uncontrolled diabetes leads to peripheral vascular disease, stroke, kidney failure, and visual disturbances. Lymphatic disruption leads to chronic edema, blocking transport because of increased diffusion distances, and also inhibits wound healing. Significant trauma or pressure injury of soft tissues causes necrosis of the skin, underling fat, and other deeper structures such as the fascia, muscle, and bone. The presence of this necrotic tissue burden delays the start of tissue proliferation.

Complex wounds are often delayed in their healing because of an acute or chronic infection, poor blood supply from peripheral arterial or venous disease, malnutrition, lymphatic injury, or extensive necrotic tissue in the wound. With an aging population, there is increasing prevalence of diabetics and peripheral vascular disease. In addition, obesity has dramatically increased in developed nations and in certain areas of the world, and penetrating trauma continues to increase. The challenge of treating these wounds is mounting both from a financial burden and provider burden to treat these patients.

Conventionally, approaching a complex wound requires a multitask approach. Clinicians identify and treat ongoing nicotine use, malnutrition, glycemic control, and peripheral edema, debride the necrotic tissue, and treat the wound infections. Reducing a person's body mass index and treating peripheral vascular disease can improve wound healing over time. Nonmodifiable factors such as age, the mechanism of injury, and the previous exposure to other

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treatments such as radiation therapy are factors that continue to pose significant challenges. For patients where risks are not able to be modified or changed, advanced wound healing methodologies can be an enormous help to patients in healing the many complex wounds seen today. New methods in reconstructive surgery can also be extremely helpful.

The surgical management of the necrotic tissue is the mainstay of the initial treatments of wound care. Excision of devitalized skin, fat, muscle, and bone to healthy bleeding is needed to allow granulation tissue to form and reduce the wound bio-burden, thereby reducing the risk of infection. In clinical situations that primary wound closure is not possible, providers have turned to microdeformational wound therapy (MDWT) which is becoming the standard of care.

MDWT was first described in the early 1990s by Dr. Argenta and Dr. Morykwas and was originally referred to as negative-pressure wound therapy (NPWT), Vacuum-Assisted Closure (VAC), and subatmospheric pressure treatment. Currently, there are many commercial products using these principles. We use the term MDWT to refer to a suction device that distributes reduced pressure to a wound through an interface material that causes microdeformations of the wound surface such as a polyurethane open-pore reticulated foam. This interface material that contacts the wound is covered with a semi-occlusive dressing and connected to a suction source. The first animal studies of this device showed dramatic wound healing with respect to granulation tissue or proliferation of new cells in the wound bed [1]. This concept was first commercialized under the trademark of Vacuum-Assisted Closure (VAC) by the KCI, San Antonio, TX, USA (Fig. 27.1).

The concept of applying controlled stable suction to wounds is not a new concept. For generations, surgeons have known about the importance of closed suction drainage of anatomical spaces. Chest tubes draining the pleural cavity keep the lung approximated to the parietal pleura and reduce the possibility of an empyema to form. Using suction drainage, catheters effectively treat mastectomy defects and have become a standard treatment. These catheters apply subatmospheric pressure, reduce the space, and collapse the surgical cavity [2]. MDWT is a related concept

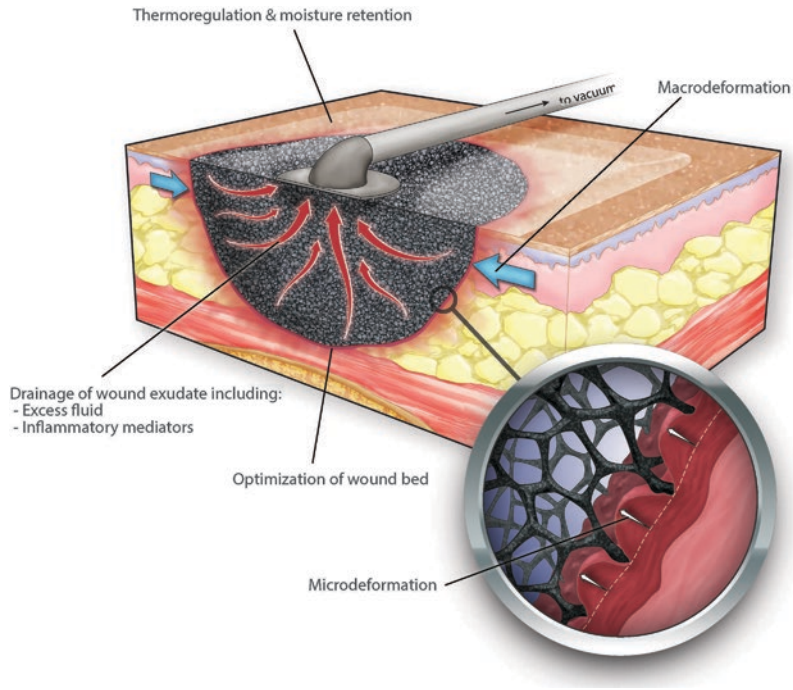

Fig. 27.1 The four primary mechanisms of action of MDWT. Reprinted with permission from Elsevier. In:. Huang **C,** Tripp L**,** Lauren RB**,** Dennis PO (eds), Effect of negative pressure wound therapy on wound healing, Current Problems in Surgery, Vol 51, edition number 7, Copyright 2014 p. 303

whereby a closed space can be drawn together by a collapsible foam where the surface of the foam acts as thousands of small suction cups applied to the wound surface.

Several other advances have improved the clinical application of this technology including portable devices that are both mechanically and electrically powered. The comparative effectiveness of a mechanically powered MDWT and an electrically driven systems is similar. The most widely used mechanical device is the Smart Negative Pressure (SNaP) Wound Care System (SNaP, Spiracur, Inc., Sunnyvale, CA, USA) which translates the mechanical force of a spring into continuous suction, thereby eliminating a costly electromechanical system [3].

Another recent advance has been the instillation of fluid into the wound bed through the foam followed by active suction. Fluid such as saline or diluted hypochlorous acid (Dakin's solution) has been used in these devices and may be effective at treating infected wounds.

Table 27.1 lists the indications and contraindications of the use of this mechanical wound treatment. Although widely applied to a variety of wounds, careful consideration of its use is needed to minimize complications.

Although the evidence for MDWT is not robust, numerous studies have reported the clinical effectiveness of this wound healing modality as compared to other conventional therapy and are discussed later in this chapter. There is widely appreciated evidence that MDWT improves granulation tissue formation, increases the rate of healing, and reduces cost of treatment [4–7].

Table 27.1 Indications and contraindications of use for microdeformational wound therapy (MDWT)

Indications	Contraindications
Chronic wounds	Directly on exposed vascular structures
Acute wounds	Untreated osteomyelitis
Traumatic wounds	Non-enteric fistulas
Subacute wounds	Necrotic wounds
Dehisced wounds	Malignancy in the wound
Partial thickness burns	
Ulcers including diabetic, pressure, or venous insufficiency	
Flaps and grafts	

27.2 Basic Principles of MDWT

The principle behind MDWT is the application of closed suction to a wound bed via an interface material. There are several mechanisms of actions that work in concert to promote and sustain wound healing. The first principal action is macrodeformation of the wound or contracting the wound together. With application of 125 mmHg of suction applied to an open-foam material, the foam contracts up to 80% of its initial volume. The wound edges are mechanically drawn together, assisting with wound closure. The physical stretch of the soft tissue causes a slight reduction in blood flow to the wound peripheral edges and causes a reaction stimulation of cell proliferation and angiogenesis through the HIF-1 alpha/VEGF pathway [8, 9].

The second principle is microdeformation that occurs at the interface of the porous foam material and the wound bed. With suction applied to the porous wound interface material, the wound bed is drawn into the material causing oscillating deforming patterns to the wound. This micromechanical oscillations cause a proliferating cellular response. This effect has been similarly seen in the peso-electric effect involved in bone regeneration. A correlation has been demonstrated between the degree of microdeformation and proliferative wound growth [10–12].

The third critical principle is the exudative management. As the suction is applied throughout the wound bed, excess fluid which has been often shown to be high in catabolites and inflammatory mediators including matrix metalloproteinases decreases in the wound bed with MDWT. An important distinction with MDWT is that bacterial load does not decrease with this therapy [13, 14].

The fourth impact is creating a sealed, thermoregulated, and moist environment preventing the regenerating wound bed from damaging desiccation [15].

Additional secondary effects are seen with the interface material between the wound bed and the suction which has been shown to be a critical component for successful wound healing. This material is often made of polyurethane foam, an inert but stable material that causes minimal reaction. This prevents external contaminants and desiccation of the wound. When wounds are exposed to air, the body forms a defensive eschar which protects the wound but also slows the re-epithelization process and provides a protective barrier for proliferating bacteria. The reticulated open-pore foam has been examined as a factor that impacts granulation tissue formation. The larger the foam size, the greater granulating tissue response has been seen in the wound bed. This is likely related to the increased deformation or strain on the wound bed [16].

27.3 Management of Chest Wounds with MDWT

Wounds of the thorax arise from many different types of injuries and diseases. The most common is cardiac surgery where the sternum is split along the midline to gain access to the heart and great vessels. Although infrequent, chest wounds are a serious complication with a reported mortality ranging from 11 to 35%. In 2011, in the USA, 213,700 patients had a cardiac surgical procedure leading to an estimated 5000 chest wound infections [17]. Cardiac surgery is becoming a less common surgery in the healthy and middle-aged patients and becoming more common in the elderly and patients with comorbid conditions such as ongoing tobacco use, poorly controlled diabetes, and end-stage coronary artery disease that is refractory to percutaneous stenting. Consequently, patients who present with sternal infections following cardiac or chest surgery are older and often more infirmed [18]. Treating this quantity and morbid complication is a challenge.

Other causes of thoracic wounds include penetrating trauma from blast injuries or stabbings. Infections such as tuberculosis or aspergillosis are chronic health problems of developing nations and may lead to draining open pleural wounds [19].

MDWT is an adjunct to complex chest wounds. The principles of treatment are similar to all complex wounds and include stabilization of the wound by debriding the devitalized and infected tissue including the skin, fat, and underlining sternal cartilage and bone. Foreign material including sternal wires and suture material should all be removed as they become a nidus for chronic infection. MDWT can stabilize the sternum, provide a protective and thermal barrier, contract the wound, and manage wound exudate. In clinical situations where there is an uncorrected coagulopathy, MDWT should be delayed until bleeding is stopped as the foam is very efficient at evacuating blood and can result in significant blood loss. Exposed critical structures such as the heart muscle, the great vessels, and the bypass grafts need to be covered by a nonadherent dressing such as Adaptic (Johnson and Johnson, Somerville, NJ, USA) or Xeroform (Medtronic, Minneapolis, MN, USA). These devices should be used in a setting that allows efficient access to operating facilities should excessive bleeding occur.

Conventional reconstruction of the sternum includes pectoralis muscle flaps, vertical rectus abdominal flaps, and omental flaps to fill in the soft tissue defects from the infections. MDWT is an alternative with many benefits including ease of use and elimination for additional complex reconstructive surgery especially in patients already severally debilitated from an infection following cardiac surgery. In addition, MDWT can be used over a longer time period and often can close these wounds over a period of weeks in patients that are not operative candidates [20].

Chest infections may be treated in several traditional manners, but MDWT provides a useful adjunct or primary method of treatment following the principles of wound care.

27.4 Open Abdominal Wounds

Open abdominal wounds have become more common with a better understanding of abdominal compartment syndrome. The abdomen is a cavity containing all the enteric components and

works as a compartment surrounded by a complex layer of fascial layer which is surrounded by abdominal wall and lumbar musculature with an overlying layer of fat and skin. The contents of the abdomen are fragile and kept isolated from the external environment. Exposed for a prolonged time to air, intestinal components may desiccate and breakdown forming fistulas and infections.

Abdominal wounds can communicate through the musculature and fascia to the visceral organs. The causes of open abdominal wounds vary but often occur after emergency life-saving surgery or complications of a planned surgery. In penetrating trauma with injury to one or more visceral structures, the abdomen may be left open to allow easy access to the abdomen for monitoring or for reexamination of the abdomen days after the initial surgical management. Commonly performed in battlefield medicine, damage control surgery repairs the open injuries for stabilization by packing the wounds to control blood loss or sealing any open intestinal injuries. A small percentage of all trauma patients who undergo an exploratory laparotomy are left with exposed visceral components with a plan for staged closure. The delay in closing the abdomen may be due to prolonged septic shock, bowel edema, or ongoing infection. In situations of a necrotizing soft tissue infection, debridement of the soft tissue, muscles, and fascia may be required to control these life-threatening infections. Serial debridements with progressive closure are a strategy commonly used for large open abdominal wounds.

Although life-saving in many situations, leaving the abdomen open leads to retraction of the abdominal wall fascia and skin edges. The lateral pull of the oblique muscles pulls the abdominal wall fascia and the rectus abdominis muscles laterally and is commonly referred to as "loss of domain." With time, moving these muscles to the midline may be challenging and require releases of the muscles in order to restore fascial and musculature coverage over the visceral organs.

A temporary abdominal closure is needed to prevent evisceration and desiccation, protect the visceral organs from damage or adhesions, provide thermoregulation, combat the contracture of the fascia and skin, manage exudates and minimize dressing changes and needs to be easily changed, and provide quick access to the open wound for inspection. Several types of temporary wound moist dressings, Bogota bags, originally described by surgeons in Bogota, Columbia, involve placing a nonadherent inert covering over the open visceral compartment. Originally described as suturing a sterilized 3 L urology saline bag to the skin edges, variations of this temporary abdominal closure are described such as applying a sterile X-ray drape, silastic drape, or any sterile nonadherent material to cover the abdominal contents. In contrast, a vacuum pack is a variation of a Bogota bag but with applied suction. The dressing includes a first layer of fenestrated nonadherent polyethylene sheet that is in contact with the bowels, layered with surgical towels with continuous suction applied from closed suction drains which is sealed with a final layer of iodoform adhesive dressing. A Vacuum Pack controls the abdominal fluid exudate and provides a thermoregulation and barrier protection. A further variation of the Vacuum Pack utilizes the open-pore foam to apply subatmospheric pressure and holds the tissue skin edges and underlining fascia in a contracted position. Marketed as VAC Granufoam (KCI, San Antonio, TX, USA), this dressing applies the open-pore foam to a nonadherent dressing that is in contact with the bowels. Numerous studies have shown the safety and clinical effectiveness of applying MDWT to this clinical problem. In addition, several studies have shown faster abdominal closure, lower complication rates, decreased length of ICU stay, and decreased cost of treatment with open abdomens treated with MDWT compared to other temporary abdominal closures [21–23].

27.5 Diabetic and Pressure Ulcer Wounds

Lower extremity wounds including diabetic ulcers of the foot and toes present an enormous medical challenge to treat. Often these patients have modifying factors of diabetes and peripheral vascular disease and wounds that need to be

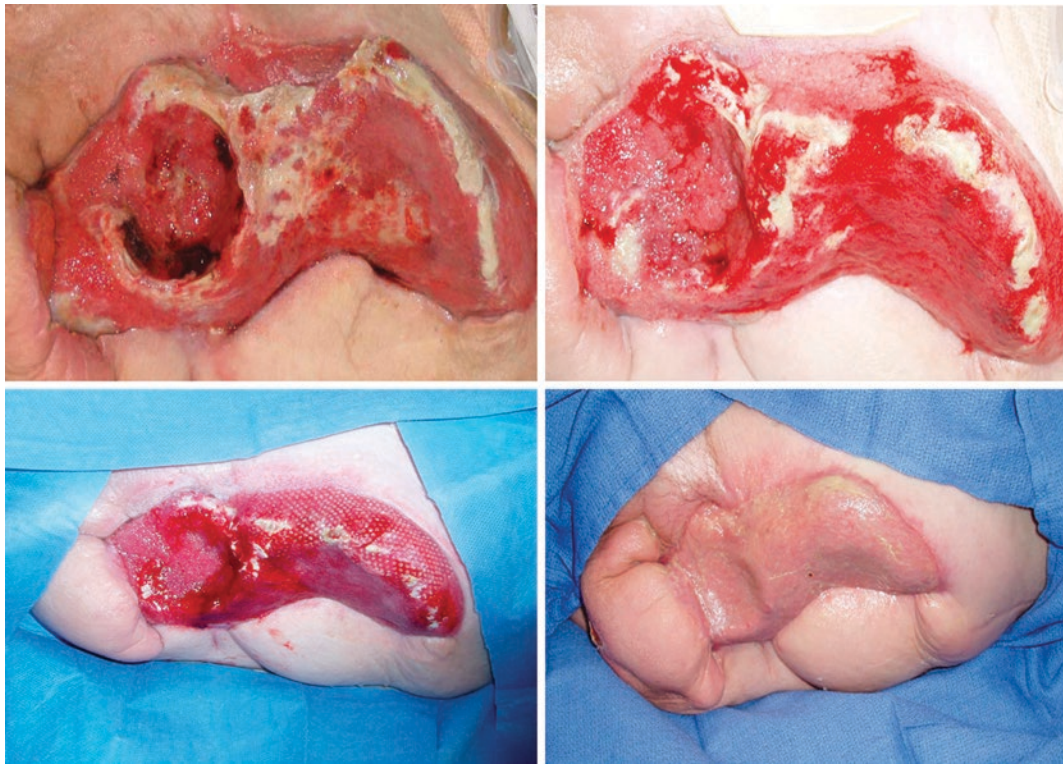

Fig. 27.2 A 52-year-old woman with severe peripheral vascular disease presented with septic shock, perforated sigmoid colon tracking to the pelvis, and gas gangrene of the left leg ultimately requiring left hip disarticulation. The open hip wound was treated with sharp debridements and negative-pressure wound therapy for 6 weeks to prepare the area for successful skin grafting

debrided of necrotic or infected tissue. Traditional healing methods often lead to prolonged healing times, costly for both the patient and providers. Treating diabetic wounds with MDWT closes wound nearly twice the rate compared to traditional therapies. The rate of mean reduction in wound size is greatly increased with MDWT as opposed to moist dressing changes with the most significant change in depth. In a similar evaluation of diabetic wound, MDWT prepared wounds faster for delayed surgical closure as compared to traditional topical wound therapy [24]. Similarly, open acute traumatic wounds or chronic pressure ulcers can also be a challenge to heal for a variety of medical reasons. MDWT accelerates the closure of these wounds as well with the greatest rate of wound volume [25]. MDWT has revolutionized the treatment of diabetic, acute complex, and chronic ulcers but does not eliminate the need to address patient's underlining diseases and the need for sharp excisional debridement (Fig. 27.2).

27.6 Safety Concerns with MDWT

Additional precautions are needed with MDWT that are similar to other wound care products. This wound device does not debride wounds and should only be applied when the wound bed is free of infection, devitalized, or necrotic tissue. Removal of all the foam material during a dressing change is critical; retained foam will prevent a wound from healing. Careful documentation and wound inspection is needed to prevent this error. Uncontrolled hemorrhage has been reported as a complication when NPWT is applied directly to blood vessels. The open-foam material may erode into vital structures leading to death or permanent disability.

27.7 Future Directions of MDWT

MDWT should be viewed as a catalyst to wound healing that requires the essential care components of controlling other morbidities, infection, and necrotic wound burden and optimizing nutrition and other factors. MDWT should be applied as an adjunct to wound healing in a coordinated plan. Although MDWT has revolutionized how clinicians approach complex problems, this technology will continue to evolve as clinicians adapt this to other problems. The limitation of this technology is that it relies on the generating new tissue from the wound itself, and the future may include using this concept to populate the wound bed initially with artificially generated progenitor cells. In addition, the role of imbedded growth factors into the foam substrate or creating a bioactive foam creates exciting possibilities for the future.

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Clinical Treatment of Hypertrophic Scars

28

Rei Ogawa

28.1 Introduction

Hypertrophic scars are caused by abnormal healing of injured skin. Common causes of injury are trauma, burn, and surgery. The scars are red and elevated and have an unappealing appearance. Moreover, they associate with intermittent pain, persistent itching, and a sensation of contraction. The inflammation in the scars is continuous and local, being mainly found in the reticular layer of the dermis of the skin [1]. In this reticular layer, there are also accelerated angiogenesis and collagen accumulation. These features suggest that the cause of hypertrophic scars is aberrant wound healing in the damaged reticular layer of the dermis. This implies that more superficial damage would not elicit hypertrophic scars. Indeed, a clinical study on human volunteers [2] showed that cutaneous injury must reach the reticular layer before it results in inflammatory scar formation.

In general, scars that eventually become hypertrophic exhibit continued inflammation after injury, and the scar starts adopting a red and elevated appearance after 2–3 months. These redness and elevation continue for more than 1 year. These features generally decrease within 3–5 years (Fig. 28.1). However, if the injury is on high-skin-tension sites such as joints, the inflammation persists for much longer, and scar contractures develop. In this case, surgery is needed to release the contractures. These observations suggest that local mechanical forces play a particularly important role in the development of hypertrophic scars. Indeed, several lines of evidence support this notion.

28.2 Preventing Hypertrophic Scar Development

A burn wound that heals in less than 10 days has a 4% risk of developing into a hypertrophic scar, whereas a burn wound that takes 21 days or more to heal has a 70% or greater risk of developing into a hypertrophic scar [3]. This means that a deep skin injury that extends to the reticular layer of the dermis needs time to heal; however, if inflammation continues for a long period, then the risk of developing a pathological scar increases. Histopathological examination of pathological scars shows that while their epidermis and the papillary layer of the dermis are almost normal apart from minor inflammation, the reticular layer shows strong inflammation with multiple blood vessels and greater collagen accumulation. Thus, to prevent the formation of pathological scars, it is essential to ensure speedy wound healing.

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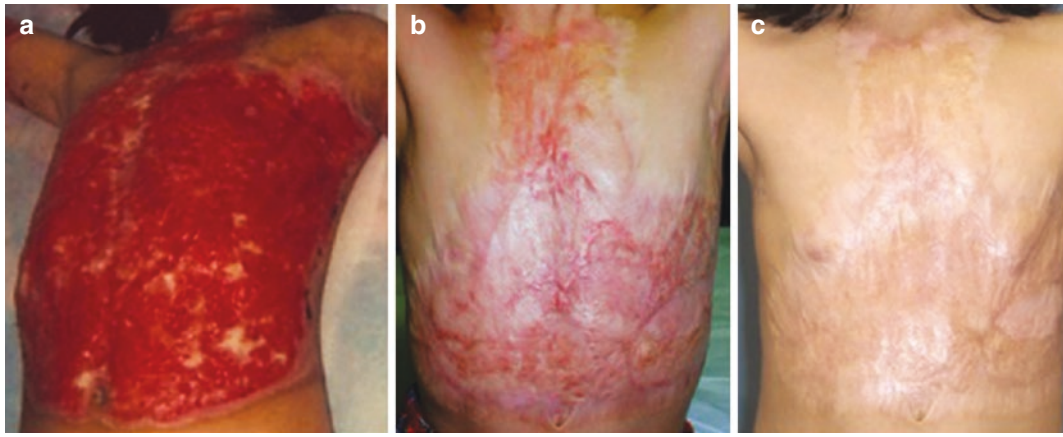

Fig. 28.1 Naturally healing hypertrophic scars. (**a**) Granulation tissues immediately after the burn wounds were sustained. (**b**) Hypertrophic scars 2 years after the burn wounds were sustained. (**c**) Mature scars 5 years after the burn wounds were sustained. Hypertrophic scars without scar contractures improve gradually during the process of scar maturation, despite minimal noninvasive treatment being applied. However, textural improvements take longer to manifest. Reprinted with permission [7]

Since stretching wounds can provoke inflammation of the dermis, wounds should be stabilized as soon as the exudate from the wound surface has stopped. The epidermis and dermis differ markedly in terms of their wound-healing rates. In the case of sutured wounds, the epidermis can regenerate within 7–10 days, leading both the patient and the physician to believe that the wound has healed completely. In fact, it may take 3 months before the dermis recovers more than 90% of its normal strength. Thus, prolonged external mechanical support using tapes, sheets, and/or garments is recommended for scar prevention. This is supported by our study, which showed that silicone gel sheets reduce the tension on the wound site [4].

Silicone tape is better than paper tape as it avoids the epidermal injuries caused by repeated taping [5]. Moreover, silicone tape keeps the scar surface moist. These tapes can be kept in place until they detach naturally. The patient does not need to change the tape after taking a bath/shower. In our experience, patients generally keep silicone tape in place for about 1–2 weeks. The exception is in summer: perspiration can reduce tape adherence.

If a patient has a clear history of pathological scars, then stabilization tapes should be exchanged for steroid plaster/tape about 1 month after epithelization has occurred. Steroid tape has been used to decrease inflammation of hypertrophic scars; this practice is particularly common in Japan and several other countries [6]. Flurandrenolide tape (Cordran® tape), fludroxycortide tape (Drenison® tape and Haelan® tape), betamethasone valerate topical patch (Betaflam®), and deprodone propionate tape (Eclar® plaster) are available worldwide. These steroid tapes/plasters should be changed every 24–48 h and should be cut so that they just cover the wound, with minimal attachment (if any) to healthy skin (unpublished data). Since these tapes differ in terms of the strength of the steroid, the most appropriate tape/plaster should be selected on a case-by-case basis.

Compression therapy of skin wounds also appears to prevent hypertrophic scar development. We speculate that it acts by occluding the blood vessels in the scar, thereby inhibiting the inflammatory signals coming from the blood vessels. An additional mechanism may be that it stabilizes the wound, thereby decreasing inflammation.

28.3 Treatment of Hypertrophic Scars

28.3.1 Surgery

Surgical treatment itself can result in the recurrence of hypertrophic scars, which are then often much bigger than the original lesions. Thus, unless the scar is a minor hypertrophic scar, the decision to surgically remove a pathological scar should be made very carefully. To reduce the risk of recurrence, it is also advisable to use particular surgical techniques, namely, Z-plasties, full-thickness skin grafting, and local/regional flap transfer [7–9].

Zigzag sutures, including Z-plasties, are good for releasing linear scar contractures and tensions [10]. A major benefit of Z-plasties is that segmented scars mature faster than long linear scars. In particular, if a scar crosses a joint, zigzag incision and suturing significantly reduce the risk of pathological scar development (Fig. 28.2).

Various local/regional flaps are also useful for releasing scar contractures [11]. Moreover, because local/regional flaps expand naturally after surgery, they are not prone to postsurgical contractures. By contrast, skin grafts do not expand, which means that skin grafting tends to generate secondary contractures that result in circular pathological scars around the grafted skin, although full-thickness grafts inhibit contraction more than split-thickness grafts (Fig. 28.3). In general, flap surgery is better for hypertrophic scars with severe contracture (Fig. 28.4).

28.3.2 Corticosteroid Administration

Corticosteroid injections rapidly reduce the volume of a scar. However, the downsides of corticosteroid injections include pain (caused by the injection itself) and difficulties associated with contraindications such as pregnancy, glaucoma, or Cushing's disease. In our experience, to prevent menstrual irregularities, the maximum dose of triamcinolone should be 5–10 mg per session. This is actually a very small dose compared to the doses used in other reports. This dose also does not cause hypopigmentation or skin atrophy and effectively reduces the thickness of pathological scars if the area to be treated at each intervention is small. Lidocaine (1%) can be used to dilute the triamcinolone if it will be used over a wide area. A narrow needle (30 gauge) and warming the solution can help to reduce the pain associated with the injection. Moreover, the injection should be placed into the edge between the scar and normal skin; if the injection is in the scar, the thick tissue will hamper the infiltration of the steroid solution. This in turn increases the pressure in the wound during the injection, which causes severe pain. When these tips are followed, the

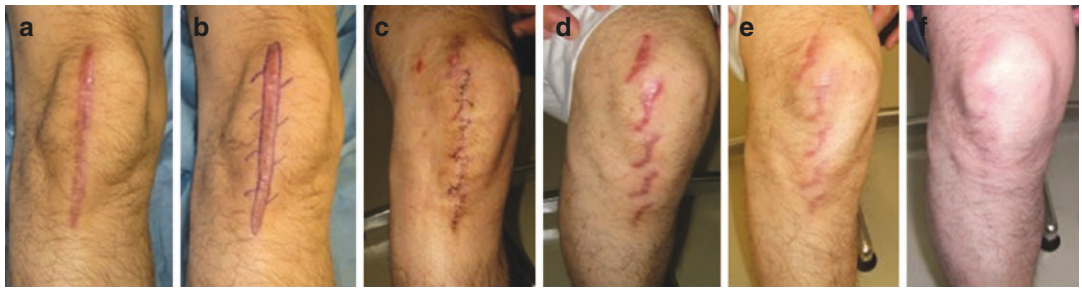

Fig. 28.2 Z-plasty releases tension and decreases inflammation. A typical case of hypertrophic scar was excised and reconstructed by using Z-plasty method. (**a**) Pretreatment. (**b**) Design of Z-plasty method. (**c**) Two weeks postoperative. (**d**) Three months postoperative. (**e**) Six months postoperative. (**f**) Twelve months postoperative. According to the releasing tension, inflammation decreases gradually, and maturation of scars accelerated. Used with permission [10]

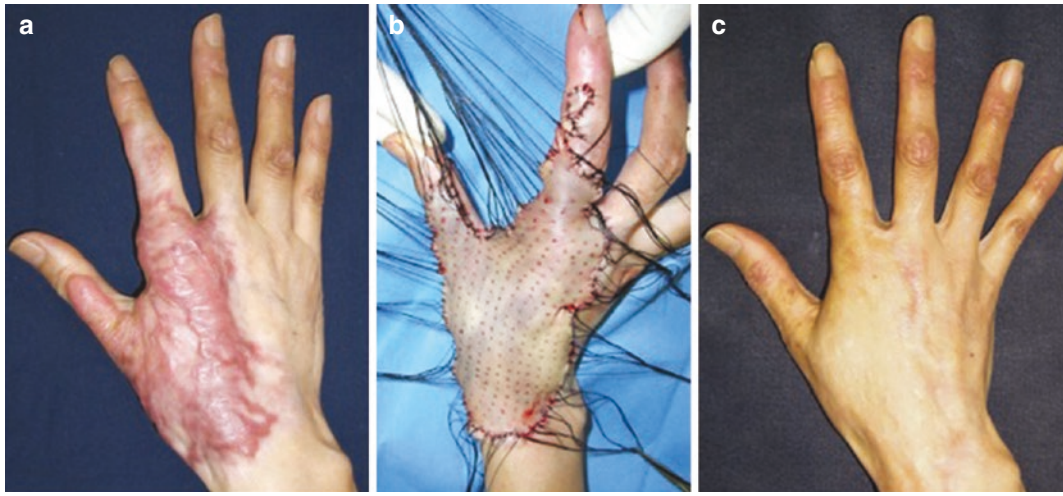

Fig. 28.3 Treatment of hypertrophic scars by skin grafting. (**a**) Pre-treatment. (**b**) Intraoperative. (**c**) A year after skin grafting. The hypertrophic scars in this case had not improved in the 2 years since the burns were sustained. Mild scar contractures developed, especially on the first web. Consequently, all scars were removed and reconstructed by full-thickness skin grafts harvested from the inguinal region. Used with permission [7]

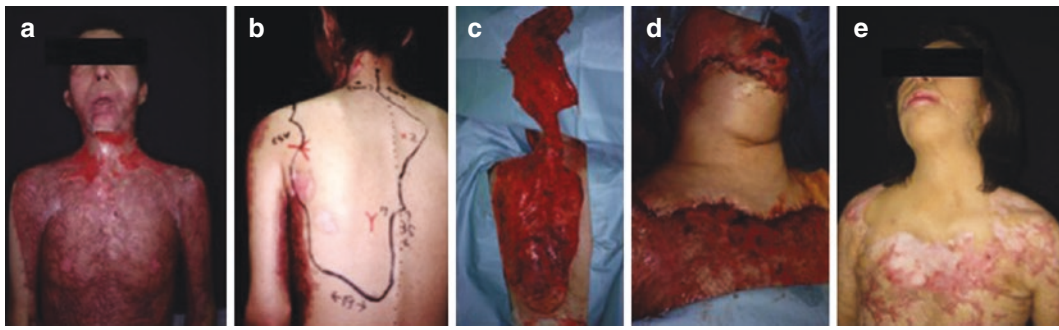

Fig. 28.4 Treatment of hypertrophic scars with scar contractures by super-thin flap. (**a**) Pre-treatment. (**b**) Design of supercharged super-thin flap. (**c**) Intraoperative. (**d**) Immediately after surgery. (**e**) Four years postoperative. Neck contracture was released by using perforator supercharged super-thin flap. Flaps expand naturally after surgery; thus, they are not prone to postsurgical contractures Used with permission [11]

patients generally tolerate monthly steroid injections for a few months, even a year. However, this is generally not long enough to achieve a complete cure. Thus, steroid injections alone may be less promising than other methods in terms of curative ability.

This problem can be overcome by using steroid tapes/plasters. Adults between the ages of 18 and 64 years can be treated with a combination of steroid injections and treatment with these tapes/plasters; once the thickness of an entire pathological scar has been reduced by several steroid injections, this effect can be maintained and augmented by using steroid tapes/plasters that the patients can apply themselves [6]. In fact, most pediatric patients can be treated by steroid tapes/plaster alone because they have much thinner skin, which means that the steroids are more easily absorbed (Fig. 28.5). Interestingly, in our experience, contact dermatitis (which is common among adult patients who use tapes) does not tend to occur in children. This may also reflect the fact that children have thinner skin through which the steroid is easily absorbed; it may also reflect the lower sebum secretion in children.

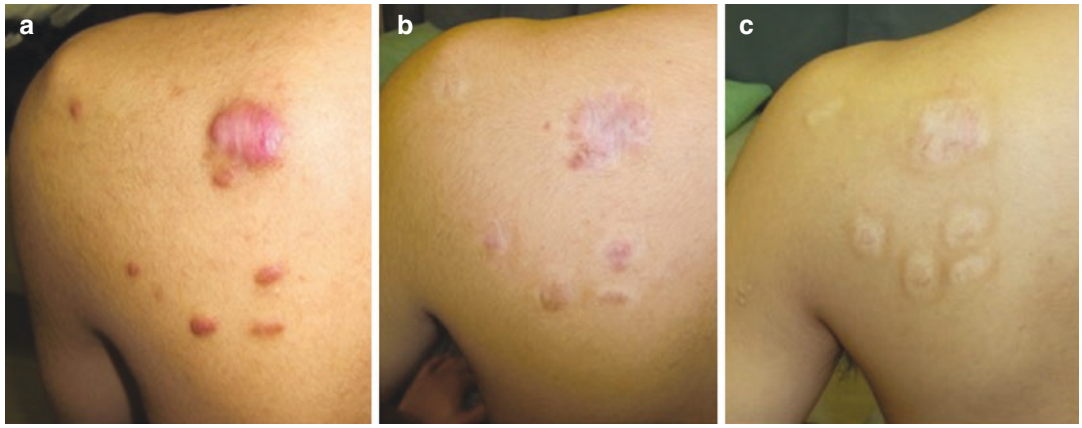

Fig. 28.5 Treatment of acne hypertrophic scarring that was treated by steroid tape. (**a**) Pre-treatment. (**b**) Six months of treatment. (**c**) Twelve months of treatment. This patient had mild scapular hypertrophic scars and was treated with fludroxycortide tape (Drenison® tape). The tape was placed on the hypertrophic scars 24 h a day and was changed daily. The inflammation resolved completely

28.3.3 Laser Therapies

Pulsed dye laser (PDL) has long been the therapy of choice for cutaneous vascular diseases, including telangiectasia, hemangioma, and vascular malformations. It has also been used to treat hypertrophic scars because they also have more blood vessels than normal skin. However, although PDL is effective for vascular diseases that affect the superficial skin layers (i.e., the epidermis and the papillary layer of the dermis), it does not penetrate deep enough to reach the deep dermal regions (i.e., the reticular layer of the dermis). Thus, PDL is not particularly effective for hypertrophic scars. By contrast, the 1064 nm Nd:YAG laser reaches more deeply than PDL. As a result, it is increasingly being used to treat keloids and hypertrophic scars. It has been suggested that it acts by suppressing neovascularization and the dilatation of blood vessels.

Long-pulsed (not Q-switched) 1064 nm Nd:YAG laser plays an important role in our treatment algorithms for hypertrophic scars (Figs. 28.6 and 28.7) [12]. The laser should generally be applied to the skin surface with the following standard treatment settings: a spot diameter of 5 mm, an energy density of 75 J/cm^2, an exposure time per pulse of 25 ms, and a repetition rate of 2 Hz. However, in the case of the hypertrophic scars on the face of pediatric patients, the treatment should start with a lower energy density (60–70 J/cm^2) to reduce the possibility of a burn injury. The best way to prevent such burn injuries is to cool the tip or air-cool the targeted skin before and immediately after irradiation. Each session should consist of three passes unless the patient feels strong pain at the first pass; in this case, the session should be stopped. Even if the patient feels no pain after the third pass, the session should be stopped. Local anesthesia is not necessary. However, if the

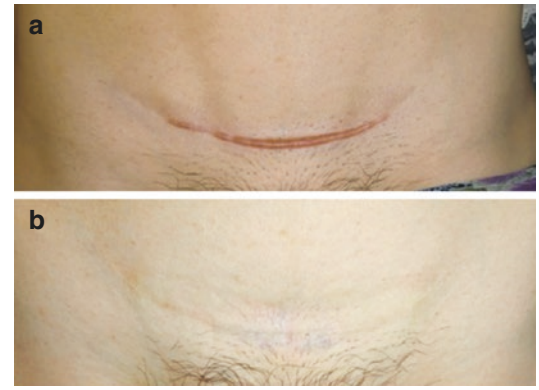

Fig. 28.6 Abdomen hypertrophic scarring that was treated by the 1064 nm Nd:YAG laser. (**a**) Pre-treatment. (**b**) One year after treatment. The 1064 nm Nd:YAG laser was used at 5 mm spot, 75 J/cm^2, 25 ms, and 2 Hz. After 1 year of treatment, the scar had almost disappeared. Used with permission [12]

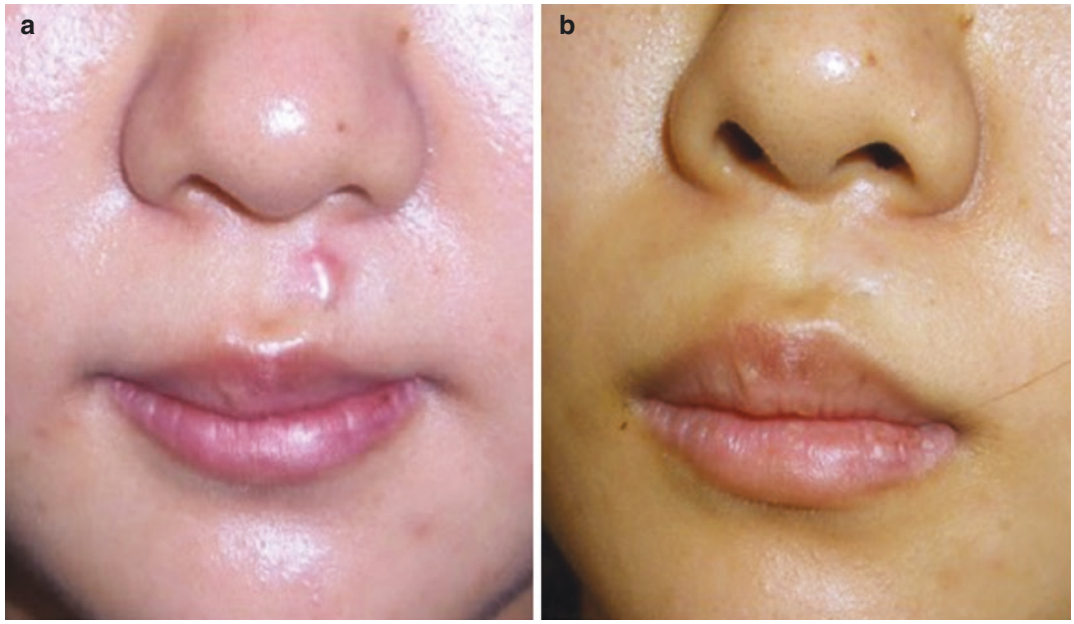

Fig. 28.7 Upper-lip hypertrophic scarring that was treated by the 1064 nm Nd:YAG laser. (**a**) Pre-treatment. (**b**) One year after treatment. The 1064 nm Nd:YAG laser was used at 5 mm spot, 65–70 J/cm^2, 25 ms, and 2 Hz. After 1 year of this treatment, the textural difference remained, but there was improvement in the redness and elevation of the scar. Used with permission [12]

patient expresses concern, anesthetic cream or tape can be used. The intervals between the sessions should generally be 2–4 weeks depending on the patient's schedule.

28.3.4 Compression and Stabilization Therapies

Compression therapy and the wound stabilization treatments described above in the section on hypertrophic scar prevention can also help treat these scars after they arise. Thus, prolonged external mechanical support using (steroid) tapes, sheets, and/or garments is recommended after surgical treatment, corticosteroid injections, and laser therapy.

28.4 Follow-up of Hypertrophic Scars

It is important that sequentially treated hypertrophic scar patients are followed up over the long term. It is also important that they are appropriately educated about scar management because if patients develop pathological scars in the first place, it suggests that they may be particularly prone to recurrence or the development of new pathological scars in response to minor stimulation or injuries. Thus, these patients should be educated in the self-management of subsequent wounds. In particular, they should be encouraged to apply steroid tape/plasters during the early stages of scar development. This will rapidly reduce the inflammation in the scar and improve its appearance. Moreover, during follow-up, laser therapy, anti-allergy agents including tranilast, anti-inflammatory agents, bleaching creams, and make-up therapies may be useful on a case-by-case basis.

28.5 Conclusions

Surgery and steroid tape/plaster therapy can manage hypertrophic scars successfully and are increasingly being used, especially in Japanese populations. Thus, there is now sufficient evidence on which to base a standard international

algorithm for treating pathological scars. Treatments are likely to improve significantly as our knowledge of scar biology increases, higher-quality clinical trials are performed, and new agents are developed.

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29 Tissue Engineering and Regenerative Medicine in Oral and Maxillofacial Surgery: The Most Important Clinical Applications of Mesenchymal Stem Cells

Marco Tatullo, Massimo Marrelli, Francesco Paduano, Francesca Palmieri, Sandro Rengo, Carlo Rengo, Gianrico Spagnuolo, and Bruna Codispoti

29.1 Introduction

29.1.1 Regenerative Medicine

Regenerative medicine is a newly developed branch of medicine, able to collect many different scientific fields including tissue engineering, physiology, surgery, cell and developmental biology, molecular biology, biochemistry, chemistry, biomaterials science, nanotechnology, physics, and bioengineering. All these technologies are aimed together to repairing or regenerating tissues and organs affected by chronic and degenerative disease or damaged by severe traumatic injuries.

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Stem cell-based therapies represent the most recent tissue engineering techniques, often tested on the most innovative biomaterials, playing a central role in the entire regenerative medicine. Stem cell transplantation, with the use of scaffolds as support, is today a routine treatment. But, the history of the transplants began in 1954, with the first successful organ transplantation performed by Joseph Murray, who replaced a kidney from a twin into his brother [1].

The advent of synthetic materials, such as Teflon and Silicone, led to the production of new devices capable to provide structural replacement of specific tissues in the human body.

Afterward, studies on extracellular matrix and its interaction with the surrounding cells gave way to an improved understanding of cell and tissue growth and differentiation; such new knowledge allowed the starting of the modern era of tissue engineering that links together materials science with cell biology.

Stem cells are undoubtedly the main characters on the regenerative medicine scenario, not only for their wide potential to proliferate and to differentiate into specialized mature cell types but also for their paracrine effects in modulating inflammatory responses.

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In the last few years, scientists are focusing their attention on the interaction that stem cells establish within the site of transplantation, with the attempt to reproduce their physiological microenvironment, known as "stem cell niche." The ex vivo reproduction of the niche conditions has primary importance for a successful engraftment of the new implanted tissue/organ.

29.1.2 Oral-Derived Mesenchymal Stem Cells

Stem cells are commonly defined by their clonogenic and self-renewing capabilities and by the potential to differentiate into multiple cell lineages. Whereas embryonic stem cells (ESCs) are derived from the blastocyst stage of the embryo and they have the ability to generate any terminally differentiated cell in the body, adult stem cells reside in the postnatal organism and retain tissue-specific potential (multipotency). In 2006, researchers created the induced pluripotent stem cells (iPSCs) from mature differentiated cells using a mix of four transcription factors (Oct4, Sox2, c-Myc, and KLF4) [2]; despite pluripotency of iPSCs (similar to ESCs), further investigations showed that the conversion of cells to iPSCs poses a substantial risk of tumorigenesis [3]. The use of adult stem cells in regenerative medicine avoids ethical implications, mainly linked to the embryonic stem cell manipulation, and reduces the possibilities to promote neoplastic processes.

Hematopoietic stem cells were the first adult stem cells isolated from bone marrow (BM) [4]; a few years later, a second population called "mesenchymal stem cells" was isolated from the same tissue [5]. Currently, it is known that adult stem cells are present in many organs and tissues, including the peripheral blood, blood vessels, skeletal muscle, skin, teeth, heart, gut, liver, brain, ovarian epithelium, and testis. The possibility of in vitro manipulation of stem cells paved the way for cell-transplantation-based therapies.

Mesenchymal stem cells (MSCs) retain the widest range of differentiating fates, such as bone, cartilage, adipose tissue, muscle, tendon, marrow stroma, and neural tissues. Thus, their great plasticity, the extensive proliferative potential, and the ability to differentiate into various cell types give to these cells a prominent role in regenerative medicine.

MSCs can be identified by expression of specific surface markers, CD73, CD90, and CD105, and lack of hematopoietic specific markers including CD34, CD45, and HLA-DR. These cells could virtually reside in all postnatal tissues. Human bone marrow (hBM) has been originally identified as the major source of MSCs; however, the harvesting of BM-derived MSCs requires an invasive surgical procedure; moreover, the collected cells from this source are typically not abundant. Recent studies aim to achieve tissue regeneration by using MSCs taken from easily accessible sites: oral and dental tissues represent highly rich and accessible sources of mesenchymal stem cells. The access to dental-derived stem cells is quite simple, and the isolation from oral tissues is highly efficient, in comparison with other stem cell niches of the adult human body [6].

A variety of cell populations with stem cell properties have been isolated from the oral cavity (Table 29.1).

The first study demonstrating the presence of MSCs in dental tissues, with bone marrow-derived stem cell (BMSC)-like phenotype, was conducted by Gronthos in 2000; in this work, the authors identified an odontogenic progenitor population, from enzymatically disaggregated adult human dental pulp. Dental pulp stem cells (DPSCs) are able to show high proliferation, clonogenicity, and multipotency in vitro and the capability to regenerate a dentin- and pulp-like structures, after xenogeneic transplantation [7]. This pilot study led to a series of researches about the huge commitment of DPSCs toward the regenerative medicine. For example, such cells have been shown to retain the ability to differentiate into hair follicle cells [8], adipocytes, chondrocytes, neurons [9], hepatocytes [10], and myocytes [11].

In 2003, MSCs have been isolated from dental pulp of stem cells from human exfoliated deciduous teeth (SHEDs): SHEDs represent a

Table 29.1 Mesenchymal stem cells from oral tissues

	Location	Institution	Multipotency	First authors
DPSC	Dental pulp	Gronthos et al.	Osteoblast	Gronthos et al. (2000)
		National Institute on Dental Research, National Institutes of Health	Neurocyte	Gronthos et al. (2002)
		Bethesda, Maryland, the USA, 2000	Odontoblast	Batouli et al. (2003)
			Adipocyte	Pierdomenico et al. (2005)
			Chondrocyte	Kerkis et al. (2006)
SHED	Human exfoliated deciduous teeth	Miura et al.	Odontoblast	Miura et al. (2003)
		National Institute on Dental Research, National Institutes of Health	Neurocyte	Miura et al. (2003)
		Bethesda, Maryland, the USA, 2003	Adipocyte	Miura et al. (2003)
			Osteoblast	Seo et al. (2008)
			Endothelocyte	Cordeiro et al. (2008)
PDLSC	Periodontal ligament	Seo et al.	Cementoblast	Seo et al. (2004)
		National Institute on Dental Research, National Institutes of Health	Adipocyte	Seo et al. (2004)
		Bethesda, Maryland, the USA, 2003	Odontoblast	Trubiani et al. (2007)
			Osteoblast	Gay et al. (2007)
			Chondrocyte	Gay et al. (2007)
DFSC	Dental follicle	Morsczeck et al.	Osteoblast	Morsczeck et al. (2005)
		Stiftung Caesar, Center of Advanced European Studies and Research	Cementoblast	Morsczeck et al. (2005)
		Bonn, Germany, 2005	Neurocyte	Vollner et al. (2009)
SCAP	Apical papilla	Sonoyama et al.	Odontoblast	Kikuchi et al. (2004)
		University of South California, Los Angeles, California, the USA Okayama University, Okayama, Japan, 2006	Osteoblast	Ikeda et al. (2006)
hPCy-MSC	Human periapical cyst	Marrelli et al.	Osteoblast	Marrelli et al. (2013)
		Calabrodental, Unit of Maxillofacial Surg; Tecnologica Research Institute Crotone, Italy, 2013	Neurocyte	Marrelli et al. (2015)

population of postnatal stem cells capable of extensive proliferation and multipotency. These cells basally expressed neuronal and glial cell markers; thus, SHEDs were proposed as therapeutic cells, used to treat neural tissue injury, to repair damaged tooth structures, and to induce bone regeneration [12].

Periodontal ligament maintains teeth in situ, by connecting dental cementum and alveolar bone; Seo et al. isolated from this specific connective tissue a cell population expressing mesenchymal stem cell-like markers, called periodontal ligament stem cells (PDLSCs). These cells were able to differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells in vitro and to contribute to periodontal tissue repairing, by generating a cementum-PDL-like structure, when transplanted into mouse model [13].

Dental follicle is a thin connective sac that surrounds the developing tooth; MSCs isolated from these ectomesenchymal tissues (dental follicle progenitor cells, DFPCs) have the potential to differentiate into neural cells and to regenerate bone and periodontal tissues [14].

Stem cells isolated from the root apical papilla of the human teeth (stem cells from apical papilla, SCAP) [15] have been shown to be able to differentiate through the odontogenic-osteogenic lineages and into adipose and neural cells [16].

Oral-derived MSCs show high self-renewal ability, clonogenicity, and multipotent differentiation capacity, similar to BMSCs [17].

The head and neck district is rich of very interesting reservoirs of stem cells: Cells from the human parotid glands exhibit both epithelial and mesenchymal stem cell-like features, including commitment potential and marker expression [18]; the periosteum surrounding the jaws also contains cells with high potential to differentiate into osteoblasts and chondrocytes [19].

Therefore, as point of strength, dental-derived stem cells can be easily obtained from teeth extracted for orthodontic reasons, as well as from impacted teeth, or following severe periodontitis and other similar reasons.

In the recent years, Marrelli et al. demonstrated for the first time that a population of cells, showing mesenchymal behavior, could be isolated from human periapical cyst-mesenchymal stem cells (hPCy-MSCs). These cells were reported to have high ability to differentiate mainly into osteogenic lineages, contributing to achieve significant progresses toward clinical application of oral and maxillofacial bone reconstruction [20, 21].

hPCy-MSCs were also demonstrated to express high levels of transcripts for neuronal markers, when exposed to specific differentiating conditions. These studies demonstrated that inflammatory cysts may serve as a new reservoir of MSCs for cell therapy, to treat human neurological disorders [22].

In conclusion, because of their peculiar characteristics related to their proliferation, differentiation, and plasticity, dental tissues are nowadays an attractive source of mesenchymal stem cells, to be used in regenerative dentistry, and also for wide application in extraoral tissue and solid organ regenerative medicine.

29.1.3 Clinical Applications of MSCs on Tissue Defects Affecting the Aesthetic Zone

The wide interest for mesenchymal stem cells in tissue engineering has promoted a number of research activities, showing very promising results.

Some case reports are particularly interesting, as they describe the clinical use of adult mesenchymal stem cell (ASCs) to successfully treat defects affecting the mandible, maxilla, and cranial bones.

Mandibular reconstruction has been investigated by means of bone marrow-derived MSCs: Cells were seeded on titanium mesh, loaded with hydroxyapatite (HA) and bone morphogenetic protein-2 (BMP-2); these scaffolds were then implanted to treat a critical-sized mandibular defect [23].

Mesimaki described how the seeding of ASCs in a microvascular flap, in combination with beta-tricalcium phosphate and bone morphogenetic protein-2 (BMP-2), showed promising outcomes up to 8 months after the implantology, in the treatment of a large defect of the maxilla [24].

Autogenous adipose stem cells, seeded on bone grafts, have been applied for the treatment of a seven-year-old patient with severe cranial trauma, by using fibrin glue, showing notable results [25].

Clinical use of mesenchymal stem cells in aesthetic procedures is slightly growing: the main limitations of stem cell use in cosmetic surgery seem to be related to the high costs of acquiring and culturing MSCs and to ethical issues.

Studies focused on cosmetic procedures with adipose-derived stem cells tried to investigate the adipose-grafting retention in the breast surgical site, especially after the oncological surgery: this grafting showed to improve the skin quality of irradiated breasts.

Yoshimura demonstrated impressive outcomes, by employing the "cell-assisted

lipotransfer" procedure, where a fat graft is transferred along with freshly isolated mesenchymal cells: this approach may be useful in cosmetic breast augmentation or after the removal of breast implants to improve the tissue healing [26].

Enrichment of adipose grafting with stem cells was reported, in order to enhance soft tissue augmentation at the face; the liposculpture could be applied for the treatment of patients with lipodystrophy and also for facial volumetric rejuvenation [27].

Clinical researches are ongoing also with the attempt to apply stem cells in the treatment of chronic wounds and burns. A recent experimental study involved 20 patients suffering from chronic burns, extensive lesions, skin ulcers, and diabetic wounds; the therapeutic effect on wound closure was demonstrated in all the patients treated with bone marrow-derived stem cells, added to collagen sponges [28].

In 2011, the US FDA approved the first personalized cell therapy, named "laViv," for the correction of thin wrinkles or nasolabial folds around the mouth and nose. Such treatment is performed by the collection of autogenous fibroblasts, cultured for 90 days and then injected into the dermis [29].

The ability of MSCs to produce an array of cytokines that promote the collagen synthesis and turnover (TGF-β, EGF, VEGF, and PDGF) makes them a good candidate for antiaging therapies; however, current evidences of MSC application on this topic are still poor.

The clinical use of MSCs in aesthetic surgery is, thus, still in the very early stage; further safety data are required to protect patient's health; moreover, the improvement of procedures for cell manipulation is highly necessary to make stem cell-based cosmetic treatments less expensive to be usable on a commercial scale.

29.1.4 Application of Mesenchymal Stem Cells in Regenerative Medicine

Mesenchymal stem cells represent an attractive candidate for clinical applications, due to their ability to self-renew and to differentiate into both mesenchymal and non-mesenchymal lineages;

MSCs secrete several immunomodulatory molecules, which provide a regenerative microenvironment that promotes self-regulated restoration of a variety of damaged tissues and organs; current data suggest that MSCs exert also trophic and anti-inflammatory effects [30].

The specific cellular and molecular mechanisms involved in the immunoregulatory activity of MSCs remain still under investigation; some evidences demonstrate that the capability to modulate immune responses depends on both paracrine effects through the release of soluble factors and also on cell contact-dependent mechanisms [31]. The fact that mesenchymal stem cells have immunomodulating properties and inhibit function of immune cells has been exploited for the treatment of autoimmune conditions including graft versus host disease, Crohn's disease, multiple sclerosis, refractory systemic lupus erythematosus, and systemic sclerosis. The therapeutic effect of MSCs in the treatment of type I and type II diabetes is suggested because of their ability to generate functional insulin-producing cells, and because of their effective role in counteracting the complications of diabetes mellitus (cardiomyopathy, diabetic nephropathy, diabetic polyneuropathy, diabetic retinopathy, and diabetic wounds), this effect is basically due to the regulatory paracrine activity of MSCs [32]. In the ischemic cardiomyopathy, MSCs seem to prefer to secrete soluble paracrine factors, with the aim to induce endogenous cardiomyogenesis and angiogenesis; however, this mechanism is yet to be explored [33].

Whole organ transplant is the most preferred solution in case of liver and kidney diseases, but donors are often missing. Under specific growth conditions, MSCs have been shown to adopt functional features of differentiated hepatocytes able to successfully engraft the mouse liver; therefore, generation of hepatocyte-like cells from MSCs could become a real alternative to liver transplantation [34]. Experimental in vivo studies reported that the exogenous administration of MSCs to mice with acute renal injury could promote both structural and functional kidney repair, while about 2.5% of the injected

MSCs showed substantial engraftment; probably, the protective and regenerative effects of MSCs to the kidney are due to the ability of MSCs to inhibit the release of pro-inflammatory cytokines and to secrete a number of trophic growth factors that promote angiogenesis and cell proliferation, reducing apoptosis [35].

There is a great interest in the use of MSCs to treat neurodegenerative diseases, through providing of secreted neurotropic factors which encourage the repairing and, potentially, the new growth of neuron-like cells.

MSCs isolated from human periapical cysts and dental pulp spontaneously expressed neuron- and astrocyte-specific proteins in their basal state, showing a predisposition toward the neural phenotype. Probably the different phenotypes of DPSCs with respect to BMSCs are related with the different developmental origins; in fact, tooth development occurs from neural crest cells rising from ectodermal embryonic layer instead of mesodermal derivation of bone marrow [6, 22].

Despite the recent widespread clinical application, the original interest on MSCs was exerted by their intrinsic ability to be easily induced to differentiate into the three main mesenchymal lineages: adipogenic, chondrogenic, and osteogenic.

Compared to the clinical applications of stem cells in other diseases, the different uses of MSCs for bone and cartilage regeneration are being developed at a higher rate. In some countries (the USA, Korea), there exist some prepared and approved mesenchymal stem cell-based products that significantly contribute to the employment of stem cells in bone and cartilage regeneration; these products are routinely used for treatment of connective tissue disorders and traumatic injuries in orthopedics.

The capability of MSCs to regenerate connective tissues found their greater and ample application in oral and maxillofacial surgery.

The area of regenerative medicine that has received the most attention for the maxillofacial region is bone regeneration; autogenous grafting has been considered, over the years, the goal standard for bone replacement in congenital deformities, acute trauma, chronic nonunion, or resection of pathology. Several studies reported that a combination of isolated MSCs, with scaffolds and growth factors, successfully repairs cranial defects in different animal models; these results shifted the focus of surgical specialists on the use of cellular techniques, biomaterial replacement, or signaling molecule for bone substitutes in the attempt to avoid donor site morbidity and provide a more convenient way to regenerate defects.

MSCs residing in the oral cavity tissues retrieve clinical interest in oral and maxillofacial surgery as a cell source for regeneration of bone and dental tissues such as cementum, dentin, and periodontal ligament.

The development and evolution of techniques with increasing efficiency and innovation for oral and maxillofacial application are described below.

29.2 Techniques

Treatment of degenerative and traumatic injuries is the main purpose of oral and maxillofacial surgeons; in order to achieve these targets, a number of techniques have been improved over time; as mentioned above, these techniques consist in the usage of the three main pillars of regenerative medicine: biomaterials, growth factors, and stem cells (Fig. 29.1).

The first surgical additives to be used were human plasma derivatives that mimic the final stages of blood coagulation, forming a fibrin clot. Fibrin sealants, fibrin glues, or fibrin tissue adhesives were commercially available in Europe since the late 1970s [36].

Platelet-rich plasma (PRP) and platelet gels were the first generation of platelet concentrates developed to combine the sealant properties of fibrin with the well-known effects of platelet growth factor as a source of healing cytokines. Growth factors regulate several cellular functions including chemotaxis, proliferation, tissue healing, and regeneration; the use of autologous platelet concentrates provides an ideal growth factor delivery system at the site of injury [37]. The evolution of PRP was platelet-rich fibrin

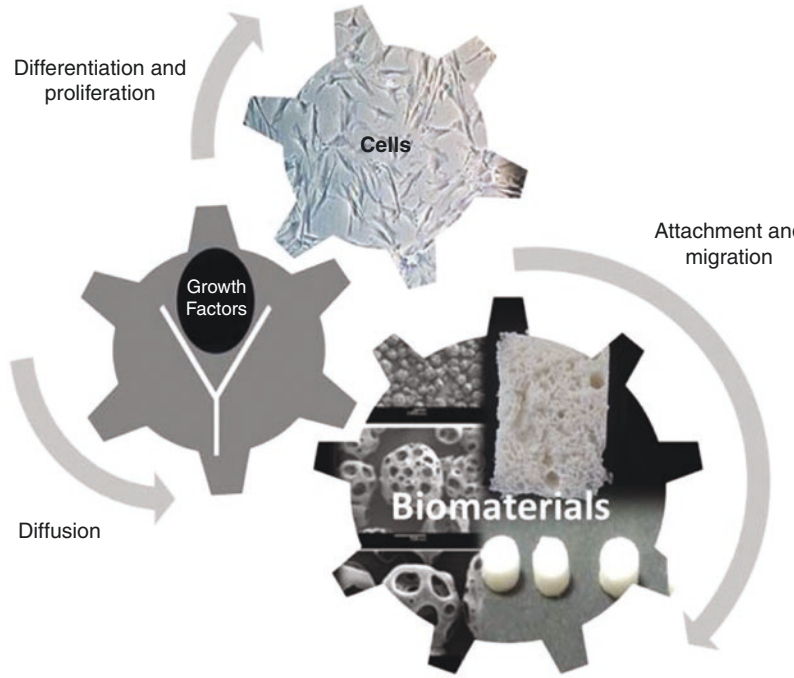

Fig. 29.1 Regenerative medicine: key elements

(PRF), developed for the first time in France by Choukroun in 2001. This second generation of platelet concentrate eliminates the risks associated with the use of bovine thrombin (exerted for gelling of PRP before application). PRF is composed of an intimate assembly of structural glycoproteins, glycanic chains, and growth factors embedded within a fibrin network slightly polymerized; these biochemical components appear to accelerate physiological healing process [38]. Numerous techniques for autologous platelet concentrate application have been investigated and applied in oral and maxillofacial surgery. In a follow-up study plan of 127 patients, requiring maxillary sinus lift, the use of platelet-rich plasma was tested as a grafting material for bone regeneration before dental implant; as a result, patients treated with PRP reached a statistically significant improvement in implant-prosthetic rehabilitation at six months after surgery, compared to control patients [39].

Platelet-rich fibrin membranes have been shown to increase long-term maintenance of crestal bone and to induce rapid healing of soft tissue around post-extractive dental implants [40]. Promising results were also obtained using PRP to support bioactivation of dental implants placed in maxillary and mandibular region [41].

Fabrication of membranes has become the standard in dental surgeries requiring space provision and in guided bone regeneration procedures. Originally, non-resorbable membranes included expanded high-density polytetrafluoroethylene (PTFE), titanium-reinforced PTFE, and titanium mesh were largely diffused; successive membranes with controlled degradation periods were generated. Resorbable membranes could be composed of organic materials originating from natural precursors such as chitosan, silk, or collagen. Collagen membranes are made of collagen types I and III, mainly derived from bovine sources. The action of collagenases allows reabsorption; interestingly the time of membrane loss can be modified by cross-linking treatment that prevents or delays degradation, so these membranes are useful when the synthesis of new bone depends on the prolonged presence of a mechanical barrier.

Synthetic membranes are formed by polylactic and polyglycolic acid (PLA, PGA); the time of reabsorption of these membranes is slower compared to collagen membranes. Different

bio-resorbable polymers and copolymers are currently used in synthetic membranes: Poly(DTE carbonate) has shown low immunological reaction and high ability to induce bone regeneration; polylactic acid-polyglycolic acid (PLLA-PGA) copolymer provides a rigid scaffolding to protect the graft materials. New membranes made by a combination of polylactic acid, aminopropyltriethoxysilane, and carbonate of calcium show greater ability to induce proliferation of bone cells compared to non-hybrid membranes.

Concerning constituents used for implants, since 1978, autologous material has been used for bone regeneration; the absence of immunological response placed these techniques as preponderant in bone grafts. But the increased surgical time and patient morbidity of autologous graft led to investigation for homologous, heterologous, and synthetic sources for graft materials.

Vital bone tissue obtained from donors is stocked in bone banks; before use, homologous tissues are tested and treated to prevent any risk of antigenicity or disease transmission. Heterologous material is obtained from bones of different animal species; the most common source is bovine bone.

A recent study demonstrates that dental pulp stem cells seeded on hydrogel scaffolds derived from decellularized and demineralized bovine bone extracellular matrix (bECM) promote mineral deposition and upregulation of osteogenic genes in basal and even more in inductive conditions in comparison with those seeded on collagen ones [42]. Furthermore, the same cells cultured on bECM hydrogels with osteogenic medium displayed a higher upregulation of the osteo-specific markers compared to collagen hydrogels [43].

Collectively, these results demonstrate that bECM hydrogel could be proposed for odontogenic or osteogenic differentiation of DPSCs for dental and bone regeneration.

Inorganic materials commonly consist of metals, alloys, or mineral compositions. Alloplastic grafts are synthetic bone substitutes presented in different forms, sizes, and textures showing great similarity with mineral bone tissue. Bioactive ceramics directly bond with living tissues when implanted; the first produced synthetic bioactive materials were specific compositions of glasses, glass ceramics, and sintered hydroxyapatite. Hydroxyapatite is a natural component of hard tissues (98% in enamel, 65% in bone tissue). Synthetic hydroxyapatite is bioinert and biocompatible and could be produced in porous and ceramic forms. Natural hydroxyapatite could be derived from corals, animal bones, eggshells, wood, and algae.

Tricalcic phosphate grafts release calcium and magnesium ions during reabsorption, creating an ideal ionic environment for bone synthesis. Synthetic glass ceramics made of silicon dioxide, sodium oxide, and phosphorus pentoxide ensure osteoconductivity and are mainly used in maxillary sinus lifts.

The identification and production of recombinant morphogens, growth factors, and cytokines are a clinical focus in tissue engineering with special regard to bone regeneration. Growth factors are critical signaling molecules that instruct cells during development and achieve tissue regeneration in the adult organism. Growth factors used in oral and maxillofacial reconstruction are clearly addressed toward the repair of mesenchymal tissues. Among the multitude of employed molecules, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), transforming growth factor beta (TGFb), vascular endothelial growth factor (VEGF), and bone morphogenetic proteins (BMPs) have been used in maxillofacial reconstruction in a large number of animal models.

In surgery, the possibility to implant stem cells, which bring the regenerative potential, in a tridimensional conformation that resemble the anatomic structures is undoubtedly advantageous.

The combination of cells, biomaterials, and growth factors into scaffolds, manufactured in a laboratory, reproduces the tridimensional architecture and the physiological composition of the desired tissue to be replaced.

A scaffold acts as a matrix and allows the attachment, migration, and differentiation of

progenitor cells. Scaffolds have been composed with a variety of natural or synthetic biomaterials (see above); their functions are to provide a structural support to the cells, to be a reservoir of growth factors, and to offer a flexible spatial environment for tissue remodeling. The properties of the ideal scaffold include biocompatibility, appropriate mechanical strength, and suitable degradation. Furthermore, porosity, permeability, and interconnectivity are also important features for an effective and efficient diffusion of gases and nutrients; these characteristics influence cell adhesion, migration, and proliferation within the scaffold. In a pilot study, focused on the biological compatibility of innovative dental biomaterials, the behavior of DPSCs grown was evaluated on silicon nanoporous and mesoporous matrices; as a result, among the 28 matrices examined, silicon scaffold functionalized with (3-aminopropyl) trimethoxysilane/toluene was found to better support the proliferation of DPSCs [44].

Depending on the clinical target, different potential tissue engineering methods could be performed. A general inductive technique consists in the delivery of soluble signaling molecules to the adjacent tissues; in this approach, growth factors are carried by a bioactive scaffold and are exploited to attract cell movement and to organize cellular behavior.

The guided tissue regeneration approach is extensively used for the treatment of periodontal diseases; in this conductive approach, the scaffold acts as a submissive tridimensional mechanical support to which cells can connect and propagate.

For the treatment of huge tissue defects, cell transplantation is more suitable. This procedure typically includes cell harvesting from a donor source and in vitro handling of the donor cells that are directly seeded onto polymers typically made up with the physical forms of fiber-based mesh, sponge, or hydrogel. The cells residing into the scaffold proliferate, forming a regenerated tissue that is subsequently established into tissue-deficient areas.

Summarizing, the bioengineered tissue constructs recapitulate the physiology, natural architecture, dynamic conditions, intercellular matrix, and cell-extracellular matrix interactions; such ex vivo constructs found extremely large application, for example, is employed for pharmacokinetic and pharmacodynamic analyses of drugs, as preclinical models for high-throughput drug screening and device testing. The advantages offered by these man-made tissues include high reproducibility and accurate control over culture conditions; these techniques represent a powerful tool in regenerative medicine; therefore, they are particularly important in medical research.

29.3 Discussion

Regenerative medicine has been defined as the application of scientific principles to the design, production, modification, and growth of living tissues using cells, growth factors, and biomaterials, either alone or in combination [45]. These three central elements, stem and progenitor cells, growth factors, and appropriate biological scaffold, have been thoroughly discussed. Tissue engineering involves the use of matrices or scaffolds that guide the implanted cells and also the host's surrounding cells during tissue regeneration or restoration.

The use of more undifferentiated cell types such as stem cells or early mesenchymal progenitors that retains self-renewal and multi-lineage potential is preferable to that of terminally differentiated cells. Differentiation of stem and progenitor cells can be obtained in vitro by changing the culture conditions after their expansion or by providing a new physiological microenvironment in the transplanted area in vivo.

Adult stem cells harvested from donor tissues could be further expanded in culture and then associated with biomaterials to form a scaffold. A biomaterial should easily integrate with the adjacent tissues and favor new tissue ingrowth (e.g., osteoconduction in bone regeneration). It should allow colonization by the host blood vessels and should be biocompatible and resorbable. Polymers include collagen that can be prepared in solution or shaped into membrane films, fibers, sponges, and hydrogels. Synthetic polymers allow a better control of

physicochemical properties and delivery kinetics. They also reduce the risk of potential biohazardous complications. Biodegradable scaffolds supply the initial structure and stability for new tissue formation but degrade as tissue forms, providing three-dimensional space for matrix deposition and tissue growth, in the aim to mimic the extracellular matrix in a regenerating environment. Thus, scaffolds have to be instructive to the cells as well as provide mechanical support. Biomaterial can be used alone or in combination with growth factors.

Growth factors are cytokines that are secreted by many cell types and function as signaling molecules; as an example, the members of the TGF-β family, particularly bone morphogenetic

proteins, are mostly relevant to bone tissue engineering; in fact, BMPs promote the proliferation of mesenchymal stem cells and induce their chondrogenic and osteogenic differentiation. For this significant role in bone development, BMPs have been quite often incorporated into tissue engineering scaffolds and delivery systems.

The choice of the cell sources depends on accessibility and frequency of cells. Oral tissues are a very accessible and abundant source of highly immature mesenchymal stem cells, with great proliferation rate and different broad range of specialized tissue, with special regard to their innate propensity to neural differentiation. Dental-derived stem cells could be easily initiated to osteo- and odonto-differentiation; therefore, these cells represent an interesting powerful tool for oral and maxillofacial regeneration.

29.4 Conclusions

Oral and maxillofacial surgery is focused on treatment of traumatic or degenerative diseases.

Scientist efforts initially were based on guided tissue regeneration and on the use of several biomaterials as graft substitutes and in the employment of a variety of growth factors; subsequently, stem cell-based transplantation and new techniques based on recruitment of host cells (cell homing) improved previous technology. The discovery of even more accessible sources of mesenchymal stem cells, such as oral tissues, allows to reduce patient's morbidity in the view of cooperating in the collective purpose to recapitulate the microenvironmental niche, for understanding cellular behavior, and moving to an integrated approach to better create innovative and effective regenerative medicine products.

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30 Reconstruction of Post-Traumatic Maxillary Ridges Using a Radial Forearm Free Flap and Allogeneic Tissue-Engineered Bone Grafts

James C. Melville, Huy Q. Tran, Jonathan W. Shum, Ramzey Tursun, and Robert E. Marx

30.1 Introduction

Many options are available to the surgeon when considering maxillofacial reconstruction. Since it was first introduced in the literature, the reconstructive ladder has upheld a solid foundation for the novice and veteran surgeons (Fig. 30.1). At the very top of the ladder, free tissue transfer is often the preferred choice for large maxillofacial defects due to the ability to reconstruct both soft and bony tissues simultaneously. In 1989, the fibula free flap was first received as a novel way of reconstructing mandibles [1]. Since then, it has remained the workhorse for reconstruction of tumor-resected maxillomandibular defects [2]. In one systematic review, the author found a 99% survival rate and 95% dental implant success rate for vascularized fibular free flap [3]. Despite its success, an osteocutaneous free flap is an invasive technique associated with serious morbidities [4]. As such, it may be inappropriate to harvest a fibula free flap to reconstruct smaller segments of defects when other alternatives can be explored.

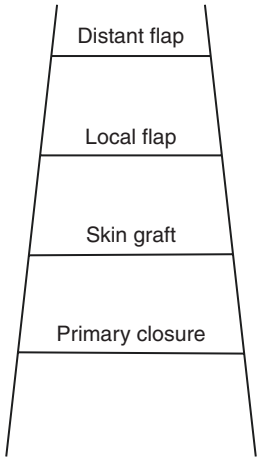

Fig. 30.1 Traditional reconstructive ladder

In consideration of reconstructing post-traumatic maxillary alveolar ridge defect, a traditional block bone graft or an onlay graft is usually

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the first treatment choice [5]. It requires adequate soft tissue for coverage, a requirement that patients of maxillofacial trauma often lack. Any communication to the intraoral environment almost certainly means a death sentence to the graft. At the other end of the spectrum, one can quickly advance the reconstructive ladder by turning to osteocutaneous microvascular flaps such as fibula free flap as an alternative. As mentioned before, they are by far the most invasive options, are too bulky, and have incorrect geometric shape for the defect [6]. Additionally, a history of surgery or trauma to the donor site is a contraindication to flap harvest.

In 1982, Song et al. [7] published the first reported case of cutaneous radial forearm free flap. The flap has long since remained one of the most reliable options for reconstruction of maxillofacial soft tissue defect. It brings the advantages of shape versatility, being thin and pliable with large- and long-caliber vessels, low donor morbidity, and relatively simple flap harvest [8]. With such tool in the bag, we can further our reconstructive approach by incorporating the concept of tissue engineering. Bone marrow aspirate has long been studied in orthopedic literature but is a relatively new concept in maxillofacial surgery [9, 10]. In immediate reconstruction of benign tumor extirpations, study showed excellent success rate in using a combination of cadaver bone, bone marrow aspirate concentrate (BMAC), and recombinant human bone morphogenetic protein 2 (rhBMP-2) [11, 12]. Unlike an open approach such as iliac crest harvest, bone marrow aspiration is far less invasive and is associated with less morbidity. In this study, all patients present with excellent regenerated bone volume and were good candidates for dental implants during their 6-month follow-up.

30.2 Indications

1. Maxillofacial defects lacking considerable soft tissue and bony structures.
2. Patient is not a candidate for onlay block graft due to significant soft tissue deficiency.
3. When fibula free flap is too excessive and results in inadequate esthetic and functional results.
4. Patient is not a candidate for vascularized fibula free flap due to peripheral vascular disease, other comorbidities, and previous trauma/surgery to the lower extremities.

30.3 Contraindications

30.3.1 To Radial Forearm Harvest

1. History of trauma/surgery to both arms
2. Negative modified Allen's test and/or abnormal duplex ultrasound

30.3.2 To BMAC Harvest

1. Bone diseases: congenital (osteogenesis imperfecta), metabolic (osteopetrosis), and malignant (multiple myeloma)
2. History of trauma or radiation to harvest site

30.4 Preparation of Bone Marrow Aspirate Concentrate

The concept of tissue engineering revolves around three familiar principles: osteoconduction, osteoinduction, and osteogenesis [13, 14]. First, the allogenic bone chips provide a conductive scaffold for the process of bone regeneration. Second, the rhBMP-2 provides the activation signal for bone regeneration and recruiting signal for migrating osteogenic cells. Last, the BMAC can provide significant amount of mesenchymal stem cells that are capable of differentiating into osteocytes and osteoblasts [15].

The bone marrow can be aspirated from the anterior ilium, the posterior ilium, or the tibia. In this chapter, we will explore the anterior iliac approach.

The patient is first positioned in the supine position. Adequate padding at pressure points is needed to avoid nerve compression and postoperative pain and numbness. The anterior spinal iliac crest (ASIC) is marked and the iliac crest is outlined. The surgical site is then surgically prepped and draped.

A Nick incision using a #11 blade is made at least 2 cm posterior to ASIC. Care must be taken to avoid dissecting anteriorly to the ASIC to

prevent damage to the lateral femoral cutaneous nerve, which may lead to debilitating complications such as meralgia paresthetica. Using a hemostat, blunt dissection is done to create a pathway through several layers of the abdominal wall until contact with the bone. A BMAC trocar system, wetted with a heparin concentrate of 1000 U/mL, is then inserted into this pathway and directed to engage the ilium. A drill can then be used to engage the trocar tip into the medullary cavity. At this point, the bone marrow is ready to be collected. The inner trocar is removed, and the outer aspirating sleeve is left in the cavity. One milliliter of the heparin solution is first loaded onto each syringe to prevent coagulation during collection and processing. The heparin solution is injected into the bone marrow cavity prior to aspiration. At least 60 mL of the bone marrow should be aspirated for sufficient quantity of mesenchymal stem cells and osteoprogenitor cells [16]. This can be done with two distant harvest sites to maximize the amount of cell concentrate. The harvest sites can be approximated with a deep dermal suture and closed with the surgeon's preference of subcuticular or simple running suture.

The bone marrow aspirate is then processed immediately after collection. The aspirate must pass through a filter into conical tubes to remove microscopic clots and debris. Subsequently, the tubes containing the aspirate are then centrifuged twice. The first centrifugation at 2400 rpm for 10 min serves to separate the acellular plasma layer from the cellular concentrate, with the latter transferred into another conical tube. Another centrifugation of 3400 rpm for 6 min serves to isolate the pellet of BMA/white cell concentrate. The pellet is then resuspended, and a final hemanalysis and complete blood count with differential are performed [10].

Once the radial forearm is harvested and anastomosed, the BMAC is then mixed with rhBMP-2 protein and crushed corticocancellous bone. The mixture is packed into the alveolar ridge defect with a resorbable mesh for stability.

30.5 The Radial Forearm Free Flap

The radial forearm free flap relies on the radial artery for its blood supply and a dual venous system for drainage through the cephalic vein or venae comitantes [17]. The flap usually includes the volar forearm skin, the antebrachial fascia, and the intermuscular fascia.

Preoperatively, it is important to assess any history of trauma or surgery to the donor arm. Modified Allen's test (MAT) is performed to determine the vascularity of the donor site. A positive MAT indicates adequate dual arterial supply of the hand by the radial artery and the ulnar artery, such that harvesting the former would not result in ischemia of the hand. A negative result, however, is an absolute contraindication to manipulation of radial artery. A secondary test such as duplex ultrasound can be used for confirmation in those with abnormal result. Fifteen percent of the population can be expected to have a positive MAT result, and approximately 11.6% of those will show abnormal duplex ultrasound examination [18]. It is important to avoid using the donor arm for IV access.

Utilizing the Doppler ultrasound, the flap design is drawn on the volar forearm with careful outlining of the vessels. The distal margin of the flap is 3 cm proximal to the wrist crease, and the proximal margin is dependent on the size needed for reconstruction. A tourniquet of 250 mmHg can be used for exsanguination of the arm. The uptime is recorded at this point. The incision is made using the flap outline. The dissection begins at the distal margin through the skin and subcutaneous tissue. Blunt dissection technique is carried down until recognition of the flexor tendons. The dissection is then advanced proximally with attention to identify the vascular pedicle commonly found in the septum between the brachioradialis and the flexor carpi radialis muscles. The deep branches of the radial artery can be ligated and divided to release the deep margin of the vascular pedicle. The proximal margin of the flap can then be incised, and at this point, the dissection can advance distally and subdermally. After locating the intersection of the brachioradialis and flexor carpi radialis, the two muscles are retracted. The intermuscular septum can then be further isolated up to the antecubital fossa. Any attached fascia or perforators should be ligated and divided. After achieving adequate vessel length for the recipient site, the tourniquet is released and downtime is recorded. The flap is then reperfused for 15 min, and the surgeon can

use this time to verify the patency of the vessels and its skin perforators using the Doppler ultrasound. Once the recipient site is ready, the vessels can be ligated and divided.

At this time, the radial artery can be anastomosed to an artery of the recipient site (usually the facial artery) and the vena comitans to a vein (usually the facial vein). Once the graft is placed with a resorbable mesh, the radial forearm can be sutured around and over the graft.

Prior to closure of the donor site, a drain is placed in the subdermal area. The donor site is then closed with dermal sutures or staples. Split-thickness skin graft is used to cover the cutaneous defect with overlying wound-VAC therapy for optimization of wound healing.

30.6 Case Presentation

A 45-year-old female presents for reconstruction of left maxillary alveolar ridge defect after a motor vehicle accident 4 months prior. The injury left her with severe vertical and horizontal alveolar bone deficiency, as well as missing teeth from the left maxillary central incisor to the second premolar. After presenting several treatment options, the patient opted for reconstruction of maxillary alveolar ridge defect using radial forearm free flap combined with a tissue-engineered bone graft, which consists of allogeneic bone, rhBMP-2, and BMAC. Careful consideration was taken into account the swelling and edema caused by BMP on the overlaying radial forearm flap. Due to the pliability and flexibility of the radial forearm skin and fascia, no venous congestion nor diminished arterial flow was encountered. We do however recommend taking a generous amount of the skin and fascia to compensate for the swelling.

A series of surgeries were planned for the complete reconstruction of her facial cosmesis, but the first planned surgery was to reconstruct her alveolar bone height and restore her dentition with endosteal implants. Due to her avulsive injury to her anterior left maxillary alveolus, she was missing teeth #9 thru #13 (Fig. 30.2). She also lacked the soft tissue for a tradition block bone graft or an only graft.

A thorough discussion of treatment options includes All-on-4 restoration option versus full mouth extraction and complete dentures versus bone grafting and placement of implants. The patient opted to go with the bone graft since she deferred to have her remaining teeth extracted. Various osteocutaneous microvascular flaps were considered such as the free fibula flap and osteocutaneous radial forearm flap, but all were too bulky and/or have incorrect geometric shape for the defect. After careful consideration and a thorough discussion with the patient, we decided on a novel reconstruction using a radial forearm free flap combined with a tissue-engineered bone graft consisting of allogeneic bone, rhBMP-2, and BMAC. A modified Allen's test was performed to see if the ulnar artery had adequate perfusion for the entire hand, which was positive (normal).

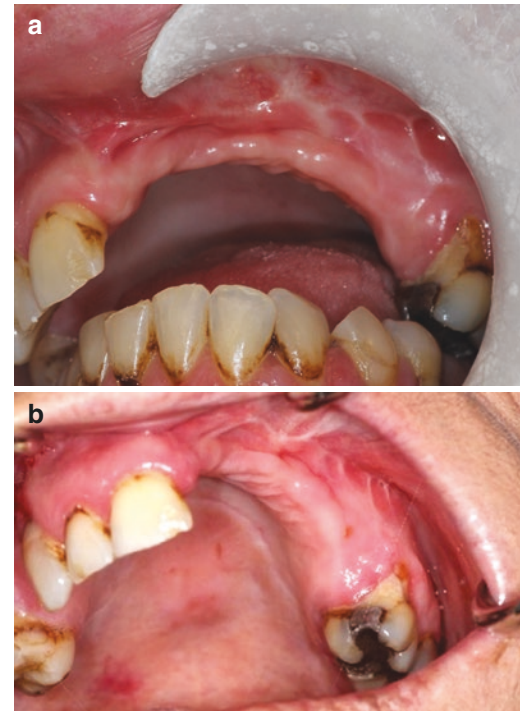

Fig. 30.2 (**a**) Loss of the left maxillary alveolar ridge with teeth #9 thru #13. (**b**) Post-traumatic defect with fibrous scar tissue

The surgery consisted of two teams, the first team exposing and preparing the alveolar defect as well as harvesting the BMAC from the iliac crest and the second team harvesting the radial forearm which is under tourniquet pressure to minimize blood loss and maximize visualization. The radial forearm skin paddle and pedicle (radial artery and vena comitans) were raised in-between

the brachioradialis muscle and flexor carpi radialis to the antecubital fossa to gain adequate pedicle length. Once the radial forearm was harvested, the radial artery was anastomosed to the left facial artery and vena comitans anastomosed to the facial vein. The area of the left maxillary ridge was denuded of its scar tissue and the remaining bone was exposed (Fig. 30.3). Thirty milliliter of crushed corticocancellous bone (University of Miami Tissue Bank, Miami, FL) and a small rhBMP-2 (Infuse) kit (Medtronic Sofamor Danek, Memphis, TN) were mixed with 60 BMAC. The tissue-engineered graft was placed and packed onto the defect with SonicWeld Resorb-X®, a 100% amorphous, noncrystalline poly-DL-lactic acid (PDLLA) mesh (KLS Martin, Jacksonville, FL) (Figs. 30.4 and 30.5). The radial forearm was then sutured around and over the graft (Fig. 30.6). With an uneventful postoperative course, the patient was discharged in 6 days. After 6 months, the bone graft demonstrated ossification and consolidation on CBCT for placement of dental implants. The flap was viable with good perfusion and Doppler signal throughout the 6 months. Clinically the reconstructed alveolar ridge showed excellent height and ridge, and the skin of the radial forearm mucosalized (Figs. 30.6 and 30.7). Three dental implants were placed in a solid bone (Figs. 30.8 and 30.9); at the same time, a vestibuloplasty was performed with split-thickness skin graft from the thigh (Fig. 30.10). The patient was referred to a maxillofacial prosthodontist for a fixed partial denture for the reconstructed ridge.

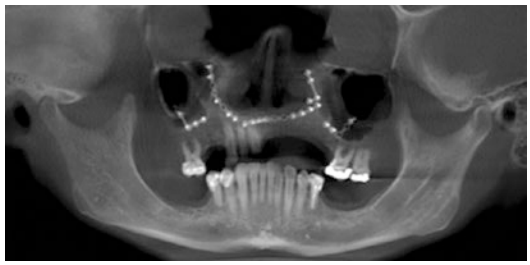

Fig. 30.3 Orthopantomogram on initial visit demonstrating deficient alveolar bone for dental rehabilitation

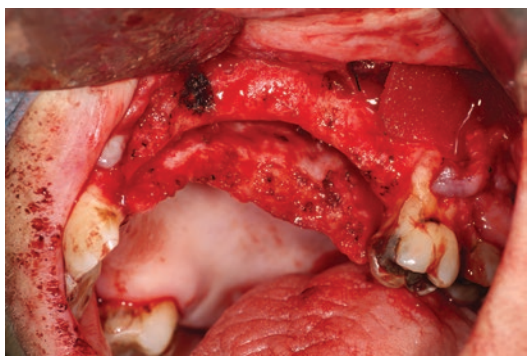

Fig. 30.4 Exposure of alveolar defect

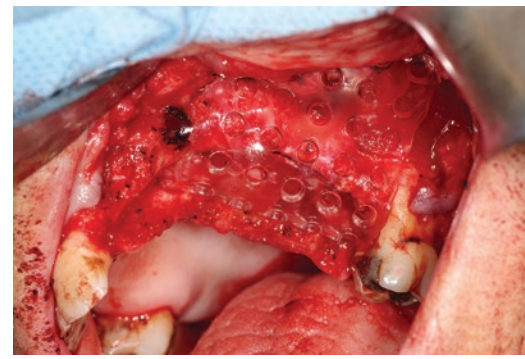

Fig. 30.5 Poly-DL-lactic acid mesh carrier

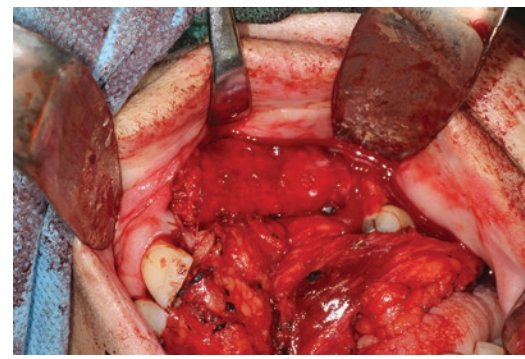

Fig. 30.6 BMP + B MAC + allogeneic bone

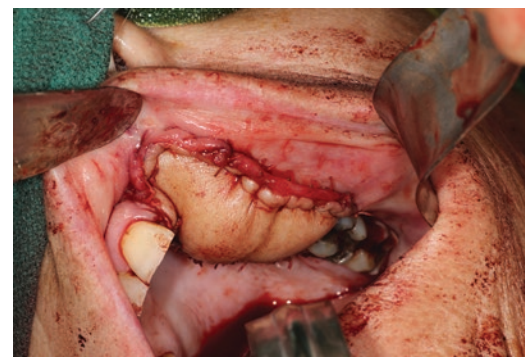

Fig. 30.7 Radial forearm flap positioned over the tissue-engineered bone graft

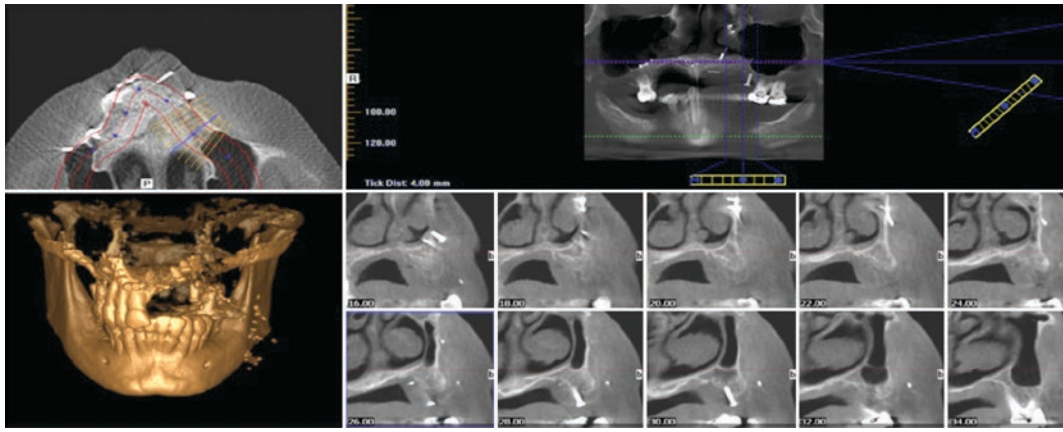

Fig. 30.8 CBCT 5 months post-op imaging demonstrating consolidation and maturation of avascular bone graft underneath radial forearm flap

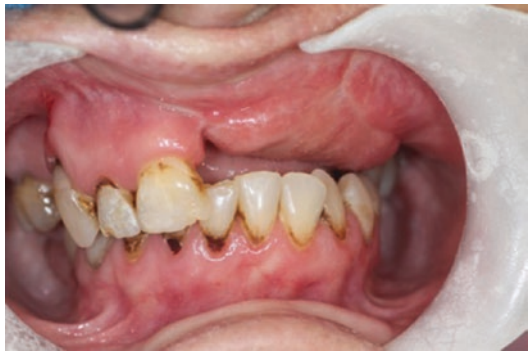

Fig. 30.9 Six months s/p RFFF + bone graft + BMP + BMAC

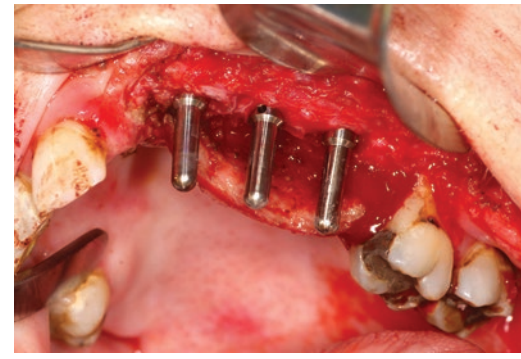

Fig. 30.11 Placement of three endosteal implants into the reconstructed alveolar ridge

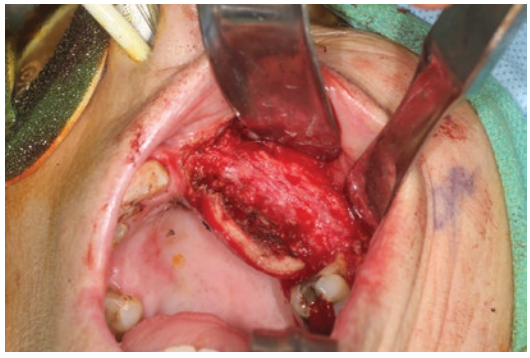

Fig. 30.10 Debulking of skin paddle showing excellent ridge formation

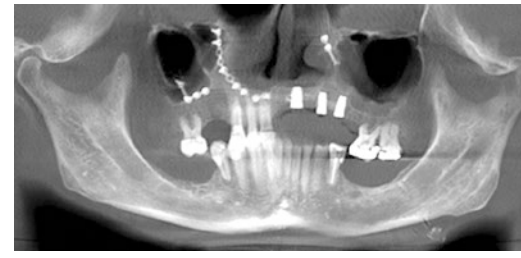

Fig. 30.12 CBCT of endosteal implants in reconstructed maxillary ridge, week postoperative

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31 Postoperative Complications of Mandibular Fracture Management

Giuseppe Spinelli, Francesco Arcuri, Domenico Valente, and Tommaso Agostini

31.1 Introduction

Mandible fractures account for 35–80% of all maxillofacial fractures. Etiologic factors of facial fracture are variable and depend on regional and social characteristics. In a review of more than ten thousand patients, mandibular fracture is most common in patients aged between 18 and 24 years and seen four times as frequent in male patients compared with female patients. Mechanism of injury is commonly assault, followed by motor vehicle accidents and falls [1].

When considering between open and closed reduction of mandibular fractures, the advantages should be weighed against the disadvantages. Considerations include the site and characteristics of the fracture and the morbidities of the treatment. Unwanted results including bony ankylosis or decreased mouth opening can be prevented by early mobilization of the mandible. Advantages of closed reduction include simplicity, decreased operative time, and avoidance of damage to adjacent structures [2]. Disadvantages of maxillomandibular fixation include inability to directly visualize the reduced fracture, need to keep the patient on a liquid diet, and difficulties with speech and respiration [3].

Closed reduction of mandibular fractures can adversely affect bone, muscles, synovial joints, and periarticular connective tissues. The effects of immobilization on bone have been recognized in the orthopedic literature for many years as "disuse osteoporosis." Rigid fixation of the mandible refers to a form of treatment that consists of applying fixation to adequately reduce the fracture and also permit active use of the mandible during the healing process [4]. The four basic principles are (1) anatomical reduction, (2) stable fixation, (3) atraumatic surgical technique, and (4) postoperative active function.

31.2 Principles of Surgical Treatment

The timing of surgery is still controversial in the literature. Some studies have suggested immediate surgery; other reports have supported to wait the decrease of the edema of the soft tissues before operating. The diagnosis is performed by plain film imaging sometimes supplemented by CT. An orthopantomogram radiograph is available in most hospital emergency departments and is the initial radiograph of choice for any patient with suspected mandibular fracture [5].

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31.2.1 Surgical Technique

Intermaxillary fixation is placed prior to reducing a fracture. This allows for use of the occlusion to aid in anatomical reduction of the fracture. Use of intermaxillary screws or full-arch bars combined with maxillomandibular fixation are the preferred methods. They maintain the occlusion postoperatively with elastic bands as needed during physiotherapy. They are usually removed after 4 weeks postoperatively.

The surgical approach depends on the site of the fracture. Either a transoral, vestibular, or transfacial approach may be performed. A facial approach provides excellent access but also produces a facial scar and adds the risk of damage to the facial nerve. Most fractures, excluding those of the condyle, can easily be approached through a transoral incision [6].

A subperiosteal dissection with a periosteal elevator provides adequate access for reduction of the fracture and placement of fixation. Attention should be given to avoid damage to the mental nerve, which exists the mental foramen near the apices of the premolar teeth. If additional exposure is needed, the nerve can be released by gently scoring the periosteum surrounding the nerve. Bone-reducing forceps are often helpful in reducing the fracture while adapting the bone plate. This also provides interfragmentary compression, making primary bone healing more likely.

The smallest bone plate that will provide adequate stability under functional loads during the healing period is chosen. The intermaxillary fixation that aided reduction of the fractures during plating is removed after the fixation is applied. A soft diet is recommended for at least 4 weeks after miniplate fixation. It is important during the postoperative period to regain preinjury function, including maximal mouth opening, with active physiotherapy [7]. An overview of the different procedures is as follows.

31.2.2 Compression Plates

Compression plates cause compression at the fracture site making primary bone healing more likely. These plates can be bent in only two dimensions because of their design, and if they are not contoured properly, they are unable to produce compression. It is important to avoid compressing oblique fractures. They also require bicortical screw engagement to produce even compression along the fracture line [6].

31.2.3 Reconstruction Plates

Reconstruction plates are recommended for comminuted fractures and also for bridging continuity gaps. These plates are rigid and have corresponding screws with a diameter of 2.3–3.0 mm. Reconstruction plates can be adapted to the underlying bone and contoured in three dimensions. A problem that may be associated with conventional reconstruction plates is loosening of the screws during the healing process leading to instability of the fracture [7].

31.2.4 Locking Reconstruction Plates

In 1987, Raveh et al. [8] introduced the titanium hollow-screw osteointegrated reconstruction plate (THORP). This system achieves stability between the screw and plate by insertion of an expansion screw into the head of the bone screw. Locking plate/screw systems offer advantages over conventional reconstruction plates.

These plates function as internal fixators by achieving stability by locking the screw to the plate and allow greater stability as compared to conventional plates. Fewer screws are required to maintain stability. The most significant advantage of this type of system is that it becomes unnecessary for the plate to intimately contact the underlying bone in all areas. As the screws are tightened, they will not draw the plate and underlying bone toward each other [9].

31.2.5 Lag Screw Fixation

Lag screws can provide osteosynthesis of mandibular fractures. They work well in oblique fractures and require a minimum of two screws. The lag screw engages the opposite cortex while fitting passively in the cortex of the outer bone

segment. This can be accomplished by using a true lag screw or by overdrilling the proximal cortex. This causes compression of the osseous segments and provides the greatest rigidity of all fixation techniques. The lag screws can be placed through the opposing cortices between the mental foramen and inferior to the teeth [10].

31.2.6 Miniplates

Miniplates typically refer to small plates with a screw diameter of 2.0 mm. These plates have been shown to be effective in treating mandibular fractures [11]. Typically a superior and inferior plate is required for adequate fixation. An advantage of these plates is that they are stable enough to obviate the need for maxillomandibular fixation.

They are less likely to be palpable, which reduces the need for subsequent plate removal. Typically screws are placed monocortically but may be placed bicortically when positioned along the inferior border of the mandible. A minimum of two screws should be placed in each osseous segment [12].

31.2.7 Bioresorbable Plates

Bioresorbable plates are manufactured from varying amounts of materials including polydioxanone (PDS), polyglycolic acid, and polylactic acid. Complications associated with these plates include inflammation and foreign body-type reactions. The common complication which we encountered during their use was screw head fracture during tightening. Consideration may be given for use in pediatric patients with the understanding of the possible complications [13].

31.2.8 Three-Dimensional Miniplates

These miniplates are based on the principle that when a geometrically closed quadrangular plate is secured with bone screws, it creates stability in three dimensions. The smallest structural component of a 3D plate is an open cube or a square stone. Clinical results and biomechanical investigations in a study have shown a good stability of the 3D plates in the osteosynthesis of mandibular fractures without major complications. The thin 1.0 mm connecting arms of the plate allow easy adaptation to the bone without distortion. The free areas between the arms permit good blood supply to the bone [14, 15].

31.3 Complications

Several factors can influence the incidence of surgical complications including inappropriate surgical technique, patient's medical status, substance abuse, concomitant injuries, and fracture location and type. Complications include postoperative malocclusion, infection and wound dehiscence, nonunion/malunion, nerve injury, scars, teeth damage, and TMJ disorder [16, 17].

Complication rates vary between studies. Paza et al. [18] reported a total complication rate of 20% with a low reoperation rate (3%). Siddiqui et al. [19] reported a higher rate of postoperative complications (58.1%), and Bormann et al. [20] described a 15% complication rate.

Complications following mandible fracture repair may be the result of the severity of the original injury, the surgical treatment, or patient non-compliance with the postoperative restriction. Complications related to mandibular fractures present challenges to even the most experienced trauma surgeon.

The consequences of complications may include problems in anatomic form (aesthetic deformity) or residual functional discomfort. Complication rates have improved since the early days of wire fixation, but even open reduction and internal fixation can produce undesirable results [19, 20].

31.3.1 Infections

It is the most common complication after mandibular fractures. A significant delay in treatment has also been associated with an increase in infection rates. Other factors include mobility of the bony segments across the fracture site or loosening of screws after osteosynthesis. Poor

plate adaptation, inadequate cooling during drilling, or placing the screw in the fracture line itself can lead to postoperative infection.

Leaving a damaged tooth in the line of fracture can also lead to an increased incidence of complications. Clinical signs are cellulitis, abscess formation, fistula, osteomyelitis, and rarely necrotizing fasciitis.

Clinical examination and plain radiography assess the status of the hardware. CT is appropriate when there is concern that the infection involves the neck. Specimens for bacterial culture and sensitivity studies should be done for antibiotic therapy.

Complication rates vary between studies: Ellis and Walker [21] reported a high infection rate (25%). Iizuka and Lindqvist [12] described an infection rate of 6.6%. Fox and Kellman [15] reported a lower rate of local wound infection and dehiscence (2.9%), whereas Seemann et al. [22] described an incidence of 5.9%.

The treatment of infected fractures involves (1) incision and drainage, (2) irrigations of the wounds, (3) systemic antibiotics, (4) removal of devitalized teeth/bone/hardware, and (5) new fixation of the fracture.

31.3.2 Nonunion

Nonunion is the failure of a fracture to unite and requiring additional treatment to achieve fracture union. Mobility is the major cause of nonunion. Infection, mobility, systemic disease, advanced age, and mandibular atrophy are contributing factors. The reoperation rate for nonunion varies between studies. Maloney et al. [23] described a rate of 6.31%, whereas Bochlogyros [24] described an incidence of 3.9%. Haug and Schwimmer [25] described a rate of 3.2%, while Mathog et al. [26] reported a rate of 9%.

Debridement of the fracture fragments; bone grafting, usually from the iliac crest; and rigid fixation with internal or external fixation are the treatments of choice [23–26].

31.3.3 Malocclusion

Improper alignment of the fracture fragments results in malocclusion and facial asymmetry. Malunions occur for at least one of several reasons: (1) inadequate occlusal and osseous reduction during surgery, (2) inadequate application of internal hardware, and (3) inadequate stability. Other contributors to fracture nonunion include impaired healing capacity secondary to comorbidities, tobacco use, and infection.

Significant malunion of the mandible will produce asymmetry and/or functional disturbances and can only be resolved through planned osteotomies [20, 22].

Treatment strategies vary from patient to patient and with each surgeon's experience in using different techniques. Comprehensive management of malocclusion and malunion requires a full orthognathic workup. Standard osteotomies are performed at a different site from the malunion for restoration of preinjury occlusion [27].

31.3.4 Nerve Injury

Sensory nerve injury (inferior alveolar and mental nerves) commonly occurs with mandibular fractures. In 11–59% of displaced mandibular fractures, there is sensory nerve injury at diagnosis. Causes of inferior alveolar or mental nerve injury are displaced fractures, delay in treatment, and improper use of drill or screws.

According to the literature, the overall prevalence of sensory disturbance after treatment of mandibular fractures is variable (53.8–76.1%) [28, 29].

Facial nerve dysfunction can result from mandibular trauma. Damage of the facial nerve after temporal bone fractures can lead to paralysis. Condylar dislocations can cause facial nerve injury distal to the stylomastoid foramen. Injury to the facial nerve branches usually takes place iatrogenically during surgical treatment, though lateral displacement of the condyle can cause facial nerve injury.

The marginal mandibular branch is the one usually injured. The surgical anatomy of this branch has been well described by Dingman, and meticulous dissection under the platysma in the region of the facial artery with identification of the branches of the marginal mandibular nerve can prevent injury to this nerve which varies between 0 and 48% [30].

31.3.5 Scars

Transfacial approaches to open reduction and internal fixation can lead to external scarring. Massages of the area with silicone topical gel are advocated to improve the appearance of the scar. Wounds contaminated by road debris like tar often produce pigmented scars that may improve with surgical treatment.

31.3.6 Teeth Damage

The immediate posttraumatic dentition status requires reliable evaluation for therapeutic management as well as the preinjury dental status of the upper and lower jaws recording the missing teeth. Osteosynthesis with screws can cause damage to the roots of the teeth with subsequent risk of tooth infection in addition to loss of vitality. A possible injury should be immediately referred to the dentist. Other dental injuries (coronal fracture, root canal, subluxation) can result from direct trauma [22, 27, 31].

31.3.7 Temporomandibular Joint (TMJ) Disorders

Mandibular fracture can cause delayed TMJ derangement (limitation of mouth opening, pain during the movement, swelling) both on the nonfractured side and on the fractured side of the mandible. The physiokinesis therapy is mandatory especially after treatment of fractures of the mandibular condyle.

In some cases arthrocentesis with intraarticular infiltration of hyaluronic acid and arthroscopy can help solve the disorders. Rarely, posttraumatic ankylosis requires surgical intervention and removal of the ankylotic block [32].

31.4 Discussion

Our data include 389 patients (258 males [66.3%] and 131 females [33.7%]) treated surgically for mandibular fracture between January 2000 and December 2011 in our Department. The mean age of patients was 28.7 years with a range of 17 to 54 years.

Daily abuse of alcohol was detected in 98 cases (25.2%); 82 patients reported drug abuse (21%). The average time between the accident and the surgery was 2.5 days. Fifty-three patients developed postoperative complications (overall complication rate: 13.6%) which were divided into major complications requiring return to the operating room (7.4%) and minor complications managed in the outpatient clinic (6.2%).

Twenty-one patients (5.4%) reported malocclusion and five patients developed nonunion (1.3%). The reoperation rate to manage malocclusion and nonunion was 1.9%. The rest of the group was managed conservatively in the outpatient clinic by prolonged guiding elastic therapy and orthodontic treatment.

Thirty-two patients reported postoperative infection (8.2%). Seventeen patients (4.3%) presented a dehiscence of the surgical wound which required a prolonged antibiotic therapy and the subsequent removal of the miniplates at least 45 days postoperatively with the resolution of the complication.

The rest of the group experienced minor complications; they were managed in the outpatient clinic by incision and drainage, irrigation of the wound, and prolonged antimicrobial therapy which solved the condition.

The complications reported in our experience included postoperative malocclusion, infection, wound dehiscence, nonunion, and reoperative

surgery [33]. Regarding postoperative infection, our rate was lower (8.2%) compared with other investigations. Ellis and Walker [21] reported a higher infection rate (25%) and an overall complication rate of 28%. Iizuka and Lindqvist [12] described an infection rate of 6.6%.We had a 4.3% rate of wound dehiscence that was similar to previously published studies.

Fox and Kellman [15] reported a lower rate of local wound infection and dehiscence (2.9%), whereas Seemann et al. [22] described an incidence of 5.9%. The reoperation rate for malocclusion and nonunion was 1.9% which is in line with our studies.

31.5 Conclusions

The global incidence of screw loosening, wound dehiscence, plate exposure, infection, reoperation, and plate removal vary among studies; however an increased rate of complications is demonstrated in patients with substance abuse or medical diseases.

Proper surgical technique (aseptic procedure, frequent intraoperative irrigation, realignment of the fracture, immobilization of the bone) associated with a strict follow-up (antibiotic, diet restriction) and early recognition of postoperative complication are mandatory for a good prognosis.

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32 Use of Porcine Urinary Bladder Matrix (UBM-ECM) in the Head and Neck Region

Bruce A. Kraemer and Amanda Gryniewicz Rowe

32.1 Introduction

The ultimate goal for head and neck wound reconstruction is restoration of a scarless, durable, symmetrical, and cosmetically normal appearance which has good color match, tissue mobility, and function. The use of extracellular matrix (ECM) wound devices has provided topical wound treatment options and enhanced healing for wounds that previously were considered amenable to only more complex surgical reconstructive procedures [1, 2]. These ECM wound devices promote healing via a process of constructive remodeling wherein the body replaces the wound device with healed tissue(s) much like what was previously present. Because this healing process requires time for the new tissue formation, specific wounds such as intra- and extra-oral or alimentary wounds are still best left to standard one-stage flap reconstruction. While some practitioners have used these devices as a primary reconstructive modality [3], in our experience we have found UBM-ECM wound devices have optimal utility in an adjunctive role in extra-mucosal head and neck reconstructions in medically challenging patients who are poor surgical candidates. This chapter reviews our clinical experience with these devices over the last 6 years of wound device use (NB—excluded from this review is UBM-ECM use in burn wound management).

32.2 Clinical Series

In this retrospective review of our initial 373 wounds treated with UBM-ECM, 35 patients with 40 wounds had the device applied to the head and neck region with 2 patients having multiple sites treated. There were 14 males and 21 females with ages ranging from 23 to 82 years. The treatment of 19 open forehead and scalp region wounds was the most common device use with nasal reconstruction; both traumatic (4 cases) and post Mohs surgery reconstruction (6 cases) comprising the next largest group. Treatment of facial scarring was done in eight wounds: severe acne scarring, scarring after a windshield injury or animal attack, old post-traumatic facial scarring, self-inflicted cheek scars, and a non-healing radiated cheek wound. One ear large keloid excision wound, one intra-oral antral fistula wound in a smoker, and a final post-radiation intraoral scarring patient completed the series.

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32.2.1 Results

This is a review of use of a new wound treatment modality over a 6-year period. As the amount and formulation of the UBM-ECM device used was based on our accumulated clinical experience, there was not consistent application of the formulations used or the amounts placed, and new device formulations were used as they became clinically available. As such, comparisons of healing rates and time to closure among these patients has little foundation, but an appreciation of the lessons learned in an attempt to establish best practice seemed warranted. Forehead and scalp wound patients as a group often needed several device applications with initial patients having alternate day powder placed and later patient have larger volumes of several formulations placed less frequently. A secondary skin graft was done in nine of these wounds. One patient in particular with a large radiated full-thickness skull wound and metastatic angiosarcoma healed poorly and died from his underlying disease with his open wound. Nasal wounds had good healing, and the forehead flap patients liked the result with a standard two-stage transfer that did not need later secondary thinning or revision. The ear keloid patient had some thickening of the healed ear scar return at 2 years, but the adjacent involved neck scar region appeared normal. Facial scarring wound patients showed improvements with open wounds, but the one patient with contracted old closed wounds showed little improvement in tissue formation or softening of the contracted skin. The two intraoral uses where the antral closure patient picked the device out of her wound and the radiated patient removed the bolster stent at postoperative day 2.5 had no long-term improvement.

32.3 Discussion

The robust blood supply of the head and neck region makes possible many local tissue reconstructive options not possible in other body regions. However, the frequency of skin cancers of this region in an ever-aging population with numerous medical comorbidities can result in patients who have exhausted standard local treatment options leaving them with few suitable local treatment options. While these patients may accept a suggested complex surgical procedure, they often do so reluctantly after being told there are no other possible treatment options. We have found that UBM-ECM wound devices have allowed us to do less complex procedures in some patients who are mindful of the increased total time of healing and pleased that they are given new options for treatment in contrast to extensive surgery. Others have found these devices useful in the head and neck region treating flap donor sites [4].

Proper wound bed preparation is an essential prerequisite for optimal healing with the use of ECM wound devices. Obtaining a wound bed void of necrotic tissue and devitalized bone must be achieved prior to placement of the UBM-ECM wound device. The wound device must also be apposed to and retained undisturbed in the wound bed so that the device can be replaced by the host's neo-tissue formation. These prerequisites are similar to Integra® Bilayer Matrix Wound Dressing use which has been available for use in the head and neck region since the mid-1980s [5–24]. However, the UBM-ECM wound devices have enhanced utility in that they been found to perform well in the face of bacterial contamination [1, 2, 25, 26]. Wounds that have a wound bed primarily comprised of bone are the most challenging tissue as one must get to a bony layer that has punctate bleeding without damaging the underlying structures such as the brain. It is important to not induce further bone injury with debridement so for larger debridements, while electric burrs may be used for the majority of the debridement, the final debridement layer is removed with rongeurs or curettes. It is also important for the outer margin of the bone debridement to extend under the soft tissue margin of the wound by several millimeters so there is adequate peripheral soft tissue at the margin to interact with the newly placed ECM wound device. Neurosurgical scalp wound patients, especially those with older meningioma procedures, can be problematic in that the variety of materials that have been historically used for treating the cranial

defect must be removed from the exposed wound regions before stable healing occurs.

ECM wound device therapy usually needs a secondary dressing that can be as simple as a head wrap or cap, silicone sheets, or sutured dressings. We have found shaving a 3–4 cm rim of hair around scalp wound helps with both keeping the wound clean and allowing for placement of a polyurethane sheet dressing which allows for retention of sufficient moisture to facilitate the constructive remodeling healing response. Alternatively, one can keep the device hydrated with a hydrogel with a secondary Telfa-type overlying dressing. One needs to be sure that the device has adequate hydration and should it appear too dry or healing is slowed, a trial of placing increased moisture should be tried before a further treatment.

Over the last 6 years, we have been using the UBM-ECM device; we have evolved in our thinking and utilization of the wound device. Initially we began using it much like other topical wound therapies only to appreciate that larger amounts of the device could be successfully applied at one setting, so we moved to placing multiple device formulations at the time of the initial operative wound bed preparation. The subsequent care was simplified as it only required placement of small amounts of hydrogel on top of a secondary dressing which caused little patient pain and discomfort. Suturing the device into the wound either directly or with a secondary dressing sutured at the margins has also facilitated postoperative care. The wound is then observed until it is healed or until the wound has filled in to a point a simple skin graft is possible.

UBM-ECM wound device healing occurs via a process referred to as constructive remodeling which occurs when the wound device is broken down at the wound bed surface and replaced with the host's native tissue(s). This newly formed tissue is typically a very close replica of the missing tissue, but as it is not identical to the missing tissue, it is improper to refer to this healing process as regeneration. Newly generated peptide fragments referred to as matricryptins, matrikinins, or matricryptic peptides which form as the host cells degrade the ECM device exert potent bioactivity with their newly exposed adhesions sites [27, 28]. These shorter fragments often have biologic responses that are distinct from, and often more potent than, those of the native parent molecule. It is the combination of all of these newly formed peptides which regulate the wide variety of injury and healing processes observed including angiogenesis, anti-angiogenesis, migration, differentiation, adhesion, as well as the associated antimicrobial activity that yields the less scarred constructive remodeling healing observed. A recent publication [29] is the most complete characterization of the composition of the EBM-ECM wound device and highlights the enhanced M-2 macrophage healing response noted in previous studies [30-32]. Over 500 proteins were identified via mass spectrometry, with 78% identified as MatriSome and MatriSome-associated proteins (Fig. 32.1). Sadtler et al. [29] found that within the MatriSome category of proteins (77% of total), 98% were collagens (especially Types 1, 3–6, and 14), 1% ECM glycoproteins, and 1% proteoglycans. Within the MatriSome-associated fraction (1% of total), 55% were ECM regulators, 44% were ECM affiliated proteins, and 2% were secreted factors. The most abundant non-MatriSome proteins included actin, desmin, and hemoglobin. Additionally, adaptive immune T and B cells were also detected suggesting a role for antigen specificity to the remodeling response. What is clear is that a deeper understanding of all of the ECM components and their interactions with the body's immune system is needed to develop best practice UBM-ECM device use.

Clinically we have noted the normal inflammatory healing response is also greatly reduced as patients experience less swelling, scarring, and pain in the treated regions both early on and in the long term. We have not observed the restoration of skin appendages; however the UBM-ECM wound device provides a healthy healed wound which is amenable to later hair follicle grafting procedures.

Mohs surgical management of skin cancers of the head and neck has become more common-

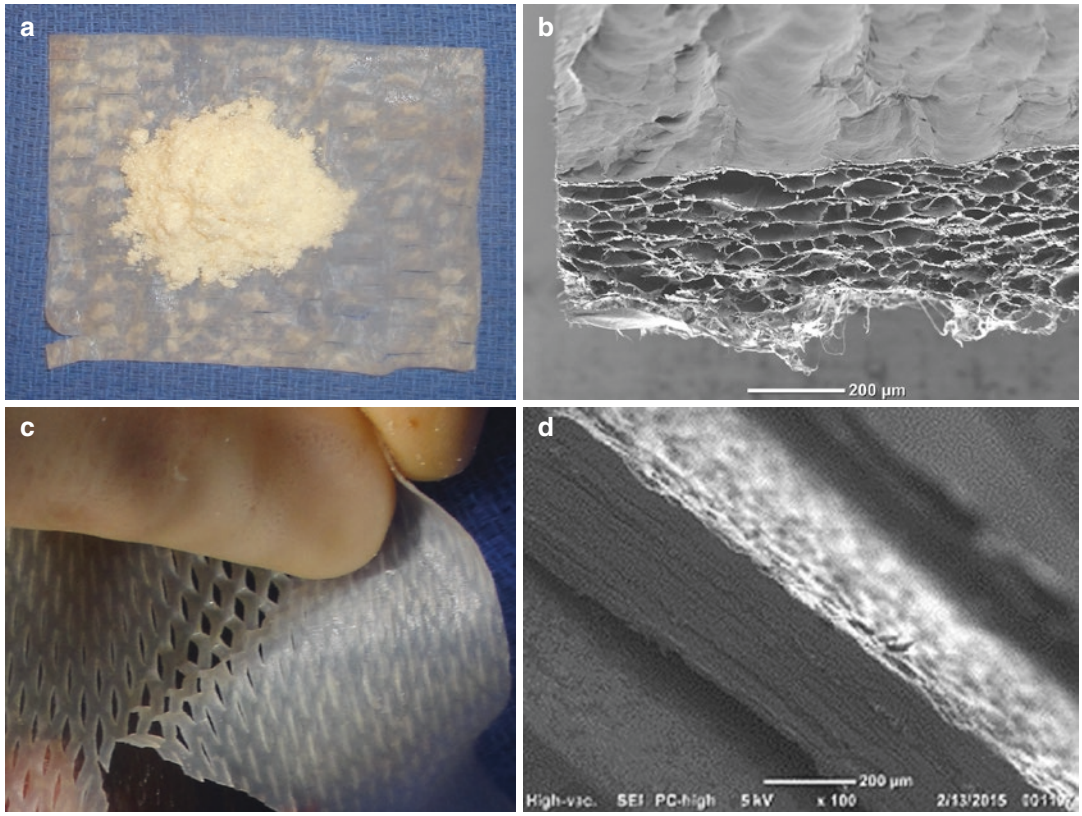


Fig. 32.1 UBM-ECM Formulations. (**a**) The freeze-dried formulation of the UBM-ECM is available as a dual-layer lyophilized sheet wound device (Cytal® Burn Matrix) or ground into a powder (MicroMatrix®, overlying powder). (**b**) Scanning electron micrograph of the lyophilized (freeze-dried) sheet shows the natural ECM matrix structure after complete decellularization. Note both the overlying intact smooth surface of the intact basement membrane layer and the honeycombed ECM matrix structure which optimally presents the ECM constituents for host tissue interaction. (**c**) The thicker fenestrated vacuum-pressed sheet formulation marketed as a 3- or 6-layer Cytal® Wound Matrix. (**d**) Scanning electron micrograph of the vacuum-pressed 6-layer sheet demonstrating the denser, compressed ECM matrix. These formulations persist in the wound bed the longest with the 6-layer device lasting the longest

place, and with micrographic margin control, the use of the ECM wound devices can have great utility. The use of an ECM wound device at the time that clear margins are obtained can help limit the scarring. Many Mohs surgeons now also perform their own wound closures and refer only the larger more complex patients for treatment. Forehead flaps are a mainstay of treatment of large nasal defects. Patient acceptance of the procedure can be an obstacle for the medically ill, the anticoagulated patient, and the younger patient who doesn't want to have a two-stage, let alone a three-stage, flap reconstructive procedure. We have also used the UBM-ECM device to manage a large nasal sidewall defect down to a smaller-sized alar rim defect which was amenable to a free ear cartilage-skin graft. A close working relationship with dermatologists is additionally useful for co-managing unusual inflammatory and healing issues of the scalp. We have treated two cases of pustular dermatosis with UBM-ECM which optimally responded to thalidomide treatment in one case and the second one which responded to FK-506.

Acute trauma degloving-type wounds can lead to large surface wound. These wounds typically heal well, but at the time of acute injury, patients want to be sure that all measures are taken to minimize scarring. For crush nasal or nasoorbitalethmoidal injuries, we have used the device in a single or multi-layer formulation depending upon the degree of injury for internal nasal lining and preservation of the nasal cavity. The high collagen content of the device also acts as a hemostatic agent to control the inevitable bleeding.

UBM-ECM wound devices are clinically available in three formulations, and an understanding of the performance characteristics of each formulation allows for the enhanced healing possible:

1. The MicroMatrix® powder (Fig. 32.1) produces a more rapid, robust healing response as the small particle size allows for rapid breakdown. It can be applied as a direct powder or mixed with saline to create a thick slurry for injection. It can be used serially with alternate day treatments or in large volumes in a single placement. We have found it useful to improved healing ("take") of skin grafts in wounds beds that are less than ideal or in patients who are poor surgical candidates.
2. The lyophilized sheet formulation (Fig. 32.1) is the device formulation prior to it being ground into powder and has a more sustained response over the period of 1 week or more as a single sheet or longer if it is "packed" into a wound.
3. The vacuum-pressed sheets (Fig. 32.1) are available in 3-layer and 6-layer sheet formulations as Cytal® Surgical Sheets. There is also an 8-layer sheet available as Gentrix® Surgical Thick sheets which is indicated for tissue reinforcement such as in hernia repair. We have found the suture-holding capacity of the vacuum-pressed sheets useful to secure the skin margins of an open wound, reduce skin closure tension as well as help to hold and retain other formulations of the UBM-ECM device placed deeper in the wound.

One of the greatest present limitations to UBM-ECM use comes from insurance companies classifying these devices as experimental and denying coverage. Also, there is limited reimbursement for outpatient use, so all of the cases in this series had the device placed in the operating room. Both of these limitations should be solved in the near future which will allow for more timely and rapid healing with use of these devices. More recently we have had success appealing the denial decisions after discussing the case with a medical director who has knowledge of surgery and wound care.

We found most patient had healed wounds with good outcomes with the following notes:

1. Scalp and forehead wounds tend to heal slower and often required several applications of the device to yield a wound able to be skin grafted or go on to complete closure.
2. Patients that initially had more aggressive bone debridement (as discussed) showed improved healing with fewer treatments.
3. The slowest healing occurred with therapeutic prior scalp radiation for cancer treatment and with pustular dermatosis of the scalp (until treated with tacrolimus – Case 10).
4. The wound device use in improving scar revisions holds great promise.
5. Contracted facial scars did not dramatically improve with placement of the device under the intact skin.
6. More research is needed to determine optimal use of the UBM-ECM wound device.

32.4 Clinical Cases

These clinical cases represent an evolution of our clinical experience trying to obtain optimal healing with the various formulations of the UBM-

ECM wound device. We found that placing larger amounts of the device at the operative setting proved to be both effective and saved patients' time and discomfort at subsequent follow-up visits. Powder was used more often in treating chronic wounds to stimulate a robust healing response in a chronic wound bed. Common to the management of all of these wounds is securing the device into the wound bed and the need for the wound devices to be kept moist with serial application of hydrogels and/or the use of moisture retentive dressings such as a polyurethane sheet dressing. Of particular note is the appearance of the lighter pink salmon-colored UBM-ECM-stimulated granulation tissue which forms (Figs. 32.2d, 32.3g, 32.8e, 32.9g, and 32.9h) and the formation of visible blood vessels in the newly formed tissue (Fig. 32.2j).

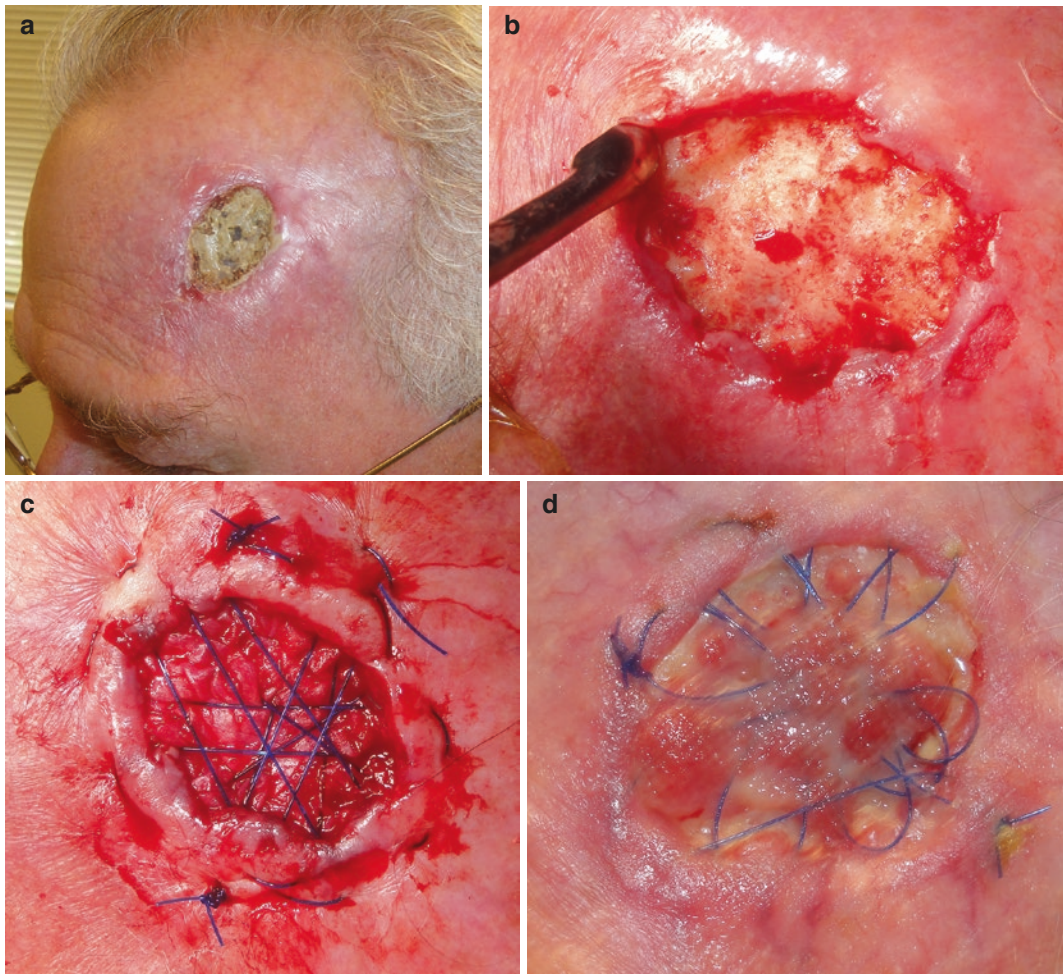


Fig. 32.2 A 65-year-old male having had a wide resection of stage 2 nodular melanoma of his left temple closed with a full-thickness skin graft, subsequent radiation therapy followed by a methicillin-resistant *Staphylococcus aureus* infection leading to this exposed bone open wound for 2 years. (**a**) Initial appearance of the left temple wound. (**b**) Wound after debridement of the outer necrotic bone. (**c**) Wound after placement of MicroMatrix® powder and Cytal® Burn Matrix and Prolene sutures placed to partially close wound and retain the wound device in the wound bed. (**d**) Wound 3 weeks postoperative. (**e**) Wound 7 weeks postoperative at time of repeat full-thickness skin graft. (**f**) Wound 2 weeks post-grafting. (**g**) Wound 6 weeks post-grafting. (**h**) Twelve weeks post-grafting, additional MicroMatrix® powder applied. (**i**) Fourteen weeks post-grafting nearly healed. (**j**) Five months later, debrided and additional MicroMatrix® powder applied. (**k**) Two months later, and after additional MicroMatrix® powder application the wound then heals—note the appearance of new blood vessels in the wound bed. (**l**) One year later. (**m**) Two years later

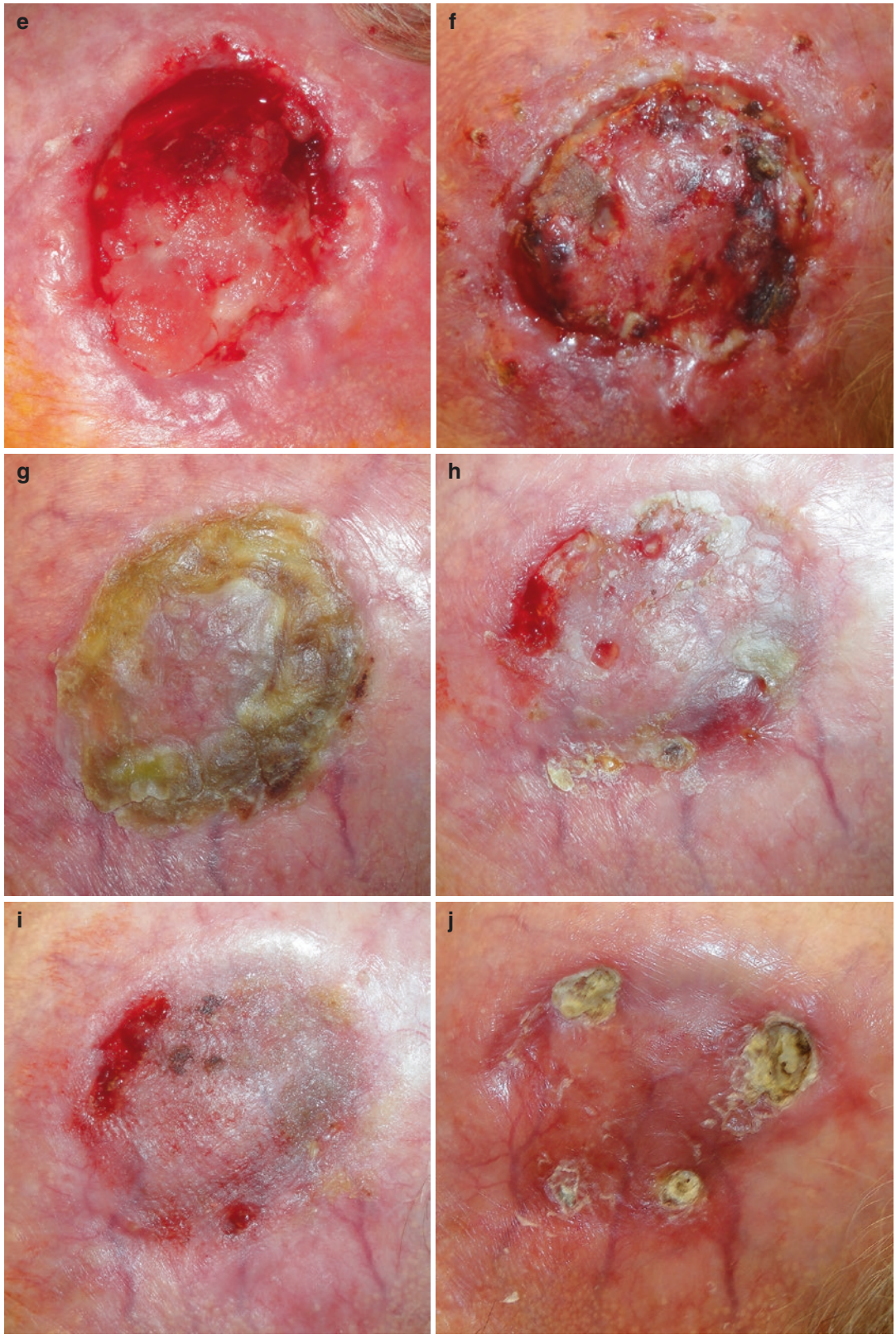


Fig. 32.2 (continued)

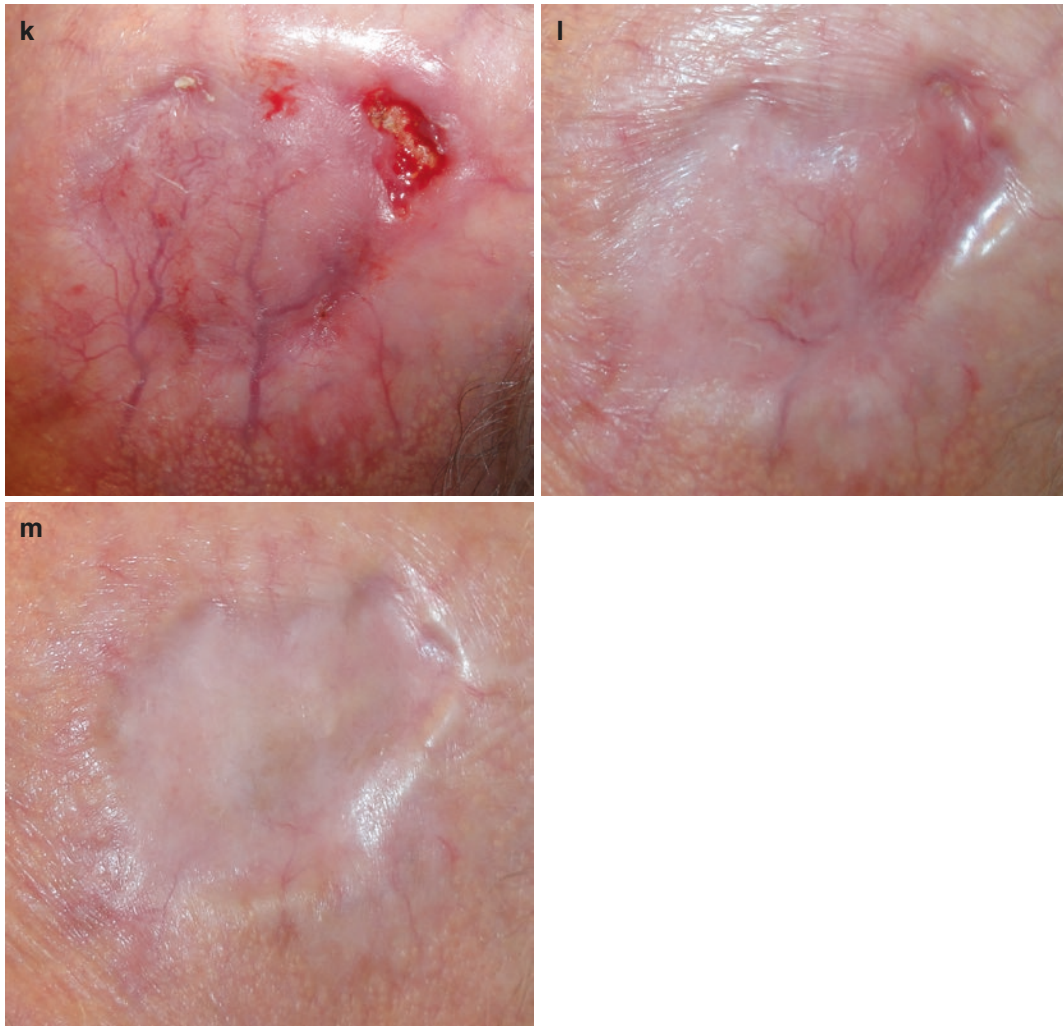


Fig. 32.2 (continued)

32.4.1 Forehead and Scalp

Initial management of this patient required removal of the obviously necrotic exposed bone prior to the placement of both MicroMatrix® powder and Cytal® Burn Matrix (Fig. 32.2). The device was retained in the wound with Prolene sutures used to partially close the circular as well as retain the wound device in the wound bed like a mesh. The healing was slow with several small foci of bone which failed to promote healing and needed removal. Of particular interest was the formation of visible new blood vessels which formed during the healing process. Integra® was not employed in this case due to the risk of infection in this chronic wound.

This patient who sustained a nearly fatal stroke had a poorly treated, infected, full-thickness skull defect with exposed, injured dura (Fig. 32.3). The dura repair device, while classi-

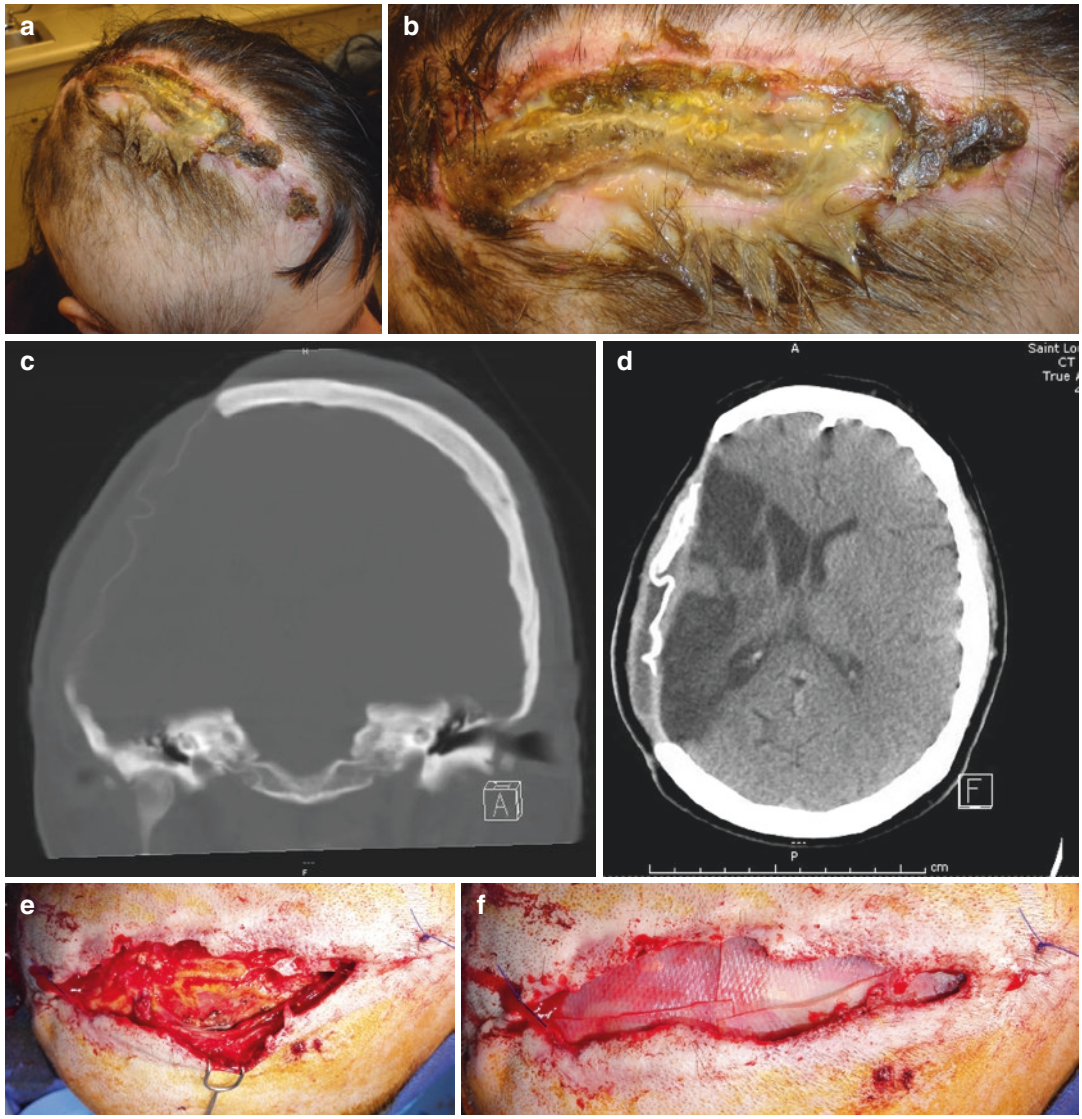


Fig. 32.3 A 42-year-old female with a scalp wound following a decompressive craniotomy for a major right middle cerebral artery stroke. (**a**, **b**) Initial wound appearance showing exposed central cranial bone edge and poor wound care. (**c**, **d**) CT scan showing the calvarial bone defect and the Durepair® dura regeneration matrix (bright wavy line in **d**) used for dural closure/reinforcement. (**e**) Debrided wound with bone debrided back to bleeding and the dural edge present just above the hooks—no CSF leak was noted. (**f**) After placement of MicroMatrix® powder and Cytal® 6-layer vacuum pressed sheet placement. (**g**) Four weeks postoperative showing residual ECM device still present in the wound. (**h**) Ten weeks postoperative with a region of bone still exposed. (**i**) One month after bone debridement and placement of additional MicroMatrix® powder. (**j**) Seven months later (11 months after initial procedure). (**k**) After neurosurgery replaced bone flap 7 months later

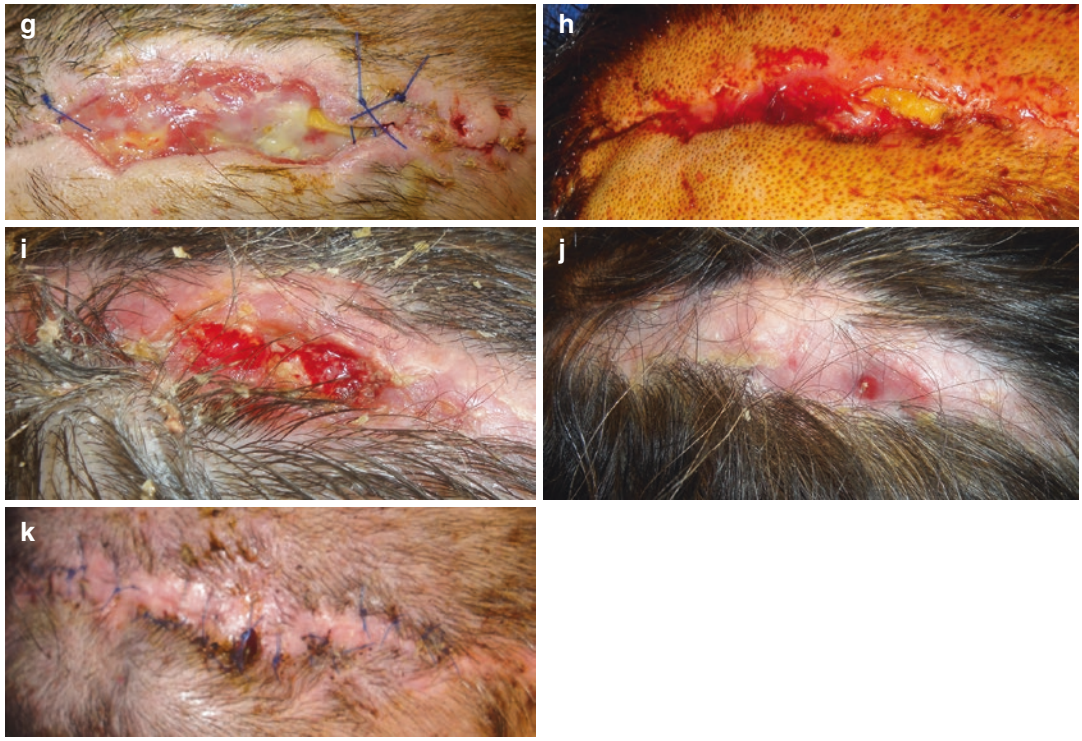


Fig. 32.3 (continued)

fied as an ECM device, is processed for strength and durability which enhances the tensile dural repair but alters the native device molecules, so it lacks the enhanced ECM wound healing response found with the UBM-ECM. Both the MicroMatrix® powder and Cytal® 6-layer Surgical Matrix was placed and closed into the wound with a small open gap treated with daily hydrogel. The initial debridement of the outer calvarial bone was inadequate and required additional debridement and placement of the UBM-ECM for final closure.

This chronic exposed skull wound which responded to debridement, ECM treatment, and subsequent skin grafting failed to completely heal until being treated with topical tacrolimus (Fig. 32.4). A large volume of MicroMatrix® powder is placed on top of several layers of the Cytal® Burn Matrix which was then secured at the periphery with staples. Once the granulation bed developed to a point it was felt the wound bed would support a skin graft, a graft was placed. Medically complex patients such as this elderly patient with rheumatoid arthritis require maximal medical management of all problems and possible dermatology consultation for unexpected complications with normal expected healing.

32.4.2 Nasal Reconstruction

The Cytal® Burn Matrix treatment of a degloving nasal tissue laceration and externally, was done in an attempt to limit the potential scarring of the nose (Fig. 32.5). Powder was not used due to the acute nature of the injury and the desired UBM-ECM healing response was desired for the first 2 weeks. The UBM-ECM device was employed due to the severity of the wounds and to avoid potential secondary internal nasal scarring which is most difficult to treat once it develops. This patient reported normal breathing and near normal sensation of the degloved nasal skin. Of particular interest is the healing of the non-treated brow region sites which developed some contracted healing (which might best have been treated with a wound sheet underlay these flaps also).

The UBM-ECM device was used in an attempt to minimize forehead flap pedicle inflammation

and distal flap swelling, thus allowing for a two-stage instead of a standard three-stage forehead flap nasal reconstruction (Fig. 32.6). We have also successfully used this in one additional patient. A single device formulation was used due to the acute nature of the surgery and desire for 2–3 weeks of desired activity. Figure 32.6c, d demonstrates the reduction of inflammation of a

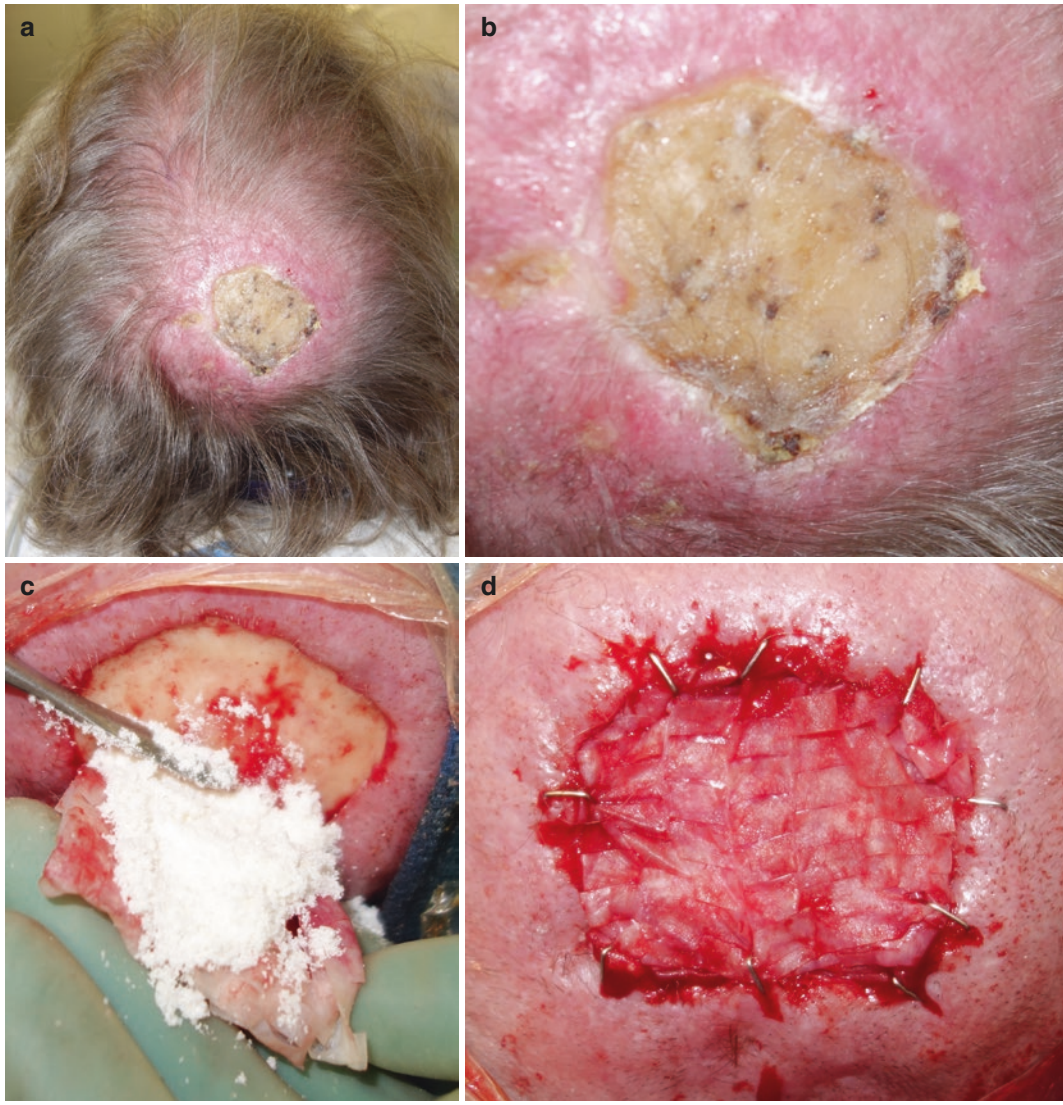
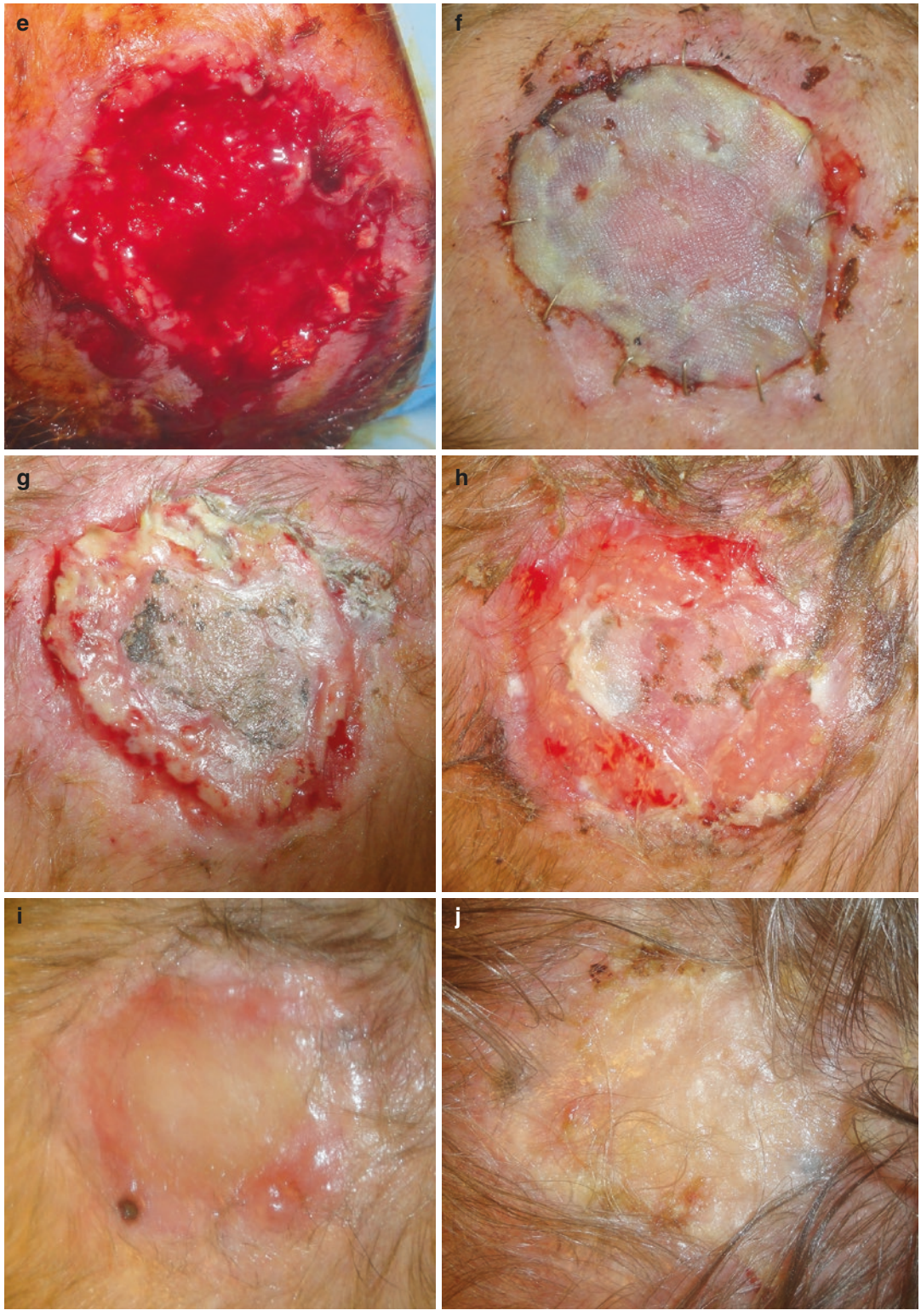


Fig. 32.4 An 82-year-old female with an open vertex scalp wound and exposed bone following Mohs surgical resection of a squamous cell cancer. This wound has been present for over 4 years, and she has had severe rheumatoid arthritis treated for over 30 years. (**a**, **b**) Initial wound appearance. (**c**) After necrotic bone debridement and placement of 200 mg MicroMatrix® powder. (**d**) Placement of a 7 × 10 cm Cytal® Burn Matrix sheet. (**e**) Four weeks later at time of full-thickness skin graft to the 4.5 × 5 cm wound. (**f**) Skin graft 1 week later. (**g**) Four weeks post-grafting. Note the peri-graft inflammation treated with silver nitrate. (**h**) Eight weeks post-grafting. (**i**) After continued poor healing she was diagnosed with pustular dermatosis that responded to topical tacrolimus treatment. (**j**) Wound remains healed 4 months later



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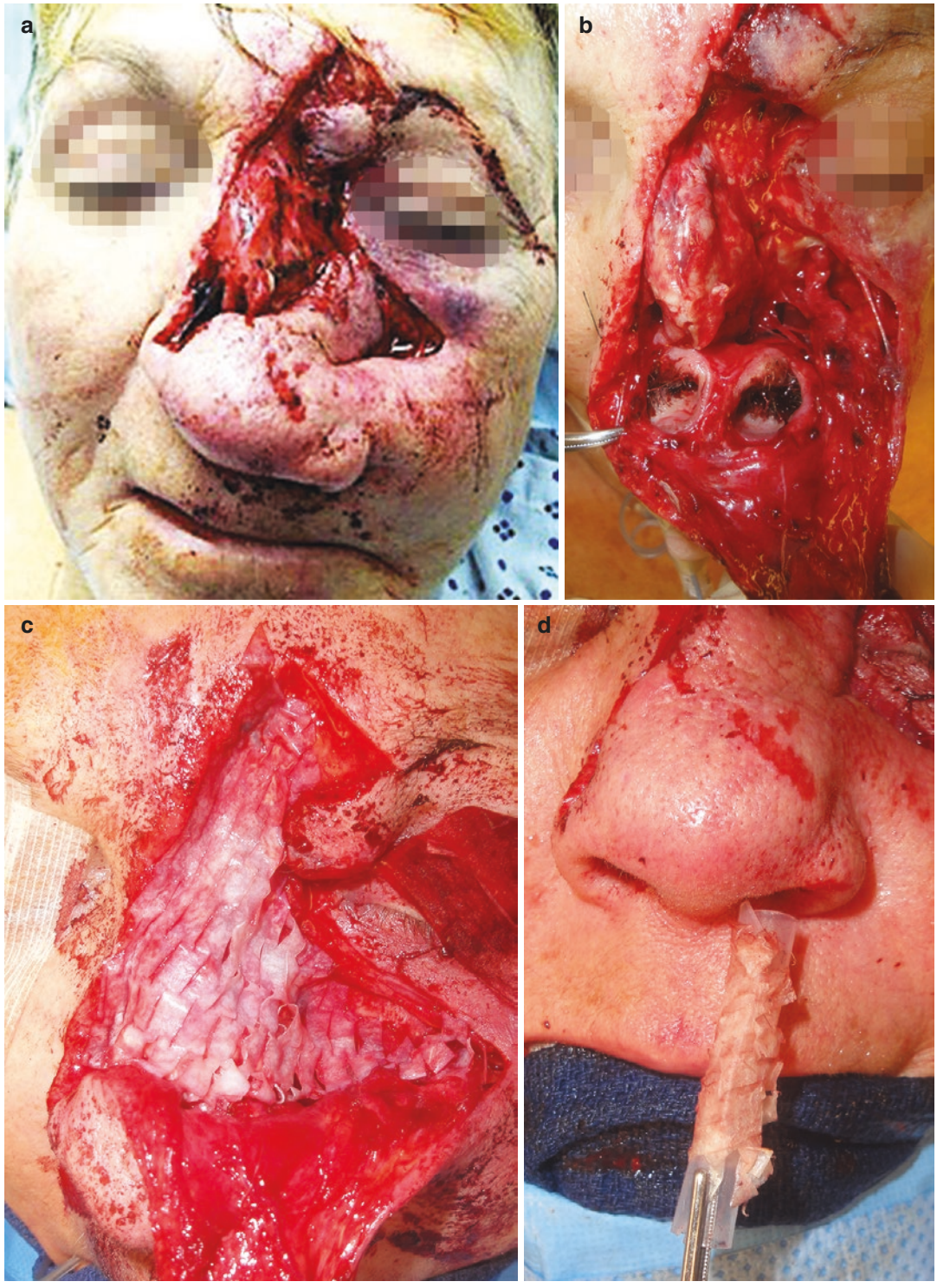
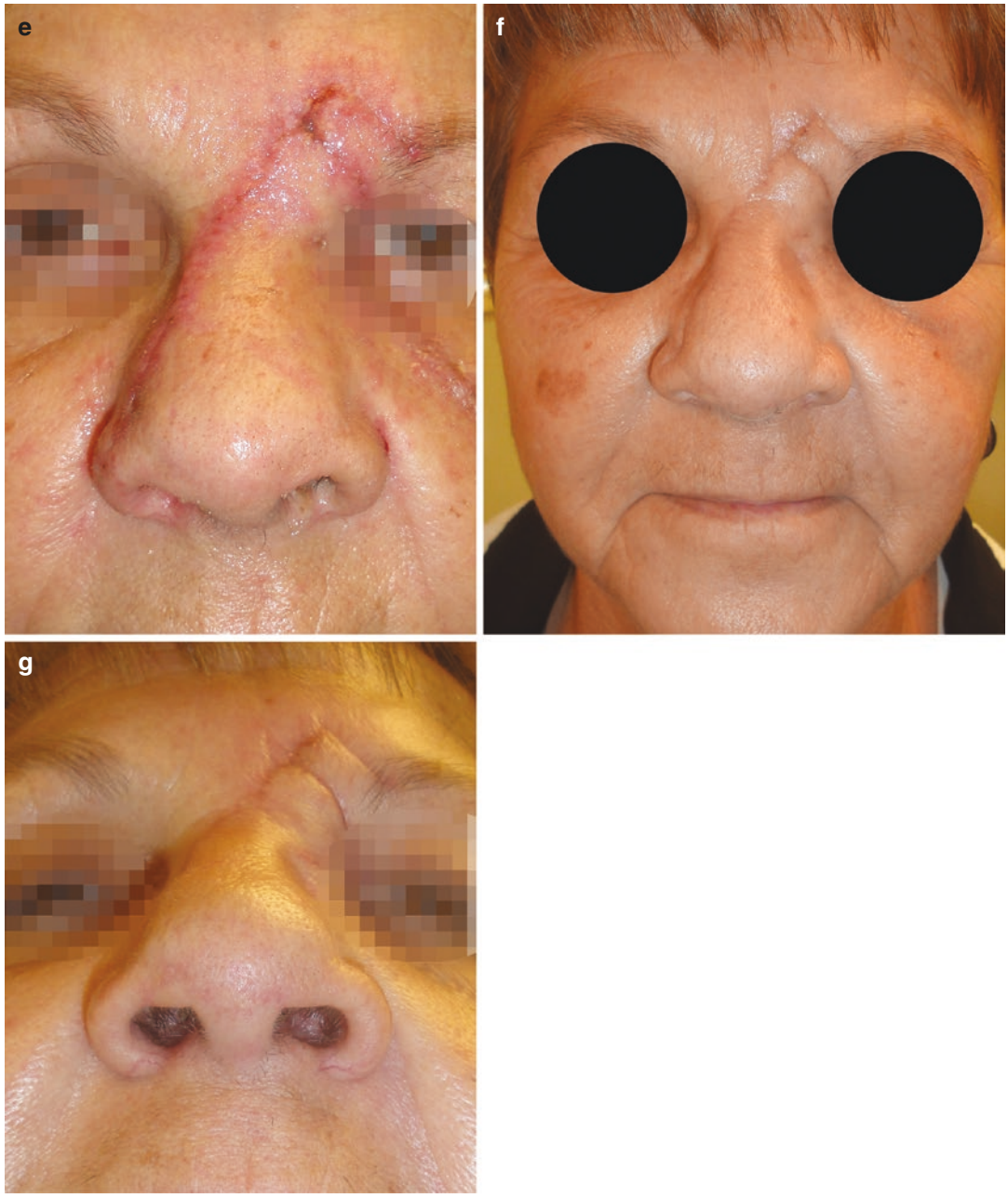


Fig. 32.5 A 71-year-old female with a degloving nose wound after slipping on the ice and falling on a sharp edge. (**a**) Nose after the initial injury. (**b**) Postoperative appearance of the transected nares. (**c**) Cytal® Burn Matrix wrapped around the nares repair and place covering the dorsal nasal cartilage and bone. (**d**) Nasal stents wrapped with Cytal® Burn Matrix to treat the internal nasal scarring. (**e**) Two weeks postoperative. (**f**) Two months later. Note the raised contracted scar healing of medial left brow which did not have underlying Burn Matrix applied. (**g**) The open nares



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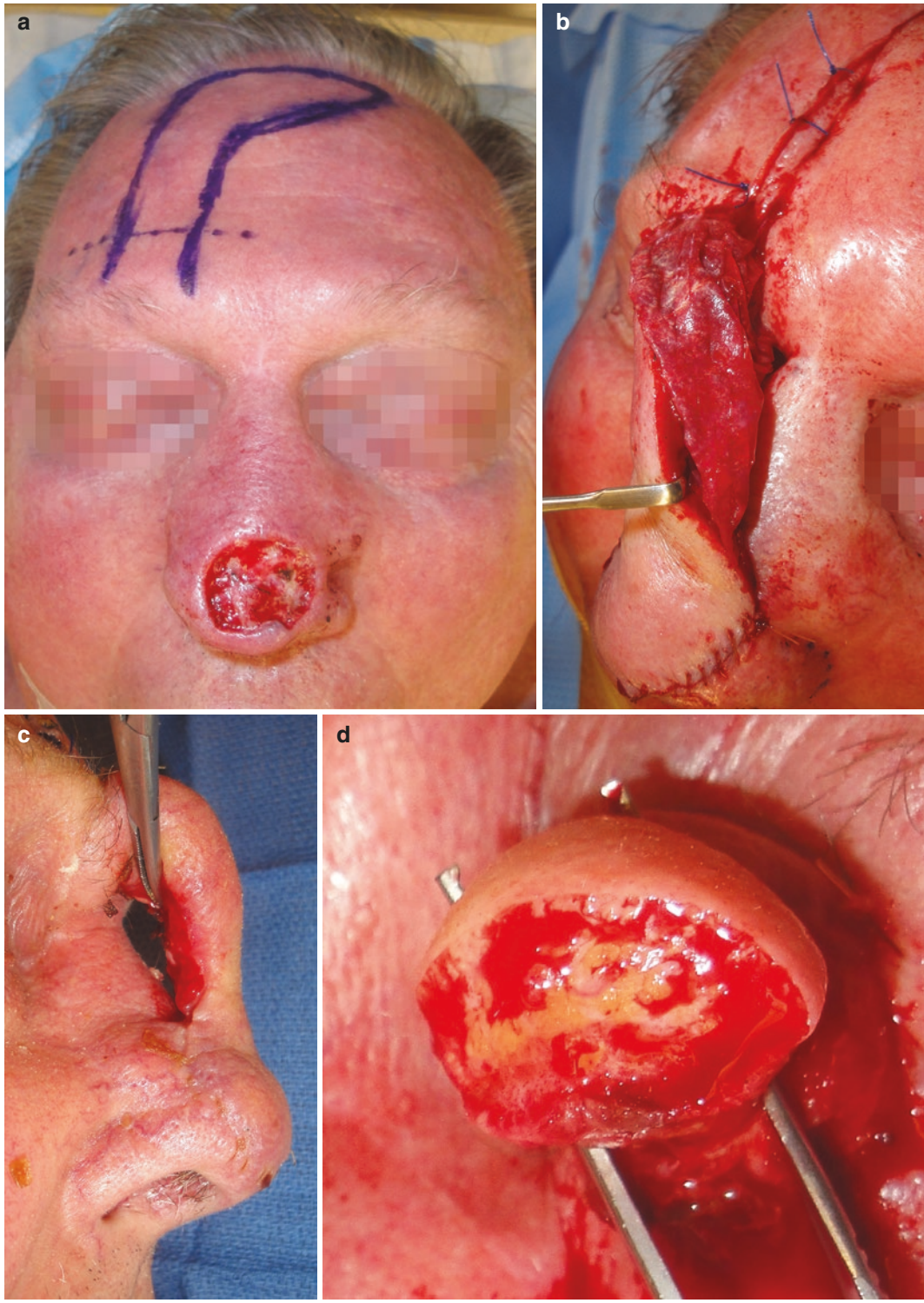
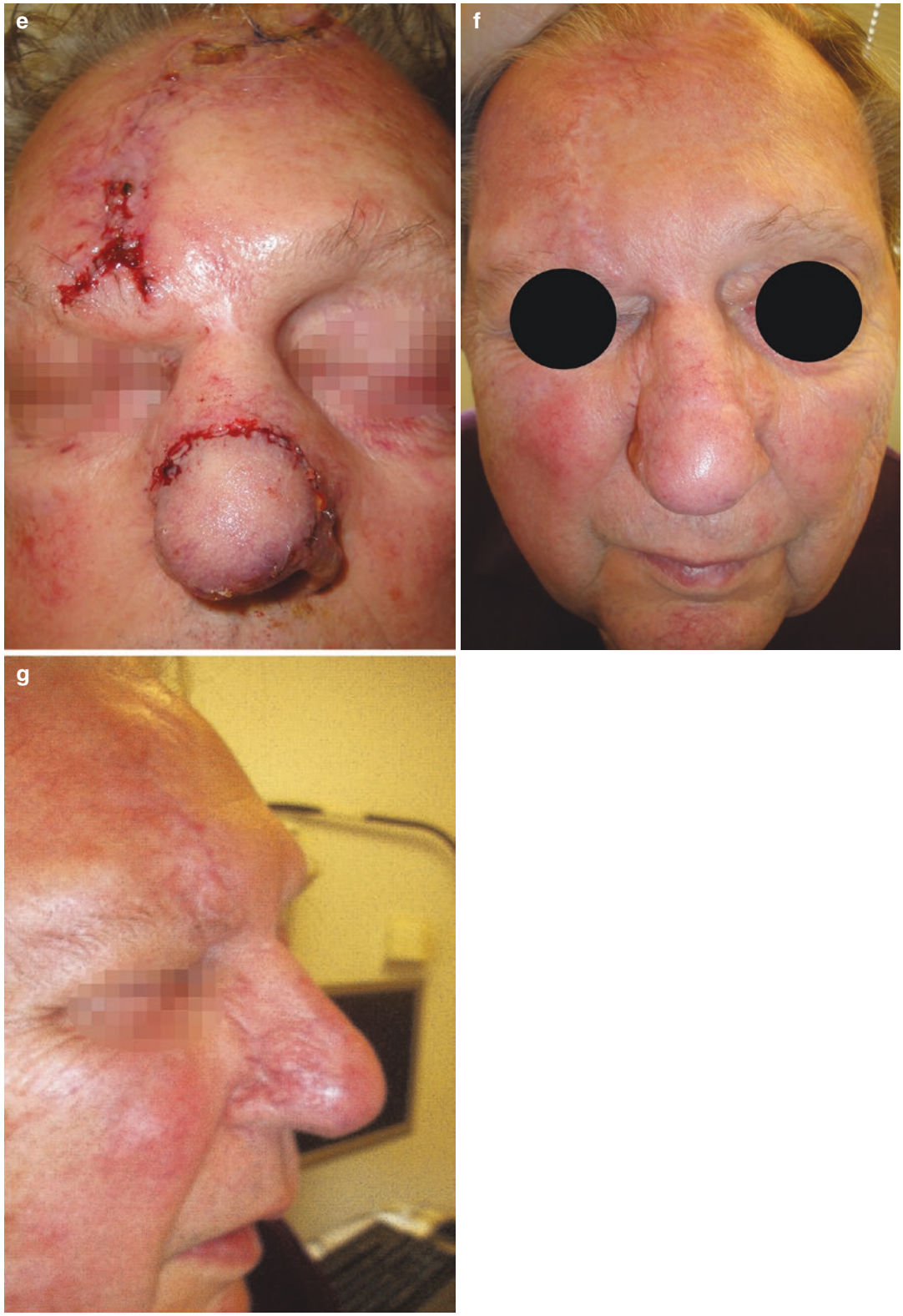


Fig. 32.6 A 75-year-old male needing closure of Mohs surgical defect of his nasal tip after resection of a basal cell skin cancer. (**a**) Initial patient appearance with forehead flap design outlined. (**b**) Use of the Cytal® Burn Matrix as a lining of the undersurface of the forehead flap pedicle. (**c**) Thin pliable forehead flap pedicle prior to flap inset. (**d**) The transected pedicle demonstrating the healed raw surface and minimal swelling and inflammation of the pedicle. (**e**) Appearance of patient at the time of inset. (**f**, **g**) Twelve months later it healed without secondary thinning or flap revision was required



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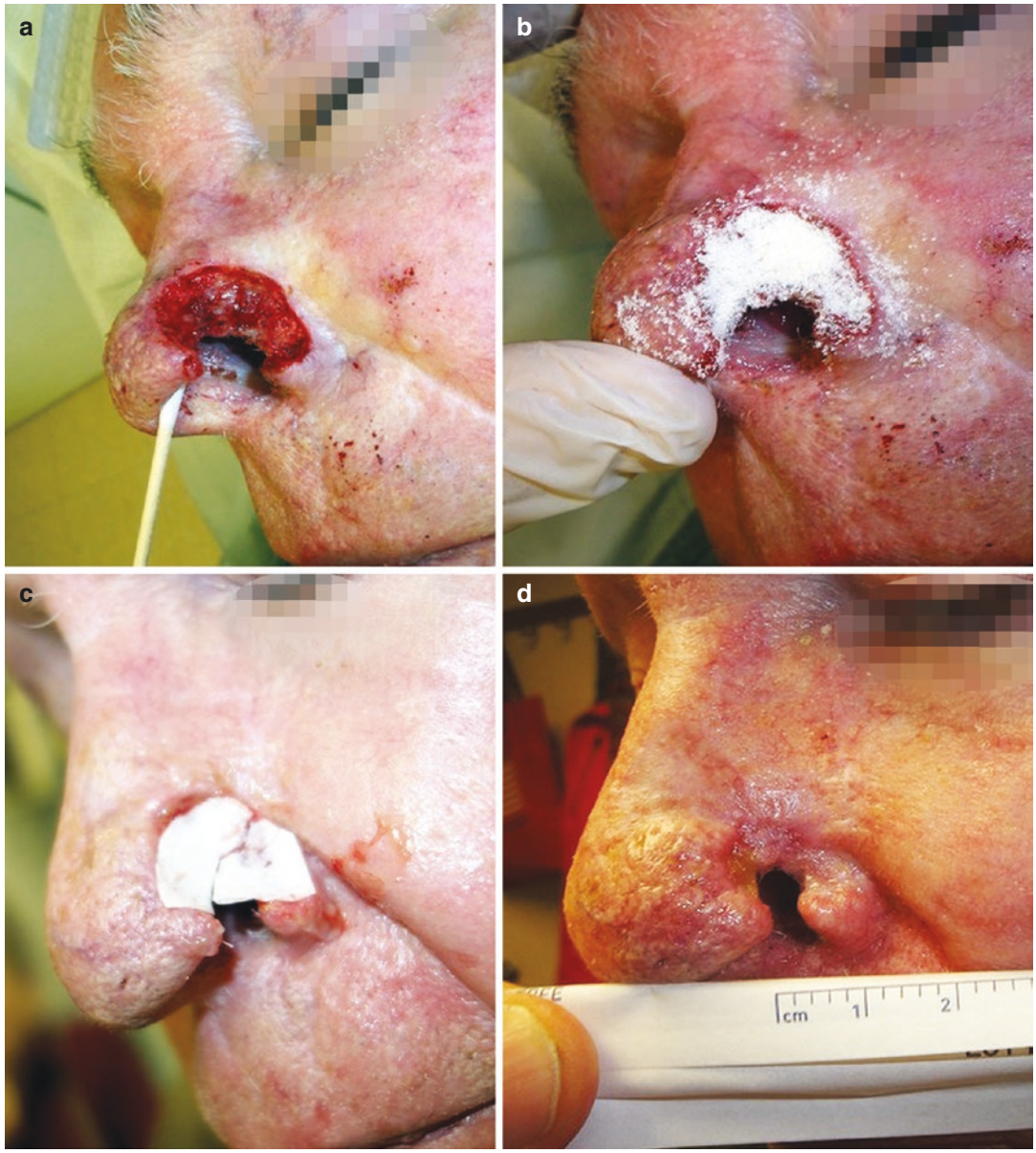


Fig. 32.7 An 81-year-old male with left nasal sidewall defect after Mohs surgical resection of a basal cell skin cancer. Patient is on Coumadin and refuses flap reconstruction due to a prior stroke. (**a**) Initial appearance of left nasal defect. (**b**) After placement of MicroMatrix® powder on the wound. (**c**) Ten days later after alternate day placement of the powder. At this point care changed to alternate day placement of Cytal® Wound Sheet. (**d**) Four weeks later. (**e**) After a composite ear skin-cartilage graft performed under local anesthesia 6 months later

forehead flap pedicle with the UBM-ECM use. This minimized distal flap swelling, so the patient avoided a second-stage thinning procedure. In addition, the device is primarily collagen, so the device minimizes the exudate from the "raw surface" of the flap and makes the post-flap care easier for these often-elderly patients.

Serial placement of the MicroMatrix® powder and then a single-layer wound sheet was used to manage this large nasal sidewall defect to a smaller size which was then amenable to a much less complicated surgical procedure (Fig. 32.7). This was one of the earlier-treated patients, and we were using smaller amounts of the device

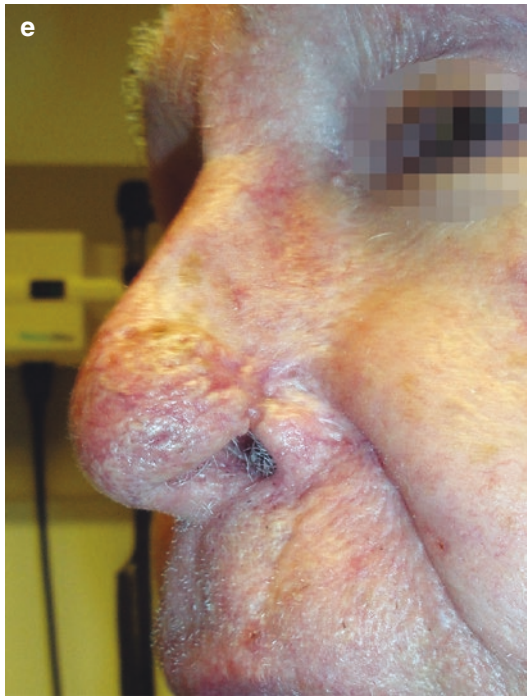


Fig. 32.7 (continued)

placed in the wound on alternate days. This patient would not stop his Coumadin and was pleased that he avoided a large procedure. He was content to live with the cleft of his lateral nose (Fig. 32.8) but agreed to a final repair as it could be done in the office under local anesthesia. The UBM-ECM lateral nasal wound healing readily supported the free composite graft from the left ear.

32.4.3 Facial Soft Tissues

This is a case of a forehead abrasion/tissue loss injury treated with a Cytal® Burn Matrix sheet applied to provide sufficient UBM-ECM wound device for the estimated several weeks it would take for the wound to heal (Fig. 32.8). While not done in this case as it was used to treat acute trauma, MicroMatrix® powder could possibly be used under the sheet as there was a severe deep tissue injury. The device was retained in the wound with a Vaseline gauze sheet secured at the margins of the wound bed. The typical pink salmon-colored granulation tissue response is noted. The healing which occurred in the most heavily injured eyebrow region was sufficient for subsequent micro-hair grafts which was critical for the final appearance. This patient returned wanting treatment of his widened temple scar with the wound device which was done resulting in a minimally noticeable smooth scar.

This patient has subacute self-inflicted bilateral cheek wounds compounded by infection (Fig. 32.9). Both the MicroMatrix® powder and Cytal® Burn Matrix were used in these semi-acute wounds left undisturbed for several weeks under a sutured Vaseline gauze dressing left undisturbed for several weeks. This dressing also limited the patient's ability to further harm herself and permitted her to focus on the treatment of her psychiatric issues and not wound care. The right chin wound at the time of operative treatment seemed a more minor injury, so it wasn't treated with the wound device. In retrospect, using the UBM-ECM treatment on the chin wound might have yielded a more favorable scar more like the much deeper and larger upper cheek scars.

This case of an elderly man with a chronic radiated skin cancer resection wound shows that an unfavorable wound bed, given enough time and a UBM-ECM device, can heal (Fig. 32.10). This was an earlier-treated patient, so only a single-layer wound sheet which was folded over several times was used. This was also one of our earlier uses of more device placed for a prolonged time. The simplified placement of a polyurethane sheet dressing weekly was easily accomplished by the patient.

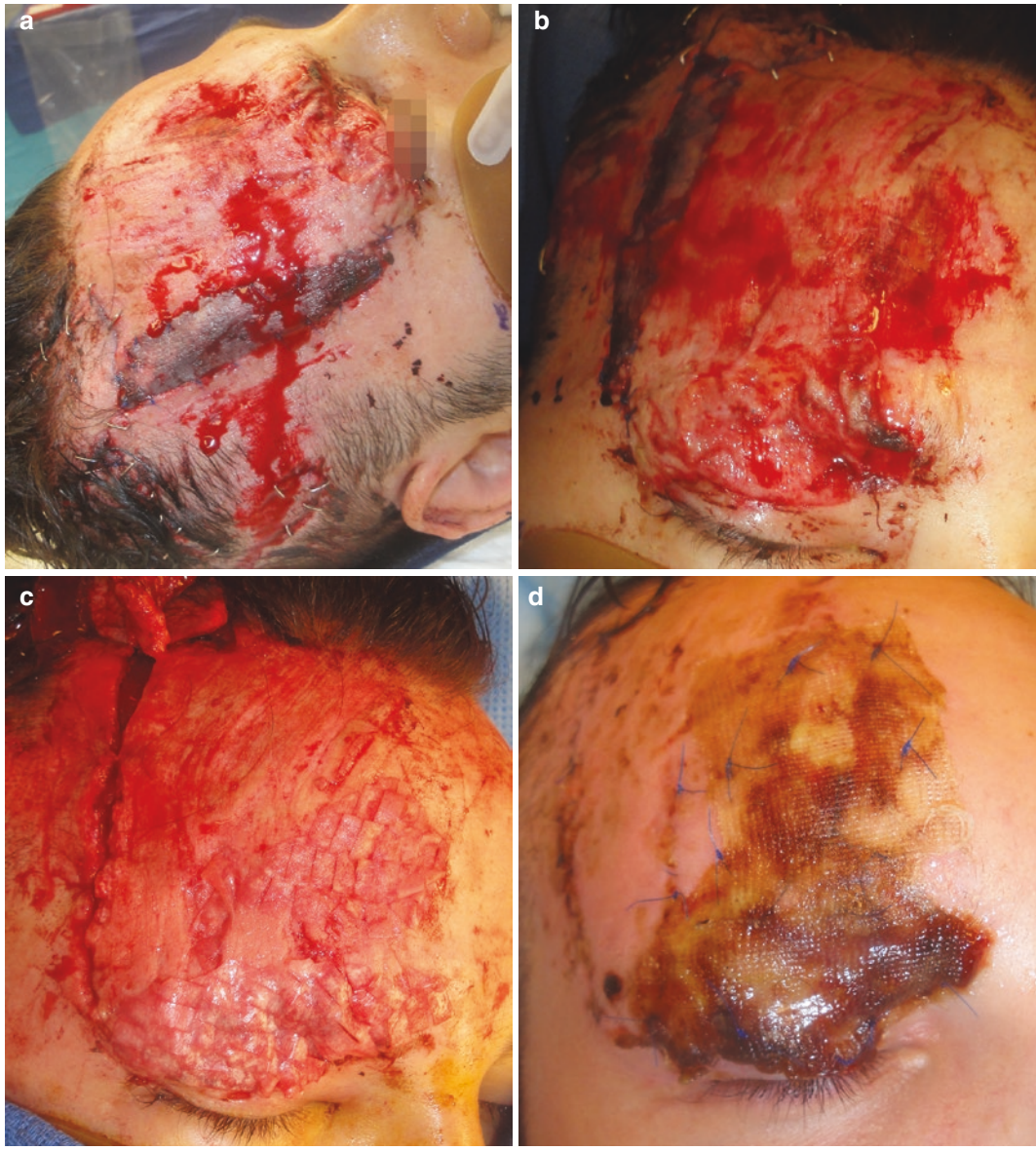
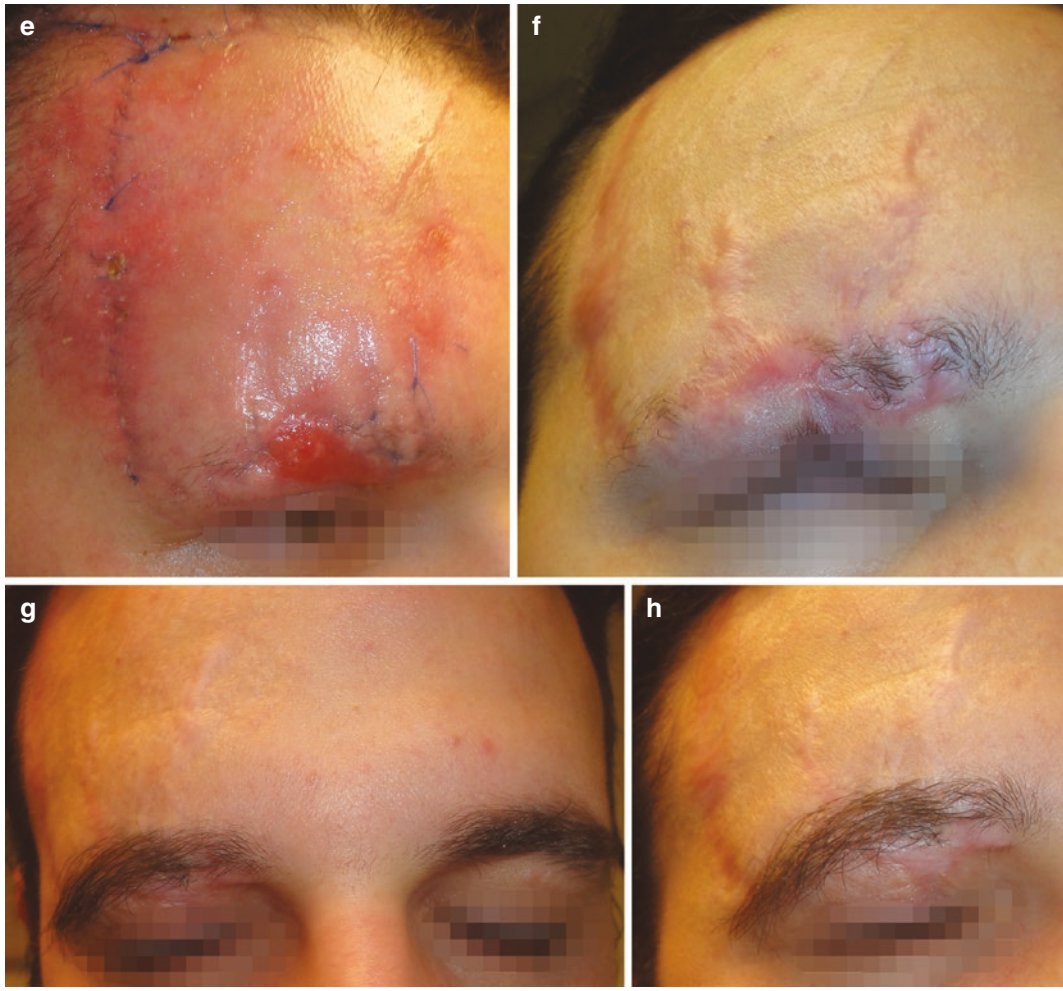


Fig. 32.8 A 23-year-old male with forehead and eyebrow wounds sustained after a windshield injury in a motor vehicle accident. (**a**, **b**) Forehead, eyebrow, upper eyelid lacerations, and tissue loss. (**c**) Cytal® Burn Matrix ECM applied to forehead and eyebrow wounds. (**d**) One week. The brown-colored ECM needs more hydration for maximal activity. (**e**) Three weeks. Note the characteristic of ECM-stimulated salmon-colored granulation tissue. (**f**) Three months post-injury. (**g**) 13 months later after microhair grafting of eyebrow. (**h**) Forehead and eyebrow appearance 27 months later. (**i**) Initial right temple wound prior to debridement of necrotic flap. (**j**) Widened healed right temple scar 27 months later. Patient desires improvement. (**k**) Excision of temple scar. (**l**) Mobility of skin edge closure. (**m**) Placement of Cytal® Burn Matrix into wound bed with deep dermal sutures imbricating the wound device up into the dermal closure. (**n**) Subcuticular Prolene suture closure with outer skin glue placement. (**o**) Healed wound appearance 6 weeks later after sutures removed



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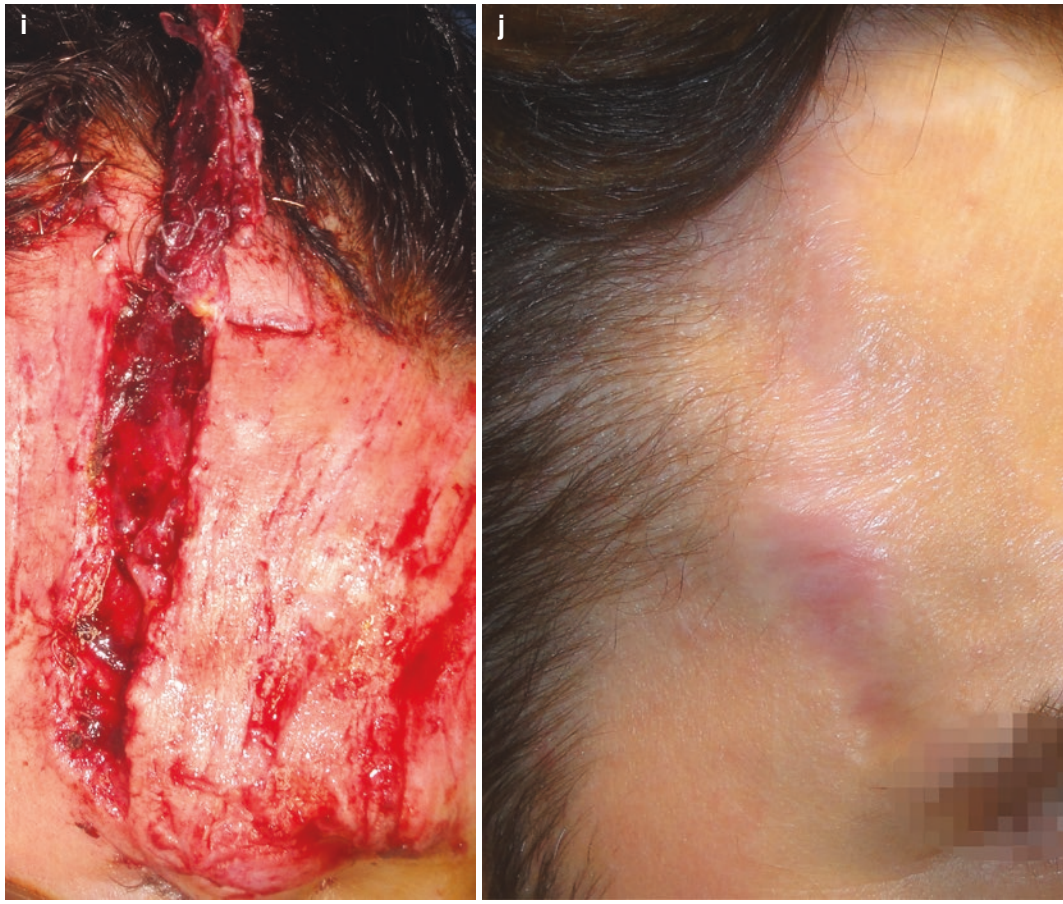

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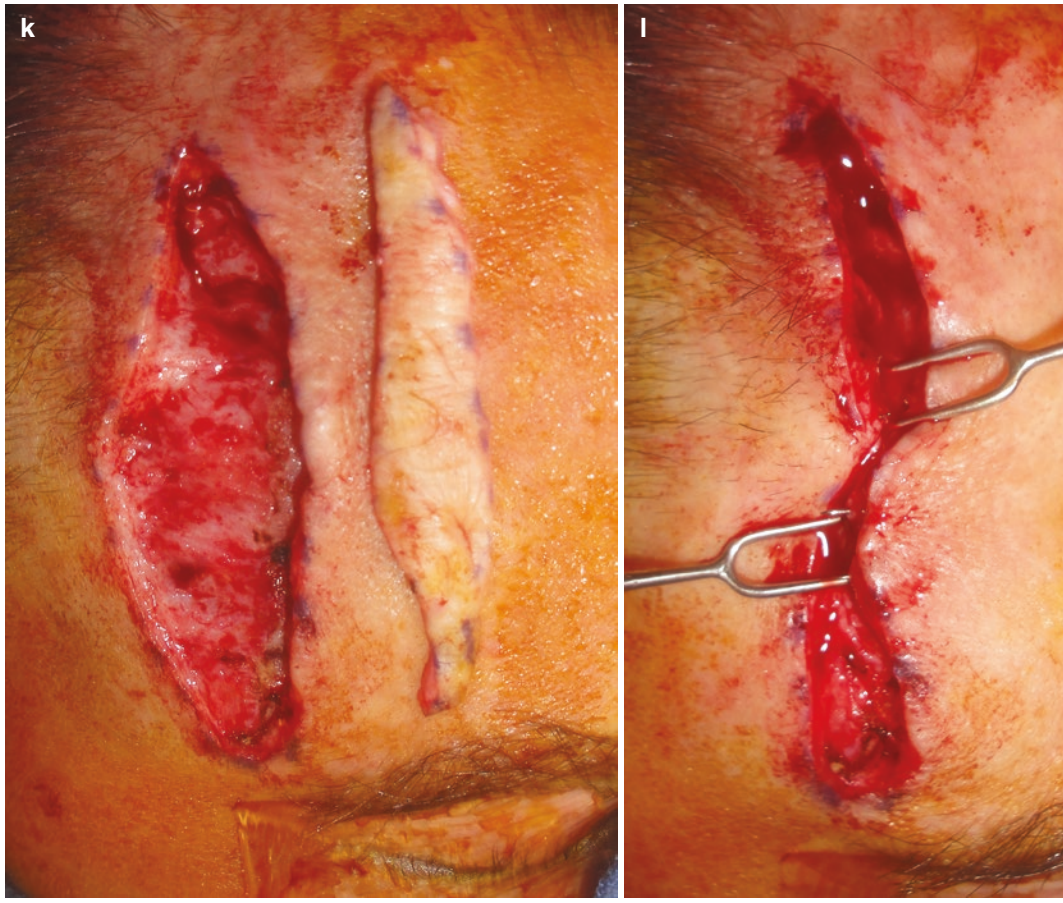

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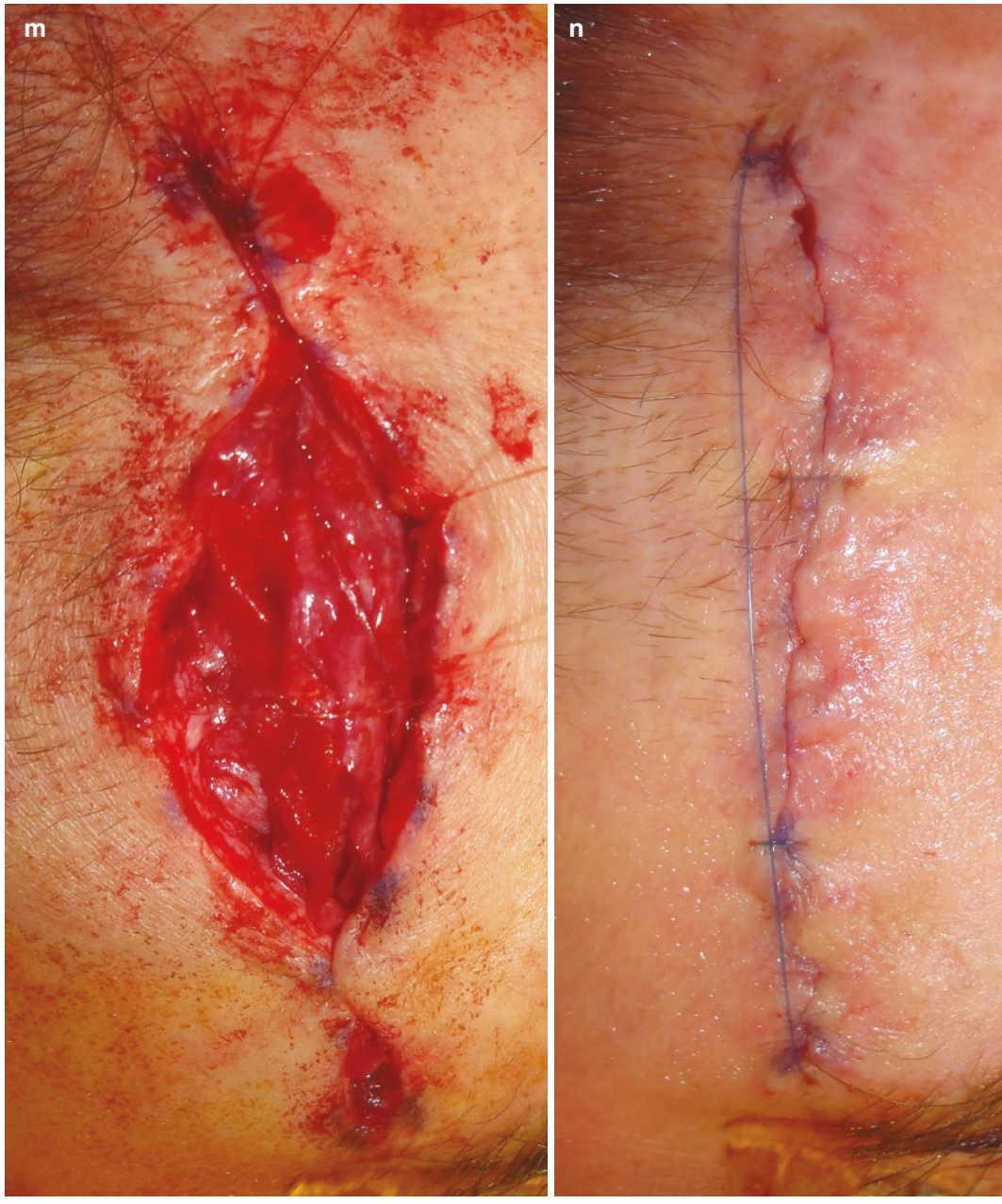

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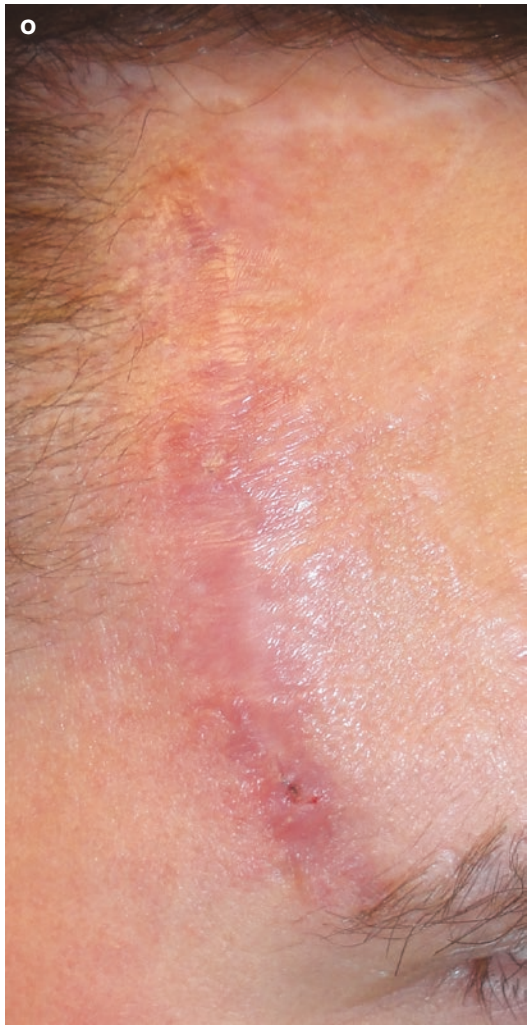

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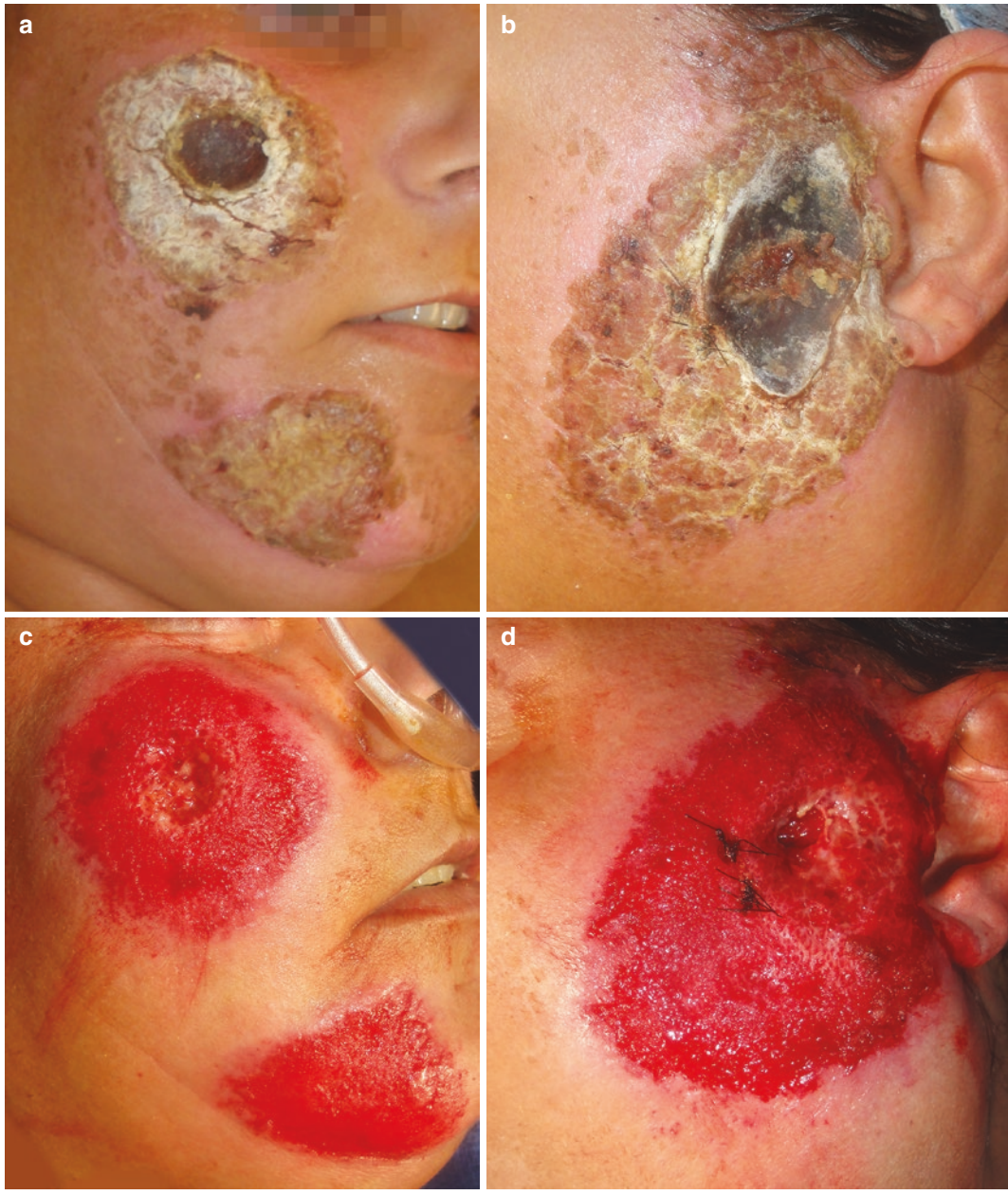
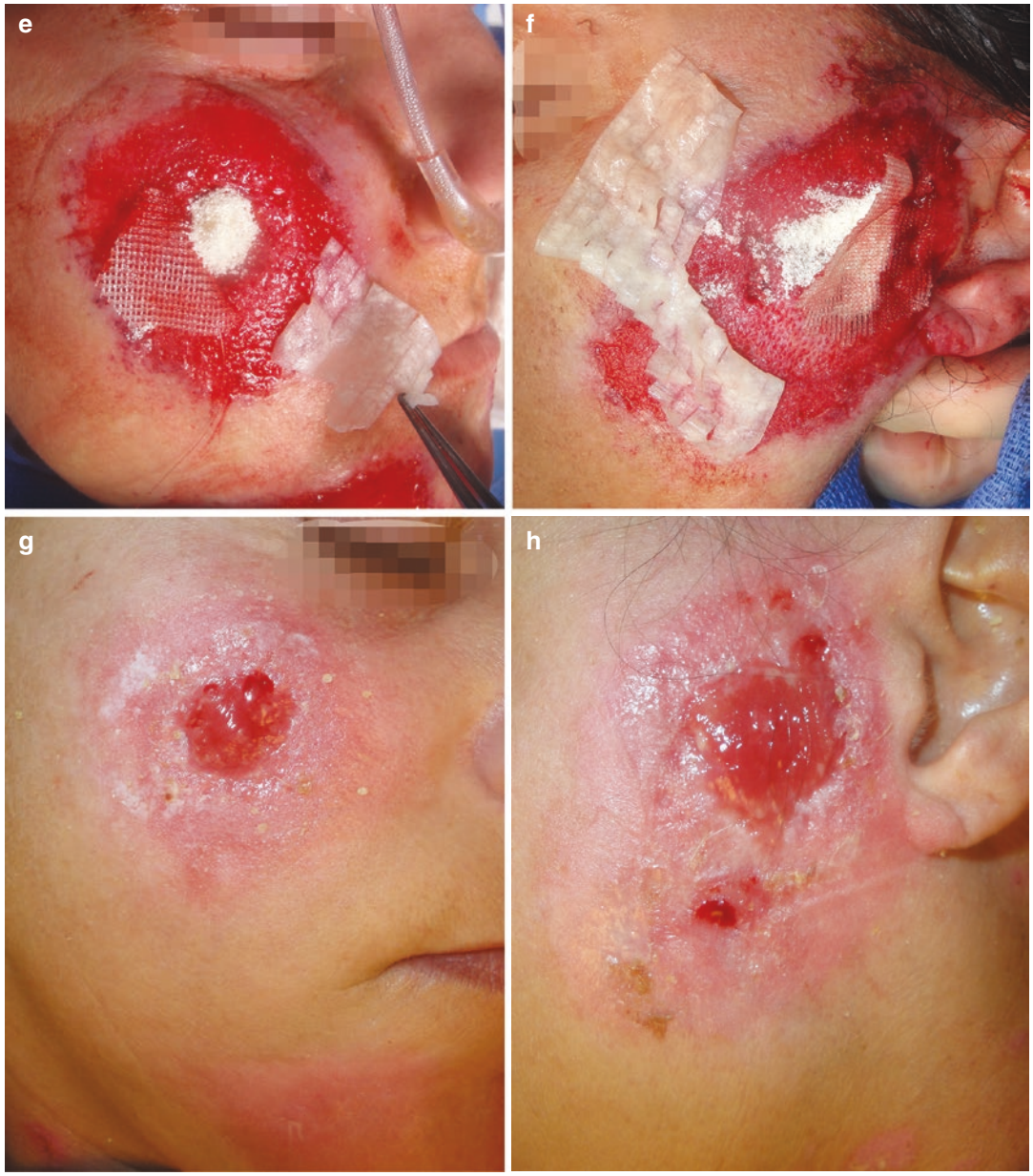


Fig. 32.9 A 42-year-old female with an anxiety disorder who self-injured her face with her fingernails. (**a**, **b**) Initial appearance of the bilateral cheeks being topically treated for necrotizing cellulitis. (**c**, **d**) Cheek wounds after wound bed preparation. (**e**, **f**) UBM-ECM being placed into cheek wounds—100 mg. MicroMatrix® powder and 5 × 5 cm. Cytal® Burn Matrix split between the two wounds. (**g**, **h**) One month later. Note the moist salmon-colored granulation tissue typical of UBM-ECM healing. (**i**, **j**) Cheek at 14 months post UBM-ECM treatment



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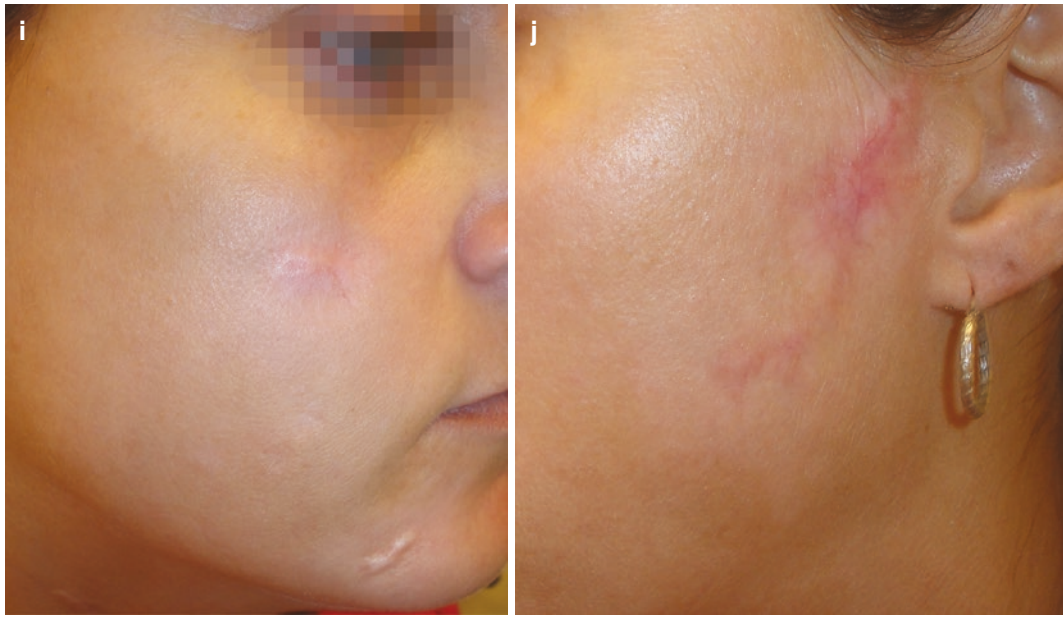

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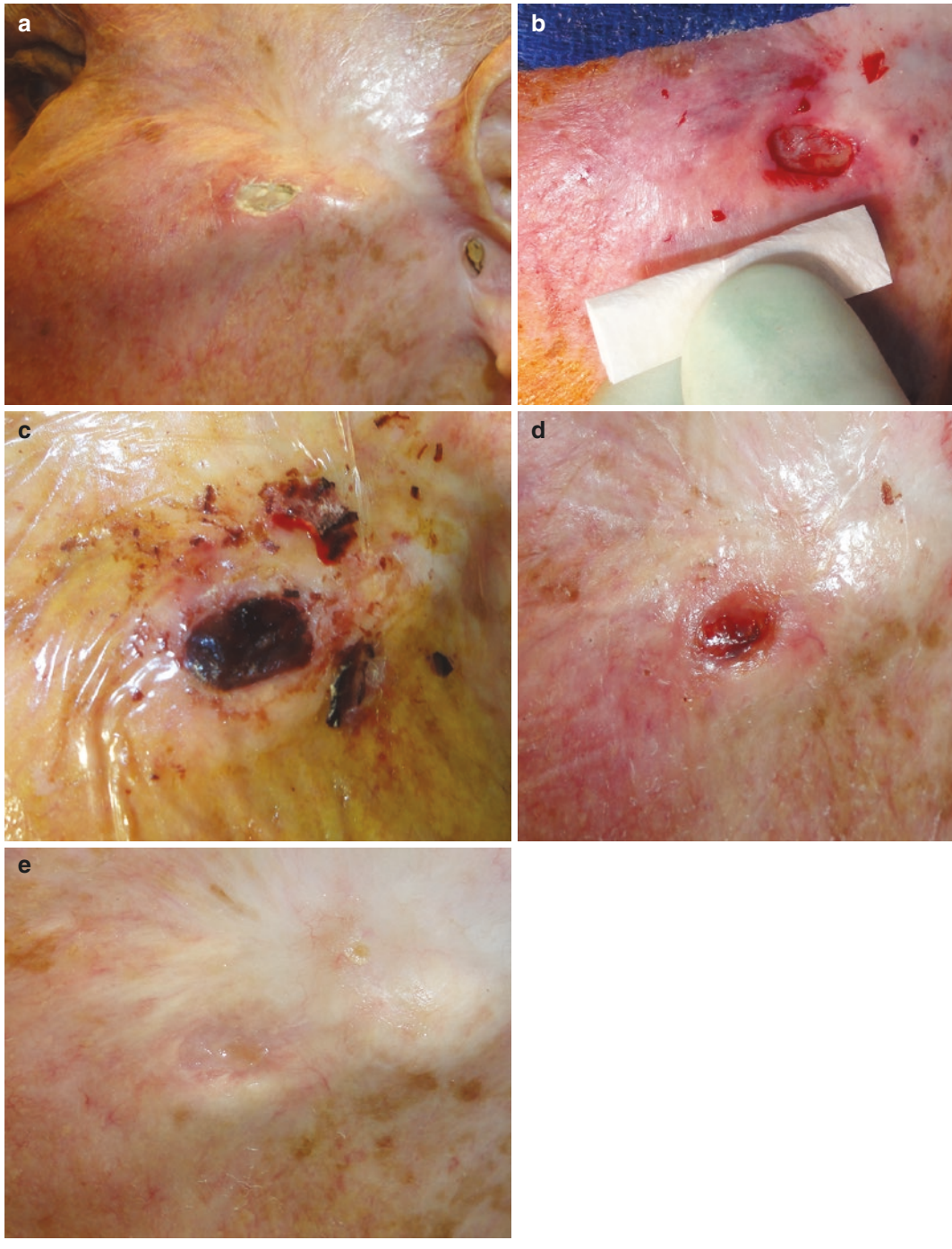

Fig. 32.10 A 91-year-old male with a non-healing left cheek wound which extends down to the zygomatic arch—after resection of a squamous cell cancer and radiation therapy. (**a**) Initial wound. (**b**) After debridement at the time of placement of several pieces of a Cytal® single layer wound sheet used to fill the wound cavity. (**c**) One week later with small overlying clot. (**d**) Seven weeks post-treatment. (**e**) Final wound 3 months post-treatment. A polyurethane sheet covering was the only secondary dressing utilized during the healing process

Acknowledgments *Disclosure*: Dr. Kraemer has been a consultant for ACell® Inc. (Columbia, MD) since 2014 and has received monies for presenting his clinical experience on the use of the UBM-ECM wound device. He began using the UBM-ECM wound devices in 2010.

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33 Advances in Facial Nerve Paralysis: Surgical Innovation, Tissue Engineering, and Emerging Technology

Julia R. Brennan, Matthew E. Spector, Jennifer C. Kim, and Michael J. Brenner

33.1 Introduction

The facial nerve is of critical importance to an individual's identity and ability to connect and communicate with others [1]. It takes part both in day-to-day functions of eating, drinking, and blinking and also in minute-to-minute expressions of emotion and social interaction [2]. The involvement of the facial nerve in eye closure makes it crucial in the prevention of corneal exposure and keratopathy and ultimately protection of vision [3]. As such, the multifaceted nature means that injury to the facial nerve can be very damaging to patients. Facial palsy has a profound impact on activities of daily living, psychosocial well-being, and quality of life measures and is thus an important focus of a vast array of both surgical and nonsurgical interventions [4].

In order to select the appropriate approach to treatment, various considerations must be taken into account. These include the cause, duration, and severity of the facial palsy, the integrity of the underlying neuromusculature, and the general goals of the patient [5]. Due to the nature of the anatomy, it is challenging to completely restore the intricacy and cooperation of the facial mimetic musculature. Current techniques aim to establish symmetry and movement. Static slings and nonsurgical approaches can offer improved resting symmetry to these patients, but they do not restore muscle tone or allow for voluntary reanimation [1]. For the purposes of this chapter, we will be focusing on the dynamic surgical techniques in reanimation and reinnervation which are currently employed to treat facial paralysis. Also included is an overview of some areas of ongoing research in facial nerve repair in which future techniques are discussed.

Facial nerve repair research has proven to be an exciting frontier for the fields of tissue engineering, nanotechnology, and bioelectrical interface design. The emerging technologies take advantage of the ongoing work in basic, clinical, and translational research that promise to advance the field even further in the coming years.

33.2 Diagnosis and Clinical Decision-Making

In order to reinstate symmetric facial movement, there must be a functional nerve that can provide input to a functional bed of muscle with intact neuromuscular junctions (Fig. 33.1). Options for neural input include the ipsilateral facial nerve, the contralateral facial nerve, or coaptation between an alternative cranial nerve and the

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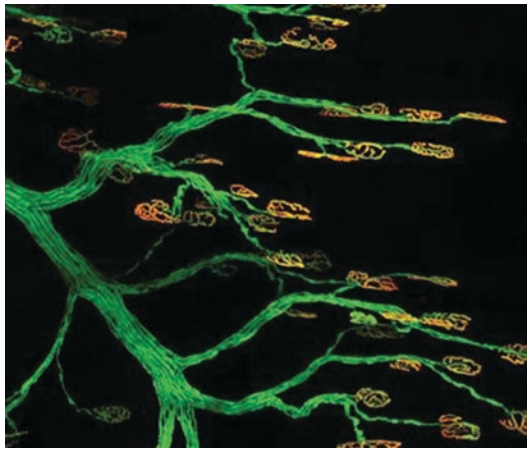

Fig. 33.1 Confocal imaging illustrating terminal axons and motor end plates, adapted from Magill CK, Tong A, Kawamura D, Hayashi A, Hunter DA, Parsadanian A, Mackinnon SE, Myckatyn TM. Reinnervation of the tibialis anterior following sciatic nerve crush injury: A confocal microscopic study in transgenic mice. Experimental Neurology. 2007;207(1)

distal facial nerve segment. The muscle bed can be that of existing facial muscles or that of a transfer, either regional or distant [5].

The choice of repair is contingent upon the duration of denervation and site of the injury. The time since injury can often indicate the viability of the underlying facial mimetic musculature. Although the exact cutoff is debated, studies demonstrate functional reinnervation can be achieved for approximately 12–18 months after injury before there is irreversible muscle atrophy and degeneration of the neuromuscular junction [6]. An electromyographic study can help evaluate the viability of the neuromuscular unit. Together with a comprehensive preoperative evaluation, these studies can assist in diagnosis and informed clinical decision-making [7]. Figure 33.2 illustrates an algorithm that can assist in evaluating a patient with facial palsy.

33.3 Approaches

33.3.1 Reinnervation

There are three available surgical techniques by which facial nerve reinnervation is achieved. For nerve transections there are primary suture repair and tissue adhesives. For any larger nerve defects, there are nerve grafts and conduits to avoid tension and bridge the gap. For any injury involving an unavailable proximal or distal nerve segment, there are nerve transfers [8].

33.3.2 Primary Suture Repair

Primary end-to-end neurorrhaphy of fresh nerve endings remains the best option for nerve transection injuries in the event that tension-free approximation is possible. A study in a primate model suggested that, for defects up to 3–4 cm, primary repair under modest tension results in better axon regeneration than grafting [9]. In the setting of larger nerve gaps, however, the higher tension on the nerve endings can result in impairments in nerve vascularity, scar tissue formation, and even nerve rupture [10, 11].

There has historically been a dispute as to the best technique for microsurgical neural repair. The advantage of fascicular repair is that it facilitates the best axonal alignment, but this requires increased operational complexity and has the potential for disruption of the nerve and the vasa nervorum. Alternatively, epineural repair is faster and less disruptive and is often the favored technique for primary neurorrhaphy [5, 12]. The epineural sleeve technique has been demonstrated to have better functional nerve recovery and is thus favored over standard end-to-end repair. In this technique, the epineural sheath of the distal nerve ending is rolled back 2 mm so as to create a sleeve over the proximal stump at the area of coaptation (Fig. 33.3). This is thought to create a chamber within which the repair site is separated from the surrounding tissues to collect axoplasmic fluid and promotes regeneration [13]. Alternatively, another technique involves end-to-end epineural repair with the use of a vein graft cuff placed over the repair to facilitate a similar isolation of the neurorrhaphy site. Of note, this graft is placed on the proximal stump before the repair and is later transposed over the repair site.

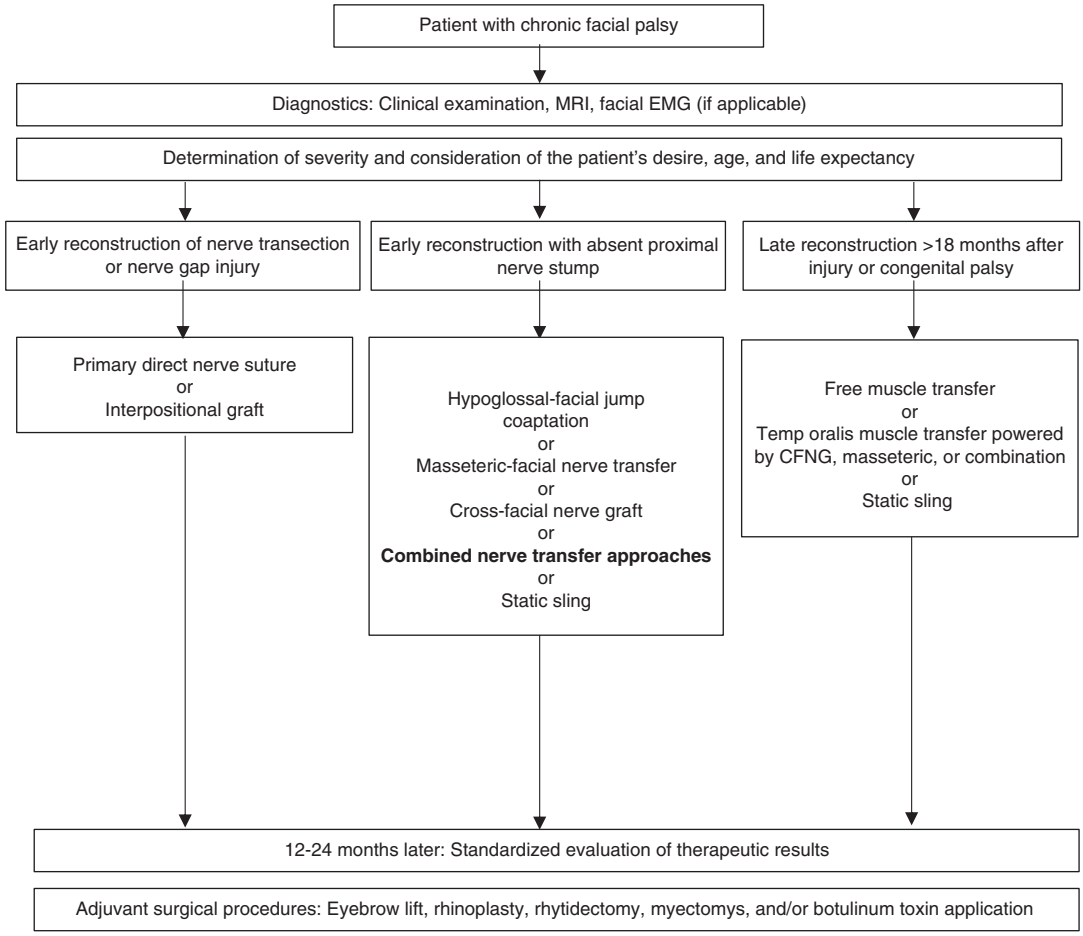

Fig. 33.2 Algorithm for approaches to repair in a patient with facial nerve palsy. It is the authors' opinion that combined nerve supply—particularly use of masseteric-to-buccal branch of facial nerve in conjunction with hypoglossal-to-facial—provides the best tone and symmetry for patients with an absent proximal stump. Adapted from Gerd FV, Pantel M, Guntinas-Lichius O. Modern Concepts in facial nerve reconstruction. Head Face Med 2010;6

33.3.3 Tissue Adhesive

Fibrin glue and other adhesives also play an increasing role in nerve repair. Studies in rodents demonstrated no difference in recovery outcomes between primary suture and fibrin adhesive coaptation, yet there was a significantly reduced operative time for the latter [14, 15]. Additionally, because the epineural sutures used in neurorrhaphy are permanent, there is the concern for a chronic foreign body reaction and increased inflammation which may inhibit axonal regeneration [16].

Historically, a potential disadvantage about fibrin glue concerns about its ability to hold the nerve endings together and maintain an adequate tensile strength at the site of repair. Multiple studies in animal models have indicated that these adhesives actually have biomechanical performances that are equivalent to that of primary suture repair, suggesting that these concerns are unfounded [17, 18]. As such, fibrin glue presents as a quicker and easier modality that may indeed be functionally comparable to primary suture repair.

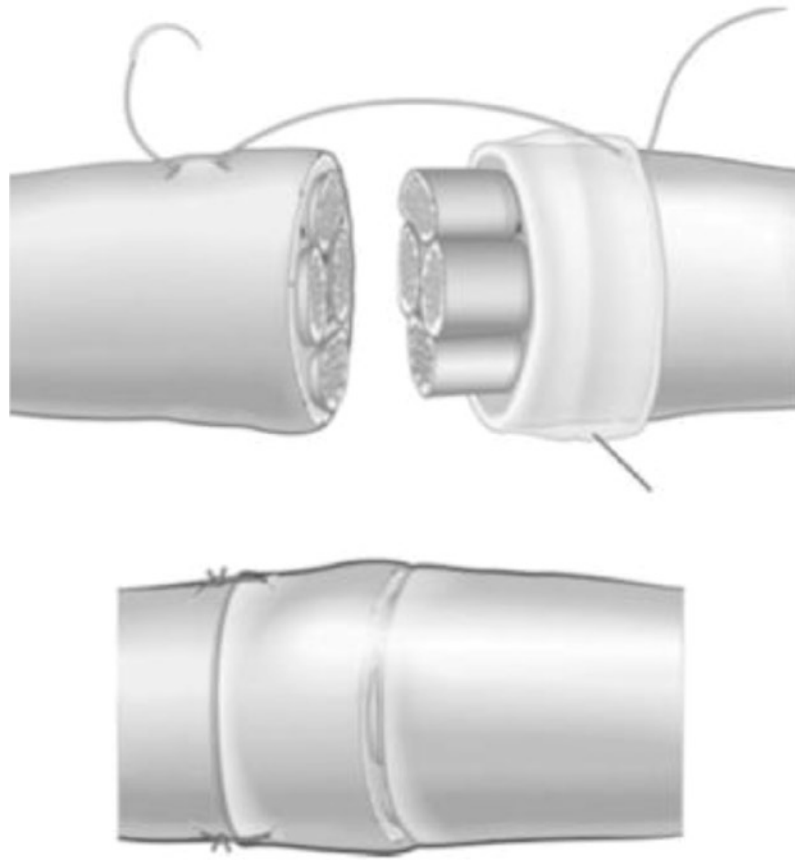

Fig. 33.3 Diagram of epineural sleeve repair, reproduced with permission from Siemionow M, Brzezicki G. Current techniques and concepts in peripheral nerve repair. Int Rev. Neurobiol 2009;87:148

33.3.4 Nerve Grafts

33.3.4.1 Autografts

For the repair of larger nerve gaps across which tension-free or minimal tension neurorrhaphy is unavailable, grafts are indicated. Nerve grafts in particular contain components, namely, Schwann cells and their extracellular matrix and growth factors, that make them the ideal scaffold for regeneration of injured nerve axons. Taken together, these constitute a microenvironment that effectively promotes the advancement of regenerating axons [19].

As compared to primary suture repair, nerve grafting results in both an increased incidence of synkinesis and a longer rehabilitation time as nerve function returns [20]. In synkinesis, aberrant neural regeneration across the graft can cause multiple muscle groups to contract when just one is activated. This can be minimized by the use of multiple neural inputs such that the upper facial musculature and lower facial musculature are innervated separately. Using a masseteric-to-buccal coaptation as an adjunct to the cable nerve graft has been shown to reduce the incidence of synkinesis and result in a faster recovery of oral commissure movement [21] (Fig. 33.4).

There has also been consideration as to the importance of nerve polarity in orienting these nerve grafts. Historically, the philosophy has been to orient the nerve graft in its physiologic orientation such that the proximal end of the graft is coapted with the proximal end of the defect. Conversely, many surgeons recommend the opposite so as to minimize the potential for arborized axons to get lost to misrouting [22]. A systematic review revealed no significant differences in functional outcomes or nerve generation between normal and reversed polarity nerve autografts [23].

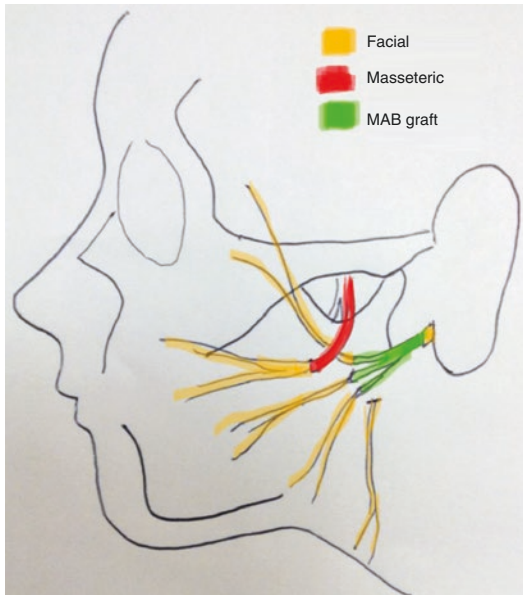

Fig. 33.4 Diagram of concurrent masseteric nerve transfer and cable graft. MAB represents the medial antebrachial nerve used as the nerve graft

33.3.5 Nerve Reconstruction and Nerve Transfers

In some instances of facial nerve injury, neither primary suture repair nor graft techniques are applicable. When the proximal facial nerve stump is resected or damaged but the distal NMJ and facial muscles are intact, a nerve substitution may be indicated. The best outcomes for these procedures have been reported when they are performed within 12–18 months, although some studies report success after up to 4 years [24, 25]. After chronic denervation, the neuromuscular junction undergoes significant atrophy at the motor end plate, and the outcomes of nerve transfers are much less successful [5].

33.3.5.1 Hypoglossal-Facial (XII-VII)

The hypoglossal nerve is a valuable option for nerve substitution of the facial nerve as it is proximal to the extratemporal portion and has a high population of myelinated motor axons. In addition, hemi-tongue weakness is more tolerable among patients than weakness of other facial musculature [26, 27].

Historically, the classic procedure entailed transection of the entire hypoglossal nerve and reattachment distally to the facial nerve stump. Since its advent, there have been several modifications in order to minimize tongue dysfunction and subsequent issues with articulation and mastication. Animal studies have sought to quantify the percentage of hypoglossal axons required to preserve acceptable tongue function. A rodent model demonstrated that at 40% preservation of the hypoglossal nerve, tongue atrophy is minimized and the facial musculature can be effectively reinnervated [28]. This has led to an early modification in which there is partial nerve sacrifice. Other modifications include the jump graft which utilizes a donor cable graft – either from the greater auricular or sural nerve – to bridge the hypoglossal nerve and the distal facial nerve trunk. Studies demonstrate that the end-to-side neurorrhaphy used in the jump graft repair is best facilitated by deliberate transection of some of the donor nerve axons to facilitate regeneration [29]. This modification serves to preserve some tongue function and decrease mass activation of the hemiface [30].

Finally, the infratemporal facial nerve can be reflected out of the mastoid bone and used for direct communication between the hypoglossal nerve and the distal facial trunk. This obviates the need for an interpositional graft and decreases axonal loss through an additional coaptation site [31]. After proximal nerve injuries such as acoustic neuroma with facial nerve sacrifice or, indeed, whenever there is adequate facial nerve available, this is a very attractive option as it necessitates just one coaptation between two intact nerves.

As of yet, the outcomes of these various modifications have not yet been studied among one another, and there is some debate as to which one balances the least morbidity with the best functional results. However, there is a consensus as to the inferiority of the classic nerve transfer relative to the three alternative procedures as the former results in hemiglossal paresis and mass activation and synkinesis [5]. The complete sacrifice of the hypoglossal nerve is no longer considered a treatment standard due to the existence of these newer techniques with significantly less morbidity.

33.3.5.2 Masseteric-Facial (V-VII)

The masseteric branch of the trigeminal nerve has more recently been popularized as an option for nerve transfer in the setting of facial nerve injury [32]. The masseteric nerve presents as a valuable option as it has reliable surgical anatomy, which provides for ease of dissection. There is minimal-to-no donor site morbidity from sacrifice. In addition, the nerve to the masseter is located in close proximity to the facial nerve, obviating the need for a cable graft, and has a high axonal count allowing for quick recovery and robust input [4, 33] (Fig. 33.5).

The success of the masseteric-facial coaptation may lie in the physiologic connectedness of cranial nerves V and VII. Some studies have indicated that patients who undergo this procedure may achieve spontaneous movement without the conscious thought required of hypoglossal nerve transfers [34]. In a study comparing hemihypoglossal nerve and masseteric nerve transpositions for rehabilitation of facial paralysis, the latter was demonstrated to result in better symmetry and faster onset of movement [35]. Other studies further suggest that masseteric nerve transfer minimizes the synkinesis, dysphagia, and dysarthria associated with hypoglossal nerve transfer and provides a robust axonal volume that allows for a speedy recovery to function [36].

A major disadvantage of the masseteric nerve transfer is the poor resting tone that results. Outcomes after this operation demonstrate a limited effect on symmetry of the oral commissure at rest, and this is particularly evident during dynamic stages such as speech during which the patient isn't clenching down [37]. In order to mitigate this, it is the authors' preference to combine the masseteric-to-buccal with a hypoglossal-to-facial coaptation, either through an end-to-side or a jump graft. In doing so, the repair is provided better resting tone from the hypoglossal while also benefiting from the fast, powerful masseteric input (Fig. 33.6).

33.3.5.3 Cross-Facial Nerve Grafting

Cross-facial nerve grafting has been described as another means of facial nerve repair in which the source of axons is the contralateral healthy facial nerve. It is the only donor nerve option that provides mimetic potential. It also allows for recov-

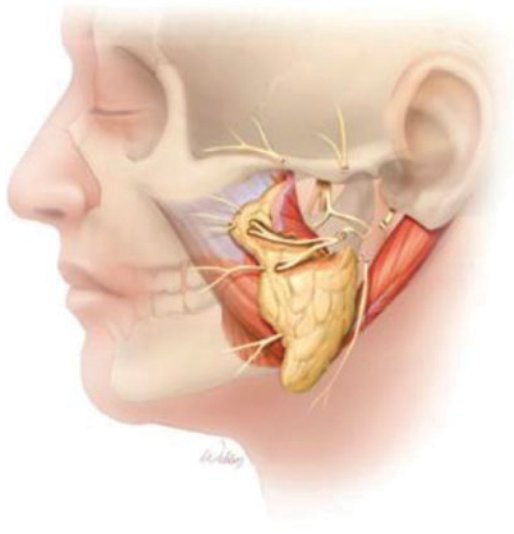

Fig. 33.5 Diagram of masseter-to-facial nerve transfer anatomy, reproduced with permission from Klebuc MJ. Facial reanimation using the masseter-to-facial nerve transfer. Plast Reconstr Surg 2011;127(5):1911

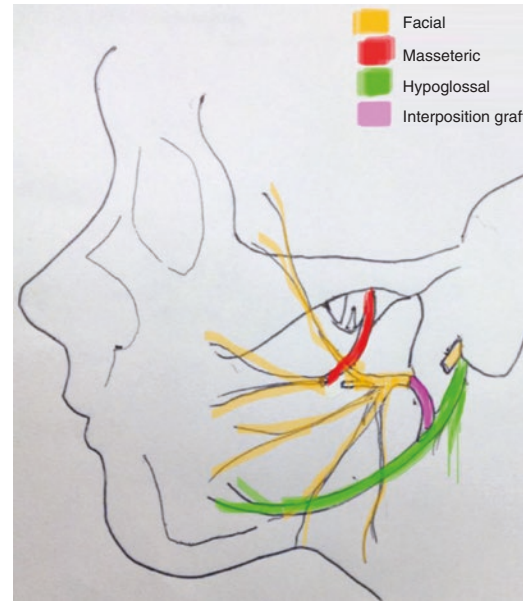

Fig. 33.6 Diagram of concurrent masseteric nerve transfer and hypoglossal nerve transfer

ery of emotion-based expression in which a patient is able to produce a smile as a natural response [38]. Due to the use of contralateral facial nerve fibers, there is no need for neuromuscular retraining or physical therapy to relearn how to activate these muscles [35].

The disadvantage to the cross-facial nerve graft procedure is that it requires a long nerve graft to reach the facial mimetic muscles and thus consists of a prolonged recovery time and requires two stages. The ensuing muscle atrophy and the limited number of axons in the repair are such that the reinnervation generates less motor power than hypoglossal nerve transfers [39].

33.3.5.4 Babysitter Procedure

The babysitter procedure is an adaptation that seeks to address some of the disadvantages of the cross-facial nerve graft technique. It capitalizes on optimal spontaneous outcomes of the procedure while mitigating the effects of longer deinnervation time. In it, a portion of the ipsilateral hypoglossal nerve is utilized first in a nerve transfer with the facial nerve trunk. Simultaneously, a nerve graft – often the sural nerve – is coapted to a healthy facial nerve branch to create the cross-facial nerve graft, which is delivered, but not connected, to the paretic side. At a later operation, once the activity of the facial nerve grows across the graft, the distal end(s) of the cross-facial nerve graft(s) is coapted to distal branches of the facial nerve [40, 41] (Fig. 33.7). The premise is that the hypoglossal nerve is a temporizing measure to prevent atrophy of the facial musculature while awaiting reinnervation [42]. By combining these two techniques, studies demonstrate that the babysitter procedure can preserve the facial musculature with an immediate, powerful nerve transfer and thus allows for optimal reanimation and synchronized facial expression in long term once the facial nerve grafts are attached [5, 43].

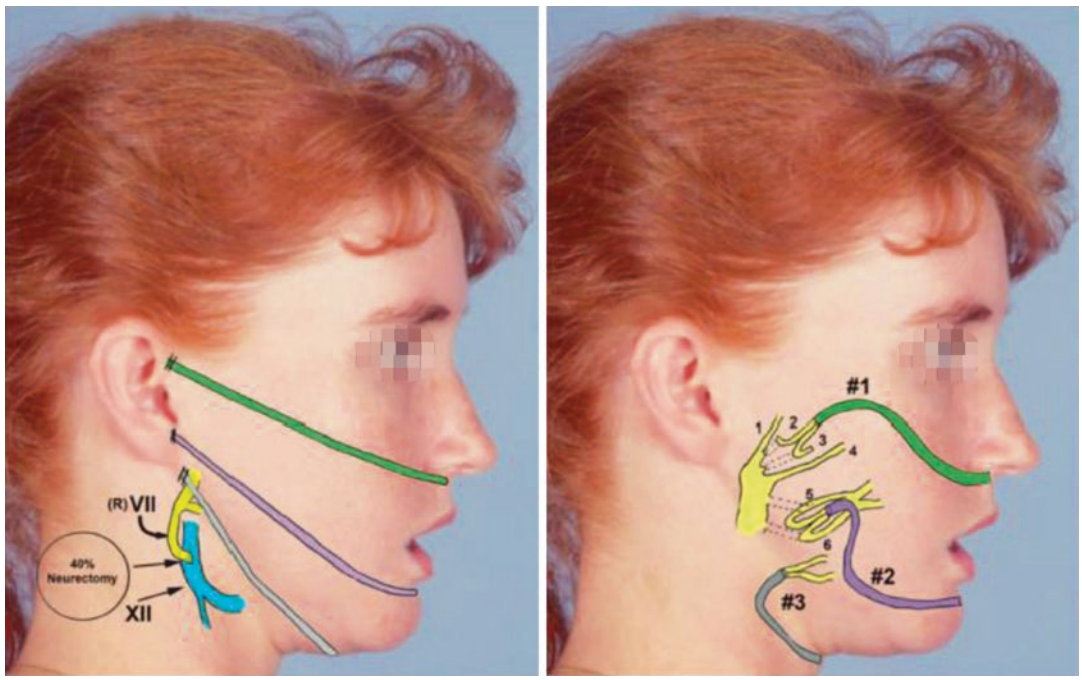

Fig. 33.7 Diagram of the babysitter procedure, stage 1 (left) and stage 2 (right), reproduced with permission from Terzis JK,Tzafetta K. The "babysitter" procedure: minihypoglossal. To facial nerve transfer and cross-facial nerve grafting. Plast Reconstr Surg 2009;123(3):872

33.4 Reanimation

33.4.1 Free Muscle Transfers

In patients who have long-standing facial paralysis, muscle transfers may be pursued. In these patients, the facial muscles are subject to irreversible atrophy and loss of function at the motor end plates and thus cannot be simply reinnervated by any of the nerve transfer procedures described above. Similarly, patients with Möbius syndrome or developmental palsy in whom the facial muscles are paralyzed from birth can be candidates for free muscle transfers as well [4].

Free muscle transfers have emerged as the premier option for these patients. The most commonly used gracilis free muscle transfer has been demonstrated to achieve quantifiable improvements in several measures including static symmetry, dynamic symmetry, and oral commissure excursion [44]. In addition, there is the potential for these patients to undergo movement-associated cortical reorganization which may allow for the development of spontaneous smiles [5, 45].

The choice of the gracilis is due to its predictable anatomy, appropriate length and contractility, and ease of harvest [43]. The procedure involves a portion of the gracilis muscle being exposed and demarcated around a neurovascular pedicle after which it is split longitudinally for transplantation. It is inset via a face-lift incision and secured to the oral commissure. For vascular supply, the facial artery and vein are used [46]. For nerve supply, there are several options. The obturator nerve has traditionally been driven by a cross-facial nerve graft which allows for a coordinated and spontaneous smile (Fig. 33.8). Alternatively, the masseteric nerve provides a significantly more powerful smile with higher axonal counts [32]. More recently, a combined approach has coupled the benefits of the two neural inputs with a double-powered transfer [47].

Due to the prolonged regeneration time of the cross-facial nerve graft, the traditional operation occurs in two parts: in the first, the cross-facial nerve graft is placed using a sural nerve donor. Axonal regeneration is followed clinically using

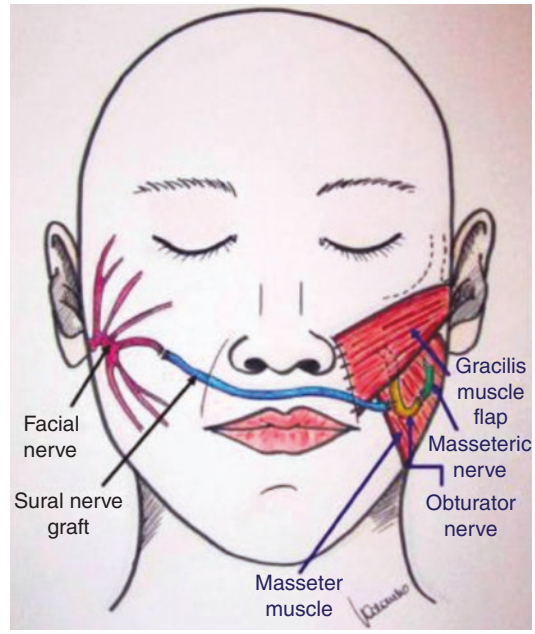

Fig. 33.8 Diagram of gracilis muscle free flap and innervation by the ipsilateral masseteric nerve and the contralateral facial nerve through an interpositional sural nerve graft, reproduced with permission from Biglioli F, Colombo V, Tarabbia F, et al. Double innervation in free-flap surgery for long-standing facial paralysis. J Plast Reconstr Aesthet Surg 2012;65:1345

Tinel's sign to determine the timing of the second stage of surgery. It may take up to a year for the regenerating axons to reach across the face. In the second operation, the gracilis muscle is transplanted as a neurovascular muscle free flap [3]. The flap is inset medial to the nasolabial crease and around the modiolus with an extension into the lower lip. Then, the vascular anastomosis is performed followed by the nerve coaptations. The flap is then affixed to the deep temporalis fascia or zygomatic periosteum along the vector of excursion [42]. The combination of this cross-facial nerve graft with input from the ipsilateral masseteric permits for spontaneous, mimetic smiles that are much more highly powered [45].

There are disadvantages associated with the gracilis muscle specifically. There is the potential for contour abnormalities and muscle bulk asymmetry that may lead to aesthetic deformities [5]. In addition, poor muscle contraction may lend to a less powerful repair if the ideal muscle tension

is not set. Studies have proposed the use of the sternohyoid muscle as an alternative as a muscle more comparable in nerve fiber makeup and bulk to the zygomaticus major. Other benefits include its nonessential function and longer recipient nerve that may provide advantages over gracilis transposition [48]. Despite this, the gracilis remains the widely accepted standard for free muscle transfer in facial reanimation.

33.4.2 Regional Muscle Transfers

As an alternative to the free muscle transfer, the donor muscle can be transferred regionally such as the temporalis, the masseter, or the anterior digastric. The temporalis muscle transfer is the most frequently used, and the favored, orthodromic approach involves removing the muscle at its insertion on the coronoid process and reattaching it to the oral commissure [43]. The advantage of regional muscle transfers is that the operation is far less complex than the free muscle transfers as there is no need for microvascular surgery; the neurovascular supply is pedicled with the regional muscle transfers [42]. In addition, the functional benefit is immediate after surgery without the long rehabilitation time associated with free flaps. Disadvantages of this operation include the potential for smile vector asymmetries, diminished excursion relative to a free flap, and lack of spontaneous smile [49].

33.5 Emerging Research

33.5.1 Advances in Nerve Regeneration

33.5.1.1 Nerve Allografts

There is a great deal of interest in finding alternatives to the use of autografts in the repair of nerve gap injuries due to secondary sensory or motor deficits incurred [50]. Allografts have been studied as an alternative, in particular acellular nerve allografts which avoid the need for long-term immunosuppression [51]. The RANGER Study has allowed for investigation into the long-term outcomes of patients who have utilized human nerve allograft in the reconstruction of the nerve. The data suggest that these allograft repairs result in high functional recovery and are a promising alternative to the traditional autograft repairs [52].

33.5.1.2 Nonneural Biological Grafts

Nonneural biological grafts have also been studied as an alternative to nerve autografts. These typically consist of artery segments, vein segments, or skeletal muscle autografts. Vein segments have been studied the most in the literature, and animal studies have reported outcomes comparable to those of nerve autografts [53, 54]. In addition, there has been some interest in the use of multiple-component conduits such as muscle-vein-combined techniques. Experimental studies have shown that combined muscle-vein conduits are rapidly colonized by Schwann cells which assist in promoting nerve regeneration [55, 56]. These, too, have demonstrated nerve repair outcomes that are nearly equivalent to autograft reconstruction [49].

33.5.1.3 Artificial Nerve Guidance Conduits

Artificial nerve guidance conduits are advantageous as they can circumvent the issues of donor site morbidity and immunogenicity that are associated with other graft techniques. Research in nerve conduits has sought to design a support structure to facilitate the direction and growth of the injured nerve and provide a barrier through which the intervening connective tissue could not cross. There are currently FDA-approved options for conduits, many of which are constructed out of type I collagen. There are a number of studies looking into techniques to control the inner conduit bioactivity in an effort to improve the efficiency and outcomes of nerve regeneration [57]. Schwann cells have been used to seed these conduits in order to mimic the native neural environment and promote nerve repair [58]. In addition, chemical influences have been studied to reduce the surrounding inflammation that can otherwise impair tissue healing in the area of the repair. Researchers have looked at using lentiviral gene

therapy to elicit a sustained IL-10 expression to modulate leukocytes in the area of spinal cord injury [59]. These and other modulators will likely play important roles in the generation of artificial nerve conduits which effectively promote guided neural regeneration.

33.5.1.4 Motor Nerve Donor Grafts

Typically, autologous nerve grafts are derived from sensory nerves, despite their role in reconstructing injuries in motor nerves. Research into Schwann cell gene expression has demonstrated that cells may express different genes and phenotypes depending on whether they are from sensory or motor nerves. Studies demonstrate that there is a phenomenon called preferential motor reinnervation in which a motor axon tends to regenerate down a motor pathway when given the option between motor and sensory pathways [60]. This is thought to be driven by the motor or sensory phenotype Schwann cells present within the nerve pathways which assist in influencing the regenerating nerves [61]. Similarly, studies in rats demonstrated preferential nerve regeneration with the use of motor nerve grafts vs sensory nerve grafts [62, 63]. There is still an absence of clinical studies in this area, but the findings thus far suggest that motor nerve defects may see optimal regeneration results with the use of dispensable motor nerve grafts.

The motor nerve to the vastus lateralis muscle is a readily accessible motor nerve during the harvest of an anterolateral thigh free flap in cases where advanced parotid malignancies require nerve grafting and soft tissue coverage. Its branching pattern is made up of 4–5 branches that arborize, and it is particularly well-suited for facial nerve repair or cable grafting [5].

33.5.2 Functional Electrical Stimulation

Studies into microelectrical system-based devices use functional electrical stimulation as a means to apply an electrical current to deinnervated muscles to stimulate muscle contraction. This technology is currently in use in several clinical applications including patients with paralyzed limbs, impaired respiratory function, and bowel and bladder dysfunction [64]. Animal studies in hemiparalysis have utilized this concept to pace intact neural signals from a healthy orbicularis oculi muscle with the contralateral, paralyzed muscle to restore eyeblink [65–67] (Fig. 33.9). There is also research to suggest that this technology may be translated into applications for other facial muscle palsies [8, 68].

33.5.3 Bioelectrical Interfaces

A major focus of research efforts targeting facial nerve injury is the study of neural interfaces that allow for the recording of a signal from a donor nerve and the stimulation of a recipient nerve.

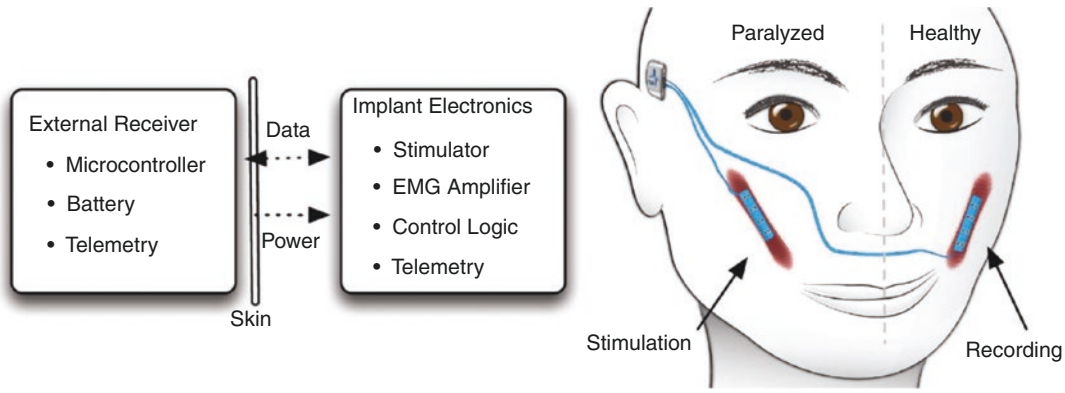

Fig. 33.9 Stimulation of paralyzed mimetic musculature using signals captured from the non-paralyzed side to generate symmetric activation, reproduced with permission from McDonnall D and Ward PD [63]

This could be applied in the setting of unilateral facial nerve injury in which the contralateral, functional facial nerve is recorded and the input is used to stimulate the side of the face ipsilateral to the injury [5]. There are a significant number of bioelectrical interfaces currently undergoing evaluation (Table 33.1).

Cuff electrodes function through the placement of an electrode placed atop the nerve that is capable of either recording neural inputs from or translating activity to the nerve. The technology is limited by the fact that the signal in question may not reflect individual nerve fascicles but rather the net activity of the entire nerve.

Table 33.1 Comparison of peripheral nerve interfaces currently being studied, reproduced with permission from Langhals et al. [5]

	Cuff electrodes	Flat-interface nerve electrodes (FINE)	Penetrating nerve arrays of electrodes	Regenerative electrodes	Regenerative peripheral nerve interface (RPNI)
Electrode structure	Exposed metal wires in silicon with monopolar, bipolar, or tripolar configuration	Advanced cuff electrode – multiple electrodes individually stimulate or record nerve activity	Either individual wires or microfabricated silicon-based electrodes that are implanted into the nerve	Silicon or polymer with holes in structure, device affixed to ends of divided nerve to allow nerve regrowth through structure	Modification of regenerative electrodes – utilized a mechanical intermediary (muscle) to maximize interface stability
Interface	Records or induces gross nerve activity from the surface of the nerve	Reshapes nerve into flatter footprint to bring individual nerve fascicles to the epineurium surface	May need pneumatic insertion tools to insert electrode intrafascicularly into the nerve	If electrodes are affixed inside the channel so that nerve regrows through, it allows interface with electrodes already in place	Muscle tissue creates a mechanical intermediary between nerve and electrode
Advantages	– Limited neural trauma, favorable biocompatibility – Long safety track record – Extensive prior use in humans	Greater selectivity and neural discrimination than standard cuff electrodes	Allows interfacing of multiple nerve fascicles and potentially individual axons	– Flexibility in size of sieves, tubes, and channels allows regeneration of nerves and fascicles – Used when the facial nerve has been divided or it is surgically acceptable to divide	– Avoids the neural trauma seen with other interfaces – Muscle tissue amplifies neural signals 10–100x – Potentially protects the nerve from direct electrical stimulation damage
Limitations	– Poor resolution of individual nerve fascicles – Biased toward axons near the epineurium/ close to cuff	– Poor resolution of individual nerve fascicles – Biased toward axons near the epineurium/ close to cuff	– 6-month life due to neural-electrical interface instability – Significant trauma and bleeding reported with cortical implantation	– Mechanical mismatch between electronics and soft neural tissue – Nerve transection required and scaffold may impede fiber growth	– Potential for interference between nearby interfaces – Optimal application-specific integrated circuit in progress

As a result, this technology cannot accurately capture the neural input to each of the individual facial muscle groups. An alternative to this is the use of the flat-interface nerve electrode to reshape the geometry of the nerve such that the individual fascicles are more superficial and thus accessible for selective stimulation [69]. The penetrating nerve array of electrodes is another option which works via intrafascicular implantation of the electrode into the nerve which allows for maximal surface area between the electrode and the neural tissue. It is a major feature in research in amputated limbs and prostheses [70]. Issues with this technology surround the incompatibility between the rigid electronic interface and the soft nerve tissue that results in rapid performance loss over the course of months [71].

Another technology on the horizon in the field of peripheral nerve repair includes the use of a regenerative peripheral nerve interface that seeks to mitigate these performance issues. Muscle tissue is used as a physiologic target into which the peripheral nerve grows, thus providing a nerve-electrode interface that prevents neuroma formation. Additionally, it facilitates a major multifold increase in signal clarity [72]. Figure 33.10 illustrates an example in which the muscle and tissue from the small intestine are used as the interface.

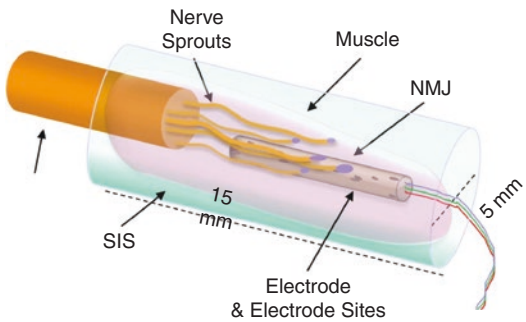

Fig. 33.10 Diagram depicting regenerative peripheral nerve interface in which the peripheral nerve has grown into the muscle and small intestine submucosa (SIS). The unlabeled arrow indicates neural input. Reproduced with permission from Langhals et al. [5]

33.6 Conclusion

Facial nerve palsy can have a significantly detrimental impact on a patient's life insofar as quality, activities of daily living, and self-image. Efficacious correction of this deficit can return a patient's ability to eat, blink, and share emotion and thus has far-reaching clinical implications. The location of the injury and the time to repair are critical factors in guiding therapeutic decision-making. From a wide variety of approaches in reinnervation and reanimation, a surgeon can effectively target the underlying neural and/or muscular deficit and return symmetry and motion to a once-paralyzed face. Important among all these techniques is the need for postoperative neuromuscular training that allows for return to function in these patients [73, 74]. Together with rehabilitation, these procedures make up a therapeutic arsenal through which critical facial nerve function can be returned to those who have suffered a very damaging paralysis.

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34 Management of Capsular Contracture: Non-surgical and Surgical Options

Jacob Haiavy and Whitney Florin

34.1 Introduction

Breast augmentation is one of the most popular cosmetic surgeries performed worldwide. In fact, breast augmentation was among the top two most commonly performed cosmetic surgical procedures in the United States in 2016 [1, 2]. The number of breast augmentations has grown 4% from 2015, with over 300,000 procedures performed in 2016 [1]. In addition, cosmetic breast augmentation has one of the highest satisfaction rates of any surgical procedure performed with reports ranging from 95% to 98%. Notably, removal of breast implants saw a 13% increase compared to 2015, with over 43,000 cases performed in 2016 [2]. In 2016, Americans spent more than 15 billion dollars on surgical and non-surgical cosmetic procedures, with a 1.5 billion dollar increase in expenditures over the past year. Surgical procedures account for 56% of the total expenditures [2].

Capsular contracture is a troublesome complication of breast implants which may require revision surgery. Capsular contracture is also one of the most common reasons for dissatisfaction with breast augmentation. Capsular contracture initially presents with firmness of the breast and can progress to pain and distortion of the breast shape and volume. When an implant is placed, a fibrous capsule forms around it. In a normal breast, the capsule is thin and soft, with no effect on the appearance of the breast. In a contracted breast, the capsule becomes thick and hard and shrinks in a way which alters the contour of the breast and the position of the implant [3, 4]. Contracture is thought to be due to a chronic inflammatory process in the implant pocket, which converts a normal foreign body response to a pathologic response. The process is not completely understood but seems to be affected by bacterial contamination or biofilm, blood, silicone gel leakage, and tissue trauma [4–6].

The Baker classification describes four grades of capsular contracture (Table 34.1), with grade I being a normal, soft breast; grade II being a minimally firm breast; grade III being a moderately firm breast with some visible deformity; and grade IV being a painful, hard, and obviously distorted breast [4, 7] (Fig. 34.1). Typically grade III and grade IV capsular contractures require surgical management [3, 4, 8, 9].

Reported rates of capsular contracture vary widely from 1% to 30% of patients who receive implants [3, 4, 9, 10]. The strongest data comes

Table 34.1 Baker classification of capsular contracture

Grade	Description
I	Normal breast
II	Minimally firm breast
III	Moderately firm breast with some visible deformity
IV	Painful, hard, and obviously distorted breast

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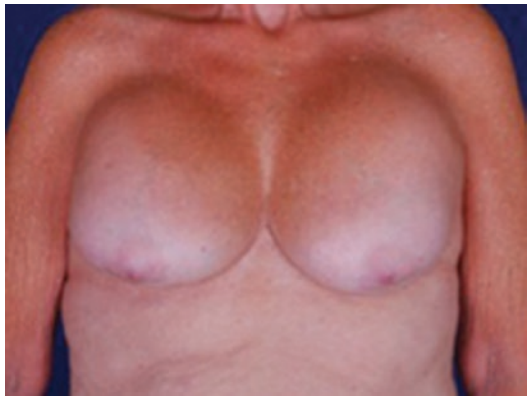

Fig. 34.1 Bilateral grade IV capsular contracture with obviously distorted breasts, which were hard and painful

from premarket approval studies. The rates of capsular contracture in these studies range from 2% to 15% after primary breast augmentation and from 5% to 22% after revision breast augmentation with a 3- to 7-year follow-up [11, 12]. Capsular contracture is often cited as one of the most common reasons for reoperation after breast augmentation [4].

Araco et al. [3] found that approximately 92% of contractures occur within the first 12 months of surgery. Others report that contracture typically develops within the first few months after implantation; however, signs of contracture may present over 5 years after breast augmentation [13].

34.2 Current Approach to Capsular Contracture

Treatment of capsular contracture is divided into non-surgical and surgical techniques. The best policy as with any complication is prevention. Measures to try to prevent capsular contracture are well described in the literature. Most surgeons agree that as with any surgical procedure, a dose of IV antibiotics administered prior to surgery is key to preventing infection. Other factors include careful hemostasis, sterile and atraumatic techniques, and local antimicrobial agents [4, 14].

It has been shown that use of the Keller funnel for insertion of the implant reduces skin contact and thus potential contamination by 27-fold ($P = 0.00059$) in a cadaver model [15]. In addition, Flugstad et al. [16] demonstrated that with the use of the funnel for insertion, the patients experienced a statistically significant reduction in the incidence of reoperations performed due to capsular contracture within 12 months of primary breast augmentation.

We all learn in surgical training that "dilution is the solution to pollution." Various irrigation solutions have been advocated over the years. In our practice we follow the triple antibiotic irrigation of the pocket advocated by Adams et al. [17]. Adams et al. demonstrated that the triple antibiotic irrigation solution decreased the incidence of capsular contracture and infection. Their solution consisted of 50,000 units of bacitracin, 1 g of cefazolin, and 80 mg of gentamycin in 500 cc of normal saline.

Textured implants [4, 18–20] and a submuscular pocket [4, 21, 22] had classically been associated with decreased incidence of capsular contracture following primary breast augmentation. We do not use textured implants for primary breast augmentation due to higher rate of reported rippling, but we do consider it as an option for secondary surgery.

Once capsular contracture has occurred, literature on non-surgical and surgical management is much less clear. Conservative or non-surgical options consist of breast exercises, high doses of vitamin E, use of prescription medication such as zafirlukast (Accolate) or montelukast (Singulair), use of herbal medication such as silymarin (milk thistle), Low-level laser (light) therapy (LLLT), and external ultrasound.

34.3 Non-surgical Options

34.3.1 Breast Exercises

To prevent and help treat early capsular contracture, many surgeons advocate postoperative compression breast exercises. The exercises are designed to keep the implant pocket and capsule larger than the size of the implant to prevent contracture. Although the authors believe that the displacement exercises do help with prevention

and treatment of capsular contracture, we did not find a well-designed study to demonstrate that.

34.3.2 Vitamin E

Baker [23] was the first to publish the effectiveness of vitamin E in reducing the incidence of capsular contracture. Vitamin E also known as alpha-tocopherol is an anti-inflammatory and a lysosomal stabilizer. It is also a biological antioxidant that protects cells from the effects of free radicals and helps stabilize their membranes. In regard to wound healing, vitamin E has been shown to decrease fibroblast and collagen formation.

In his study, Baker [23] advocated 1000 I.U. of vitamin E to be taken twice daily starting 1 week prior to surgery. If capsular contracture developed, the dose was increased to 1000 I.U. four times daily. He demonstrated a decreased rate of capsular contracture in the group that took the vitamin E. However, the incidence of severe capsular contracture (Baker IV) was the same in both groups. He advocated taking this high dose for 2 years and then reducing it to 1000 I.U. daily.

High-dose vitamin E although still used by some practitioners never gained widespread use due to different reports on effectiveness, patient compliance, and possible side effects such as dermatitis.

34.3.3 Zafirlukast (Accolate)

Having recognized that capsular contracture is a result of an accelerated or prolonged inflammatory response, the idea of modulating the immune response has been at the forefront for many investigators. One such drug is zafirlukast, which is a leukotriene receptor antagonist (LTRA). Zafirlukast was FDA approved in 1996 as a preventative and long-term treatment of asthma. Studies have shown that zafirlukast inhibits the eosinophilic influx and contractile activity of smooth muscle in all three leukotrienes (LTC_4, LTD_4, and LTE_4) in both humans and laboratory animals. This, in turn, results in decreased bronchial hyper-responsiveness to prevent an asthma attack rather than treat an attack after it has occurred.

It seems that in the case of capsular contracture, early intervention and prevention appear to be the best means of decreasing the incidence of significant capsular contracture. Schlesinger et al. [24] noted incidental improvement in their patients' capsules that were taking zafirlukast. They and a few other plastic surgeons reported their experience with a series of cases where they treated augmentation or reconstructive patients with zafirlukast 20 mg by mouth twice daily for 3 months.

Niessen et al. [25] suggest that the macrophage is a pivotal intermediary between the inflammatory phase and scar formation. The macrophage release of fibroblast-activating cytokines, transforming growth factor beta, platelet-derived growth factor, and interleukins is important in collagen production, organization, and extracellular matrix degradation. Niessen also notes that mast cell response is characterized by histamine-like activity, which is capable of stimulating more collagen formation. This results in an increase in collagen matrix in scars. Schlesinger et al. noted that zafirlukast and montelukast directly inhibit this response and therefore can reduce the rate of capsular contracture.

Schlesinger et al. [24] postulated that both myofibroblast smooth muscle contractility and prolonged collagen deposition might be involved in the development of capsular contracture. LTRAs reduce the initial inflammatory process led by macrophages and mast cells, which contribute to cellular mechanism of capsular contracture. Schlesinger noted a significant decrease in the rates of capsular contracture in his practice from 4% to 1% when using zafirlukast. He recommended the use of zafirlukast in early capsular contracture of less than 6 months or in high-risk patients such as previous history of encapsulation, periprosthetic infection, or hematoma. Ried et al. [26] in a study of 37 patients followed for an average of 16 months found a 75.7% positive response rate.

The senior author (JH) used zafirlukast as primary treatment for capsular contracture in his practice as recommended by Schlesinger et al. [24]. The response rate noted in our practice was 50% resolution of significant capsular contracture (Baker III–IV) with prevention of surgery. Of note, zafirlukast does have a reported liver toxicity side effect [27], and therefore, whenever we prescribe this medication, we first draw a liver function test panel to have a baseline level of liver enzymes. We discuss this with our patients prior to starting the medication. Over the years we have reduced the use of this medication as other medications and more natural substances that have similar anti-inflammatory effects with less adverse effects have come to light.

34.3.4 Montelukast (Singulair)

Montelukast is another leukotriene receptor antagonist that was approved for treatment of asthma. Montelukast inhibits leukotriene D_4 and is prescribed as a single 10 mg dose usually taken at night. Schlesinger et al. [24] reported an improvement in symptoms in patients with severe capsular contracture but felt that zafirlukast had a better response rate. With the concerns of liver-related adverse effects reported with zafirlukast, practitioners turned to montelukast. The adverse event profile of montelukast is comparable to a placebo, with the most common side effects being headache, influenza-like symptoms, abdominal pain, cough, and dyspepsia.

Huang and Handle [28] did a retrospective study of patients that were treated in their practice with montelukast for capsular contracture. Although a small series of 19 patients were treated, they reported 37% of their patients having completely improved, 26% having improved, 16% having no change, and 11% becoming worse or progressing with their capsular contracture. They observed that breasts with mild capsular contracture (Baker score less than III) had a greater likelihood of improvement with montelukast than those with more severe capsular contracture (Baker III–IV).

34.3.5 Milk Thistle (Silymarin)

Milk thistle, a natural herb that has antioxidant and anti-inflammatory properties, is commonly used to detoxify the body, especially the liver. *Silybum marianum* or milk thistle is the most well-researched plant in the treatment of liver disease. The active complex of milk thistle is a lipophilic extract from the seeds of the plant and is composed of three isomer flavonolignans (silybin, silydianin, and silychristin) collectively known as silymarin. Silybin is a component with the greatest degree of biological activity and makes up 50–70% of silymarin. Silymarin is found in the entire plant, but it is concentrated in the fruit and seeds. Silymarin acts as an antioxidant by reducing free radical production and lipid peroxidation, has anti-fibrotic activity, and may act as a toxin blockade agent by inhibiting binding of toxins to the hepatocyte cell membrane receptors [29].

Studies also suggest that they protect against genomic injury, increase hepatocyte protein synthesis, decrease the activity of tumor promoters, stabilize mast cells, chelate iron, and slow calcium metabolism [30].

The antioxidant properties of milk thistle are similar to vitamin E, vitamin C, and bioflavonoids in that they reduce and slow down oxidative damage. Senior author first learned about the effects of milk thistle in a live breast surgery course where another surgeon was using it for early capsular contracture instead of prescription medication due to low incidence of side effects, tolerability, and liver-protective profile. The most common side effects of milk thistle are gastrointestinal in nature with GI upset and loose bowels. The incidence is low and similar to placebo effect in studies.

Milk thistle comes in variable doses anywhere from 70 to 2000 mg. We initially treated our patients with 200 mg dose twice a day for 3 months. The response rate at this dose was low. As with any supplement, the response is dose dependent. We started recommending higher doses to our patients and found out that the optimal dose for treatment of capsular contracture is 750–1000 mg taken twice a day. The earlier the

therapy was started, the more successful were the results. Patients were educated about capsular contracture and possible therapies from our first meeting at the consultation. The importance of starting therapy as soon as tightening is perceived for higher success rate was stressed.

34.3.6 External Ultrasound

External ultrasound has been suggested as a possible treatment for capsular contracture since the late 1970s. Initially there were case reports of encapsulated breasts softening after ultrasound treatment alone. Then there were individual reports of better results combining ultrasound and closed capsulotomy [31].

In 1997 Planas et al. [32] reported a series of 24 patients with 34 encapsulations treated with external ultrasound after closed capsulotomy. The ultrasonic device used was based on a 2-MHz generator with timing adjustable power emission connected to eight transducers designed for breast anatomy. They reported 82% with significant improvement of closed capsulotomy and persistent stability achieved up to 12 months.

In 2002 Planas [33] reported his findings and recommendations for prophylactic use of external ultrasound for treatment of capsular contracture. He theorized that early application of ultrasound facilitates healing, diminishes edema, and regulates inflammation, thereby diminishing the possibility of a future capsular contracture. The mechanical effects ultrasound produces micro-massages that improve lymphatic drainage and help to resolve the edema. The biochemical effects help vascular proliferation and increase tissue oxygenation, the release of cellular mediators of inflammation, and fibrolytic processes. His modified protocol of application of ultrasound is as follows: session 1, 7 days after surgery when removing the stitches; session 2, 15 days after surgery; and session 3, 21 days after surgery. His preliminary results 18 months after starting this regimen demonstrated faster reduction of edema and inflammation, faster absorption of small bruises and ecchymoses, and a decrease of postsurgical discomfort. Most important is that from the first patients receiving this treatment to the current patients, none had experienced the formation of capsular contracture at that point.

We have not used ultrasound therapy prophylactically in our practice, but we do use for treatment of capsular contracture once patient has reported tightening of their capsule. All patients do report immediate softening of their breast after the treatments, but they seem to have rebound tightening, and we have not seen complete resolution of severe encapsulation with external ultrasound alone.

34.3.7 Low-Level Laser (Light) Therapy

The use of low-level laser (light) therapy (LLLT) for reducing pain, inflammation, and edema; promoting healing of wounds, deeper tissue, and nerves; and preventing tissue damage has been known for almost 40 years since the invention of lasers. Many animal studies and randomized clinical trials have shown the photobiomodulation and biostimulation effects of non-coherent light. Despite the evidence, LLLT has yet to receive wide acceptance in the scientific community.

Low-level laser (light) therapy is also known as "cold laser" and "soft laser" and is practiced as part of physical therapy in many parts of the world. The question is no longer whether light has biological effects, but rather how energy from therapeutic lasers and light-emitting diodes (LED) work at the cellular level and what are the optimal parameters for different uses of these light sources.

The energy of a dose of light depends only on the number of photons and their wavelength or color. Photons absorbed into living tissue can be either absorbed or scattered. The photons that are absorbed interact with an organic molecule or chromophore located within the tissue [34].

Mitochondria are thought to be a likely site for the initial effects of light [35–38], leading to increased ATP production, modulation of reactive oxygen species, and induction of transcription factors. These effects in turn lead to increased cell proliferation and migration, modulation of levels of cytokines, growth factors and

inflammatory mediators, and increased tissue oxygenation. The results of these cellular changes in animals and humans include such benefits as increased healing of chronic wounds, improvement in sports injuries, and pain reduction in arthritis and neuropathies.

There are multiple critical parameters that promote the biological responses described; however, the most critical are the wavelength, energy density, and duration of treatment. The wavelength needs to match the absorbance of the desired photo-accepting molecule and also determines the depth of penetration. The energy density will need to be high enough to elicit the desired effect, but low enough not to induce toxic or adverse effects. Generally, the clinical literature demonstrated that treatments delivered multiple times a week over several weeks result in greater efficacy. In addition, pulsing of light is known to increase penetration depth [39].

Some known parameters are that wavelengths in the 600–700 nm range are chosen for treating superficial tissues and wavelengths between 780 and 950 nm are chosen for deeper tissues, due to longer optical penetration distances through tissue.

Johnson et al. [40] conducted a study that was published in 2015. They used a handheld device called the LTU-904 laser (RianCorp Pty Ltd., Richmond, South Australia), a class 1 laser that delivers a controlled series of bursts (200 ns) of near-infrared (904 nm) laser beam pulses. The US Food and Drug Administration first approved Low-level laser (light) therapy for the treatment of lymphedema in 2006.

In their study, 33 patients with grade III and IV capsular contracture completed the 6 full treatments of LLLT. The treated area was subdivided into a 2 × 2 cm square grid pattern. Each square received 1 min of treatment for a total of 10 min (300 mJ/1 min treatment = 4.5 J/cm^2). Surgical intervention was avoided in 93.9% of treated patients (31 of 33 patients). They also administered patient surveys. Of the 31 patients who avoided surgery, the laser improved patient perceived breast stiffness by 10–95% (average, 43.6%), with the overall improvement in comfort ranging from 10 to 95% (average, 48.2%). Of the ten previously irradiated patients, only one patient stated there was no improvement.

All patients self-reported that their result was improved enough to avoid corrective surgery and stated that they would undergo further LLLT treatments again if deemed necessary.

Jackson et al. [41] performed a randomized, double-blinded study to determine the effectiveness of LLLT in decreasing postoperative pain following breast augmentation. Using LLLT both preoperatively and postoperatively, they found a significant decrease in postoperative pain and the amount of pain medication needed following breast augmentation at 1 day and 1 week.

A systematic review of eight studies was conducted by Omar et al. [42] to review the effectiveness of LLLT in the management of breast cancer-related lymphedema. They concluded that there is moderate to strong evidence for the effectiveness of LLLT at a dose of 1–2 J/cm^2 per point applied to several points covering the fibrotic area to reduce lymphedema and improve shoulder mobility.

Furthermore, Jackson et al. [41] performed a double-blind, randomized, placebo-controlled study comparing LLLT 635 nm to sham therapy for body contouring of the waist, hips, and thighs. Following six treatments over a 2-week period, the patients treated with a non-invasive laser demonstrated reduced overall circumference measurements of the specific treated regions [43, 44].

Freitas et al. [45] tested the efficacy of LLLT over a 5-week period on scar tissue in nine volunteers and found a positive effect on the macroscopic appearance of the treated scars and a decrease in the scar thickness.

Having learned all of these effects of low-level laser, we set out to find a device that would be practical for the treatment of capsular contracture. I wanted the device to be easy to use, economical, and practical for our practice and patients. One such device was the Celluma from BioPhotas (BioPhotas, Inc., Anaheim, CA) (Fig. 34.2). Celluma is a safe, affordable, and easy to use flexible LED array. The Celluma has been FDA approved for multiple indications such as arthritis, muscle spasm, muscle and joint pain,

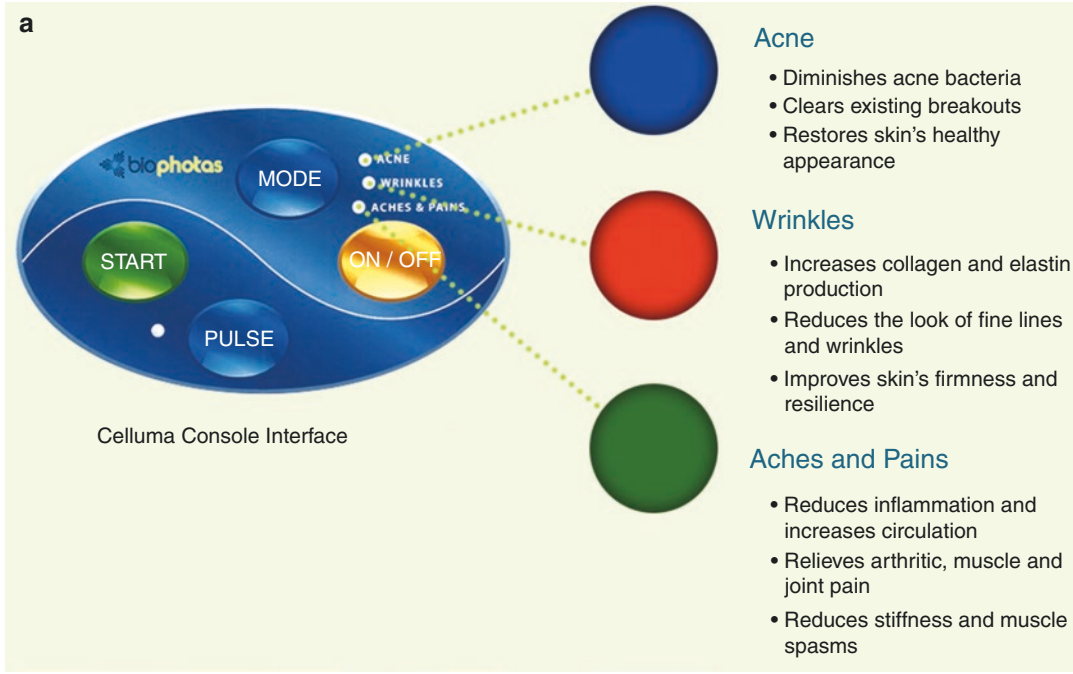

Fig. 34.2 (**a**) The Celluma console interface showing different modes. (**b**) Patient during a Celluma treatment for capsular contracture showing the flexibility of the device in conforming to the patient's body

muscle tissue tension, joint and muscle stiffness, diminished local circulation, and inflammatory acne vulgaris. The Celluma has 345 light-emitting diodes that emit energy at blue (465 nm), red (640 nm), and near-infrared (880 nm) wavelengths with frequencies of 80 Hz, 680 Hz, and 800 Hz, respectively, for duration of 30 minutes per treatment. The device comes programmed with multiple operating modes for each clinical application.

Two key clinical advantages to this device are the flexibility and ease of adaptation for optimal fitting to the contours of the body and the fact that it offers longer duration of treatment. Properly fitting the contours of the body is key to optimal energy absorption. A longer treatment duration time allows the body more time to respond to the therapeutic effects of LLLT.

Currently in our practice at the earliest sign of capsular contracture, we start our conservative treatment protocol. Our patients are educated about the risk of developing capsular contracture at the initial consultation and the importance of starting conservative therapy as soon as possible. We further explain that the earlier the treatments are started, the higher the rate of success in softening the breast and preventing the need for surgery.

Since the etiology of capsular contracture may be multifactorial and related to inflammatory changes, our conservative protocol combines multiple modalities as well. Our protocol includes:

1. Milk thistle 1000 mg twice a day for 3–6 months
2. Low-level laser light treatment with the Celluma once or twice a week for 6–12 weeks
3. Ultrasound treatments once or twice a week for 6–12 weeks

In addition, all of our breast augmentation patients are also given breast displacement exercises from postoperative day one. With the above protocol, we have been able to treat capsular contracture and prevent the need for surgical intervention in 80% of our patients. We further postulate that if milk thistle and LED treatments are started immediately after surgery and combined with ultrasound treatments, we could reduce the rate of capsular contracture even more. Ideally, to validate this hypothesis there should be a prospective randomized double-blind study that measures the rates of capsular contracture as well as the success in treatment on this protocol versus doing nothing.

34.4 Surgical Options of Treatment

Once capsular contracture has occurred, literature on surgical management is much less clear. Management has evolved from closed and open capsulotomies in the 1970s–1980s [46, 47] to capsulectomy, site change, and implant exchange in the 1990s [48–51].

Today, capsulectomy, site change, and implant exchange are considered the gold standard treatments of clinically significant capsular contracture [5, 52, 53]. This is based on high recurrence rates after capsulotomy alone, the theory of biofilm calling for complete removal of the prosthesis and all bacteria-harboring capsule [14, 54], and limiting periprosthetic scar and inflammation by changing the pocket [4]. However, the actual clinical evidence behind this treatment plan is not entirely clear.

Moreover, the indications for partial versus complete capsulectomy are elusive. The surgeon is therefore left with the decision of whether to perform a partial or complete capsulectomy and open capsulotomy or to leave the capsule in place. Despite the impressive number of breast surgeries performed in the United States, there have not been clear guidelines from implant manufacturers or professional societies regarding capsulectomies.

A statement released by the American Society of Plastic and Reconstructive Surgeons in 1995 outlines the risks and benefits of capsulectomy, but for many surgeons, this was not comprehensive enough [50]. Up until an article by Young in 1998 [55], there were only a few articles discussing capsulectomy [52, 56, 57] and case reports discussing the problems associated with retained implant capsules [57, 58]. Since then, there have been several systematic reviews of management of capsular contracture [3, 4].

A systematic review of 24 observational articles published by Wan and Rohrich [4] in 2016 found that there is no definitive evidence that capsulectomy is more effective than capsulotomy in preventing recurrence of capsular contracture. However, the data is limited in that many studies

did not specify the extent of capsulectomy performed. Therefore it remains unclear if the extent of capsulectomy affects recurrence rate of capsular contracture. Data by Collis and Sharpe [49] shows lower recurrence rate of capsular contracture for total versus anterior capsulectomy in subglandular contracture. However, after controlling for implant type, the significance of this finding is unclear [4]. Costagliola et al. [59] found no difference in recurrence of capsular contracture whether total or anterior capsulectomy was performed. However, total capsulectomy was performed for all subglandular contractures, and anterior capsulectomy was performed for all submuscular contractures. Therefore the significance of this data is unclear as well [4].

34.4.1 General Indications for Capsulectomy

Given that there is inadequate evidence to suggest that total capsulectomy is superior to anterior capsulectomy in the treatment of contracture, we allow the clinical scenario to guide our management of the capsule. Above all, the benefit of capsulectomy must outweigh the risk to the patient. The factors that affect the decision to remove a capsule extend beyond the type of implant: implant pocket and quality of the capsule (Table 34.2).

Table 34.2 Indications for partial or total capsulectomy in conjunction with implant removal

• No replacement of an explanted implant or tissue expander
• Exchange of an existing implant in one tissue plane for a new implant in a different tissue plane
• Capsular contracture (Baker grades III and IV)
• Calcified or thick, fibrous capsule
• Removal of a ruptured implant, especially one filled with silicone gel
• Removal of silicone granulomas
• Exchange of an implant for one with a larger volume
• Replacement of a smooth implant with a textured implant (regardless of filler material in existing or new implant)

34.4.2 Position of Existing and Replacement Implants

A capsulectomy should be performed when no implant will be replacing the explanted implant or when the replacement implant will be placed in a different tissue plane (i.e., changing from subglandular to submuscular position or submuscular to subglandular pocket). Retained capsules in a subglandular position are more likely to present as palpable masses or artifacts on mammography, which may lead to an unnecessary biopsy to rule out malignancy. Therefore capsules in the subglandular position should be removed assuming this can be done with minimal risk to the patient. However, implants which have been placed after subcutaneous mastectomy or breast reconstruction often lead to capsules, which are quite close to the skin. Injury to the skin or devascularization can occur when attempting to remove these capsules. Therefore, capsules which are adherent to the skin should be left in place to minimize risk of skin injury. In these cases, partial capsulectomy to the posterior portion of the capsule can be performed [4, 50].

Capsulectomy in the submuscular space provides its own set of concerns. It can be difficult to remove the capsule from the deep surface of the pectoralis major muscle due to contraction of the muscle. There can be injury to the muscle leading to excessive bleeding which can be difficult to control. Moreover, the capsule is often adherent to the chest wall. When the capsule is normal (thin and flimsy), it can be particularly difficult to remove from the chest wall. Aggressive attempts at a total capsulectomy can lead to pneumothorax. Therefore, capsules in the submuscular plane, which are not thickened or calcified, do not necessarily need to be removed. Thin capsules will likely be resorbed spontaneously and will likely not cause palpable masses or interfere with mammography [4, 50].

Another difficult scenario is when the capsule extends into the axilla. This tends to occur with older silicone implants in a submuscular position with extracapsular rupture. When attempting capsulectomy, pulling inferiorly on the capsule

with instruments can bring the axillary contents into the operative field, putting them at risk of injury. Attempt to remove the capsule, which is in the axilla risks injury to the brachial plexus or axillary vessels. Controlling bleeding or repairing damaged nerves would likely require an additional axillary incision, as well as increased operative time. In most situations, it is not advisable to aggressively remove capsule, which extends into the axilla. If it is considered necessary to remove this portion of the capsule (i.e., due to patient's insistence or a palpable mass), it is prudent to create a separate axillary incision to gain exposure to the site and minimize injury to surrounding structures [50].

34.4.3 Position of Existing and Replacement Implants

There are no indications for capsulectomy, which are specific to saline implants, whereas capsulectomy is considered more important for silicone implants. Research has found silicone in the capsules of silicone implants [13, 55, 60, 61]. Capsulectomy is thought to remove potential for residual, radiopaque silicone to interfere with mammography [50].

Most cosmetic surgeons will agree that ruptured silicone implants can lead to difficulty in the operating room. Ruptured implants in the subglandular space tend to be more confined than ruptured implants in the submuscular space, which can extend into the axilla, especially if the rupture is extracapsular. Total capsulectomy can facilitate removal of silicone material when the implant is ruptured. Capsulectomy, however, does not guarantee removal of all silicone material. Some gel may be present in tissue beyond the capsule and may not be visible or palpable. Moreover, silicone cannot be dissolved so it is not possible to completely remove all gel even with copious irrigation. It is also difficult to completely wipe away silicone in an extracapsular rupture. The surgeon can only remove as much as gel as possible, without causing unnecessary harm to the patient. In cases of ruptured silicone implants, capsulectomy is warranted unless other factors outweigh the benefits of capsulectomy [4, 50].

34.4.3.1 Silicone Granulomas

Silicone can induce the formation of foreign body granulomas [50, 62]. When silicone granulomas are present, capsulectomy is usually indicated. While there is no clear evidence that granulomas cause a systemic response, excision of granulomas will lead to more complete removal of silicone. Granulomas can also present as a palpable mass or a radiopacity on mammography. Therefore, when granulomas are accessible to the surgeon, they should be removed. A capsulectomy facilitates removal of granulomas, as they are typically adjacent to the capsule in an extracapsular rupture. Removing the capsule also permits greater exposure to identify granulomas. Large granulomas are typically easy to find with inspection and palpation. Small granulomas (<5 mm) can be missed in surgery but later become evident on mammography or MRI. Careful examination and palpation of breast tissue, pectoralis major muscle, chest wall, and axilla can lead to identification of small granulomas, which are typically harder than the surrounding tissue. An intact implant does not rule out silicone granulomas, as they may have been missed when a previous ruptured implant was removed [50].

34.4.4 Capsule Thickness and Presence of Capsular Contracture

It is not entirely clear which capsules will resorb on their own. However, it seems that thin capsules in the submuscular plane tend to resorb. Therefore, thin, flimsy capsules can be left in place since they are difficult to remove and most likely will be resorbed. Thick, fibrous capsules, on the other hand, are unlikely to be resorbed and may lead to palpable masses and/or abnormalities noted on mammography. Therefore, thick

capsules should be removed at the time of explantation of the implant. If complete capsulectomy is considered too risky, then partial capsulectomy should be performed [4, 50].

Some authors have suggested that any capsule with a Baker grade III or IV capsular contracture should be removed, regardless of whether the implant will be replaced [13, 50]. A severely contracted capsule, which is left in place, can produce a breast deformity and palpable mass. There are concerns that this residual capsule can also interfere with mammography. Bacteria may also colonize grade III or IV capsules. Removal of the capsules can decrease the bacterial load and lower the risk of developing a subsequent capsular contracture if the implants are replaced [13, 50]. However, it is important to always consider the risks of total capsulectomy, including damage to surrounding structures. The surgeon must use clinical judgment to decide the extent of capsulectomy to be performed, even in the setting of capsular contracture.

34.4.5 Calcification of the Implant Capsule

Calcification of the implant capsule can occur as well [50, 62–67]. Destouet et al. [62] reported calcification in up to 30% of women who had breast implants for 10 years or longer. The cause of calcification of the capsule is unknown. Siggelkow et al. [65] reported on 53 capsules around silicone breast implants from 43 patients (23 smooth and 30 textured devices). A higher Baker score was found with increasing patient age, implant duration, and thickness of capsule. Calcification was associated with duration of implant and age of patient. Focal calcification was noted mostly on the inner side of the breast capsule. In this study, calcification was only found around smooth implants in the subglandular site following cosmetic augmentation.

Calcified capsules make the breast very hard and abnormally round and cause discomfort. Mammographers typically do not have difficulty distinguishing between calcifications in a capsule and microcalcifications associated with carcinoma. However, a calcified capsule can obscure areas of breast tissue. Therefore, every attempt should be made to completely remove calcified capsules. Typically these capsules are easy to remove, even when they are in the submuscular plane, because there is a distinct tissue plane [50].

34.4.6 Smooth Shell Versus Textured Shell of Explanted Implant

Considerations for capsulectomy depend on whether the implant being explanted has a smooth or textured shell and the type of implant being used to replace it. Implants with a smooth elastomer shell tend to cause a relatively uniform and smooth capsule. The decision to remove this capsule depends on the factors discussed previously, i.e., positioning of the existing and replacement implant, filler material, capsule thickness, and severity of capsular contracture. Also, if an implant with a smooth surface is to be replaced with an implant with a textured surface, a capsulectomy should be performed to allow the textured shell to interact with a fresh tissue surface. This may decrease the risk of capsular contracture in the future [50].

When removing textured implants, the capsule can be left intact if a replacement implant is placed in the same position. However if a textured silicone implant is removed, it is reasonable to perform a capsulectomy in order to remove any gel, which may be present in the capsule [50].

Both saline and silicone gel-textured implants can lead to synovial-like metaplasia [13, 50, 65]. Synovial-like metaplasia is benign, but it can lead to dense hyaline collagenous fibrosis after implant duration of more than 2 years. Synovial-like metaplasia is more prominent in pockets surrounding textured implants which have been in place for a longer amount of time [65]. This may also lead to

fluid formation in the intracapsular space which can result in seroma formation. Therefore it may be wise to perform a capsulectomy when removing textured implants in order to decrease the risk of synovial metaplasia and seroma [50].

34.4.7 Change in Volume of the Implant

When an existing implant is being replaced with a larger implant, a capsulotomy or capsulectomy should be performed. If the capsule is a grade I or grade II and normal in appearance, open capsulotomy can be performed. It is obvious that using a larger implant will require some change to enlarge the implant pocket. Some surgeons do prefer to do a complete capsulectomy in order to have a fresh tissue surface against the new implant. When an existing implant is being replaced with a smaller implant, a capsulectomy may not be necessary, but can be performed, depending on the factors discussed in this article [50].

34.4.8 Considerations for Implant Replacement

Implant exchange is associated with lower recurrence rates of capsular contracture (0–26%) versus with no implant exchange (0–54%) [4]. This is particularly notable when the replacement implant is placed in the same plane. Replacing old implants in the same pocket is misguided as it is associated with the highest risk of recurrence of contracture.

There were no obvious trends in recurrence rate of contracture with textured, saline, or silicone replacement implants. However, smooth implants were associated with overall lower recurrence rates of capsular contracture. This is in contrast with the established clinical association of higher rates of capsular contracture with smooth implants versus textured implants. However, that association was based exclusively on primary breast augmentation data and may not apply to revision surgery [4].

Selection of the replacement implant should ultimately be based on the patient's tissue characteristics. Textured implants have a higher risk of rippling and palpability compared to smooth implants, especially in the subglandular plane. Saline implants also have a higher risk of rippling compared to silicone implants. Therefore, it is reasonable to use smooth gel implants to minimize rippling and palpability in the patient with thin overlying breast tissue [4].

Acellular dermal matrix is associated with a lower recurrence rate of capsular contracture (0–7%) compared to recurrence rate with reaugmentation without acellular dermal matrix (5–19%). However these studies are limited by their short follow-up periods (average, 1.4–3.6 years) [4].

34.4.9 Operative Time and Technique

Capsulectomy adds approximately an hour to the operative time, which means increased cost to the patient. Moreover, adequate exposure for capsulectomy may require a larger incision than if implantation alone was being performed. Some surgeons prefer to remove the implant and capsule together, without entering the implant capsule. The thought is that this technique results in a more complete removal of silicone gel, especially in the case of a ruptured silicone implant. Some surgeons also find this method to be easier. However, this technique requires a larger incision, and there is no clear evidence that the benefits of this method outweigh the morbidity [50].

Moreover, the capsule may still be entered despite the best efforts of the surgeon. Thus, ruptured silicone material enters the extracapsular space and must be manually removed. The authors of this article begin the dissection around the capsule and implant, especially in the case of a known ruptured silicone implant. As much dissection as can safely be performed is carried out. Sometimes the entire implant and capsule can be removed without entering the implant capsule. Other times the capsule is entered and ruptured silicone material extravasates. At this point the capsule is removed in pieces along with the implant and implant material (Fig. 34.3). In the

Fig. 34.3 Ruptured silicone implants and fragments of the capsules, which were removed bilaterally

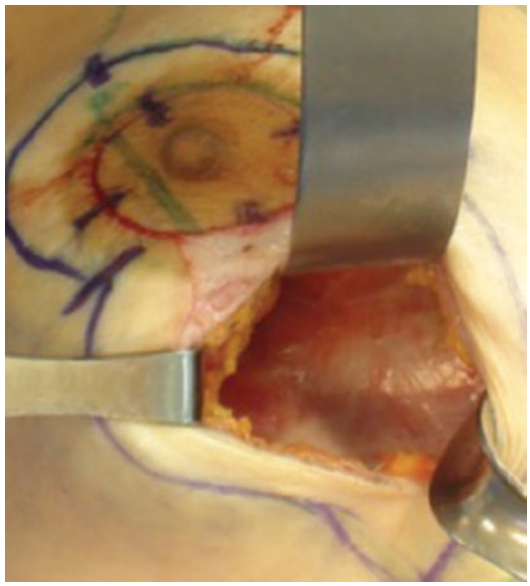

Fig. 34.4 Thin, normal-appearing capsule, which was adherent to the chest wall and left in place in order to prevent damage to surrounding structures

case of submuscular pockets, entering the implant capsule can lead to greater exposure to remove the capsule which is adherent to the chest wall (Fig. 34.4).

34.4.10 Preoperative Discussion and Informed Consent

Typically an inframammary or periareolar incision is best for implant explantation and capsulectomy. For small areola (<4 to 5 cm in diameter), an inframammary incision is advised. For extension into the axilla, an additional axillary incision may be needed for better accessibility. Preoperatively, the surgeon should have an open discussion with the patient regarding the need for new, larger, or multiple incisions. The benefits of capsulectomy versus the risks and additional expense must be discussed with the patient. Patients undergoing removal of implants for severe capsular contraction should be explained that capsulectomy is recommended to eliminate a possible palpable and visible mass, mammographic artifact, possible bacterial colonization, and risk of a poor aesthetic result [50]. This discussion must include an explanation that if the capsule is adherent to the chest wall (in a submuscular plane) or to the skin (in a subglandular plane), a partial capsulectomy will be performed to minimize risk to the patient.

Most patients request a capsulectomy once they understand the risks and benefits. This is particularly true with women who are having silicone implants removed because of the perceived risk of silicone material. Patients should be informed that the current scientific evidence does not support a risk of retained silicone gel material in the implant pocket. It is also important to clarify that removal of silicone material or the implant capsule may not improve systemic symptoms that some patients attribute to the presence of breast implants. When a patient does request a capsulectomy, it should be performed assuming it does not pose significant risk to the patient [50].

34.4.11 Open Capsulotomy

Open capsulotomy is reasonable in certain situations, such as modification of the capsule for a larger implant, correction of a malpositioned implant, modification of the shape of the breast, and conversion of a tissue expander to a permanent implant [4, 50]. This is assuming the breast is soft and the capsule is thin and normal in appearance. In the case of a malpositioned implant in which the implant is intact, the same implant can be reinserted after capsulotomy and pocket modification. However, the implant manufacturers state that implants are for single use only, which precludes implant reuse after

capsulectomy or capsulotomy. The surgeon should be aware of these recommendations and be prepared to defend the decision to reuse an implant [50].

34.4.12 Delayed Capsulectomy

Delayed capsulectomy may be required for retained capsules that produce an unaesthetic result, palpable mass, mammographic abnormality, source of fluid accumulation, or infection. A surgeon will ideally avoid the need for a delayed capsulectomy by performing a capsulectomy at the time of implant removal or replacement. However, the patient may have had the implant removed by another surgeon and then presents later with the need for capsulectomy. This most commonly occurs in the patient with capsular contracture who did not have capsulectomy at the time of the initial surgery. In this case, delayed capsulectomy should be performed [50].

34.4.13 Contraindications to Capsulectomy

Below is a summary of situations in which total capsulectomy should be avoided in order to prevent unnecessary harm to the patient.

1. A thin and flimsy capsule can be difficult to remove, and capsulectomy can cause damage to surrounding tissue.
2. In a submuscular implant, when the posterior capsule is tightly adherent to the ribs and intercostal muscles causing a risk of chest wall perforation and pneumothorax, a partial capsulectomy should be performed.
3. The risk of capsulectomy of subglandular implants in a thin patient usually outweighs the benefit. Removing a subglandular capsule can injure the skin by compromising blood supply or cause a perforation through the skin. A partial capsulectomy of the posterior portion of the capsule can be performed.
4. Patients with very thin overlying breast tissue who are replacing a saline-filled implant may benefit from the tissue padding of the capsule. In situations in which the breast is soft and the capsule is normal-appearing, the capsule can be left in place to decrease risk of rippling of the implant.
5. In the case of a malpositioned implant, such as one that is laterally or inferiorly displaced without capsular contracture, a normal-appearing capsule can be used for capsulorrhaphy.

34.5 Special Considerations

34.5.1 Contraindications to Capsulectomy

There has been a long history of speculation about the safety of breast augmentation, specifically regarding increased risk of carcinoma and/or autoimmune disorders with silicone. The National Institutes of Health found no associations between breast implants and cancer, autoimmune disorder, neurologic disorder, or other systemic diseases [68]. Moreover, the risk of breast cancer is not higher for silicone implants compared to saline-filled implants [69, 70].

However, there have been a few reports of carcinoma which seemed to arise from the breast implant capsule [71–73]. Paletta et al. [71] reported a squamous cell carcinoma which apparently arose from the implant capsule 15 years after breast augmentation. Kitchen et al. [72] reported an implant capsule lined by benign squamous epithelium and another case of squamous cell carcinoma in the implant capsule. It is reasonable to assume that squamous cell carcinoma is preceded by benign squamous epithelium. However prior to this report, there were no cases of epithelialization of breast implant capsules. The origin of the epithelial cells in the breast implant capsule is unclear. The usual histological findings in the breast implant capsule have been well documented. Host tissue reactions around the implant include formation of a fibrous capsule, foreign body giant cell reaction, and infiltration of chronic inflammatory cells [3, 4, 63, 64, 72]. In addition, calcification of the fibrous capsule has been reported [50, 63, 64].

There are several possibilities as to the origin of the epithelial cells in the capsule. One is that microscopic skin fragments could be implanted in the incision at the time of implant placement. These epithelial fragments could subsequently form an epithelial lining. Another theory is that dermal adnexal structures could proliferate into an epithelial lining. However, the most plausible theory is that ductal epithelium undergoes squamous metaplasia. Ducts are invariably transected during placement of an implant. It is recognized that endoderm-derived epithelium in the bronchus, thyroid, urethra, and prostate can undergo squamous metaplasia in the setting of chronic irritation. Therefore, it is possible that epithelium from transected ducts proliferated within the implant capsule and became metaplastic in response to chronic irritation from the breast implant [71, 72].

When carcinoma is present in or adjacent to the implant capsule, it is recommended to remove the implant, the capsule in its entirety, and any abnormal surrounding tissue to submit as a pathology specimen [50, 73].

34.5.2 Anaplastic Large Cell Lymphoma

There has also been concern over the association of breast implants and anaplastic large cell lymphoma (ALCL) [74, 75]. In 1995, a case series of three women with breast implants and cutaneous T-cell lymphoma was reported [76]. Since then, there are 63 documented cases of primary breast implant-associated ALCL [74]. While breast cancer is the most frequent cancer affecting women, primary lymphoma of the breast is exceedingly rare, accounting for only 0.04 to 0.5 percent of malignant breast tumors, 1–2% of extranodal lymphomas, and less than 1 percent of all non-Hodgkin lymphomas [77]. In 2011 the US Food and Drug Administration released an alert that women with breast implants have an increased, although very low, risk of developing breast implant-associated ALCL [78]. This form of ALCL appears to have a more benign course than systemic ALCL. Treatment of ALCL is removal of the implant and complete capsulectomy, along with oncologic consultation to investigate other sites of disease [75]. Most patients with breast-confined disease achieve complete remission after surgical management. Women with more extensive disease benefit may also benefit from chemotherapy [74, 75]. While ALCL is extremely rare, clinicians must be vigilant. Patients with late-onset seroma, sudden breast swelling, and/or pain should be suspected of having ALCL and be worked up appropriately [74].

34.5.3 Infection

Many capsules are culture positive for microorganisms, such as *Staphylococcus epidermidis*, which is associated with capsular contracture [13, 24, 68, 79–81]. Colonization of bacteria is usually an incidental finding, found during removal of implants [50]. However Pajkos et al. [14] reported a *S. epidermidis* biofilm in a patient with recurrent capsular contracture. The thought is that once a biofilm forms on the outer surface of the implant surface, it can be a source of chronic inflammation and irritation, which can lead to capsular contracture. The theory of subclinical infection may contribute to why implants placed above the muscle have higher contraction rates than submuscular implant. Implants above the muscle are in close proximity to the breast ducts which carry bacteria more than 90% of the time [13, 82].

An acute suppurative infection, on the other hand, is an uncommon complication of breast implants. An acute infection is manifested by pain, swelling, erythema, and fever. Once an infection is diagnosed, explantation with complete removal of the capsule is always indicated. This will speed the resolution of the infection and allow the normal healing process to proceed. It is advised to place a drain when a capsulectomy is performed in the setting of an infection. Failure to remove the capsule when an infection is present will lead to a dead space colonized by bacteria which antibiotics may not be able to sufficiently penetrate. This will lead to delayed healing and increased time before the implant can be replaced [50, 54, 80, 81].

There are simple preventive measures that have been found to decrease bacterial load, such as a preoperative dose of intravenous antibiotics, irrigation of the implant pocket with antimicrobials before placement of the implant, and minimizing contact between the implant and the surrounding skin and breast tissue [14–16].

34.6 Conclusions

The treatment of capsular contracture is most certainly multifactorial. The ultimate goal is to prevent recurrence of capsular contracture, minimize risk to the patient, and obtain esthetic results. The non-surgical options of treatment discussed are certainly successful in managing early capsular contracture and avoiding the need for surgery. Capsulectomy is indicated in the majority of cases when breast implants are being removed or replaced in the setting of severe capsular contracture. However, the surgeon must always weigh risks and benefits of capsulectomy. The removal of a capsule should not warrant significant risk to the patient, such as pneumothorax, devascularized skin, or injury to nerves or vessels. It is unclear if a total capsulectomy is advantageous over a partial capsulectomy in preventing recurrence of contracture. Therefore, it is up to the surgeon to use clinical judgment to guide management of the implant capsule.

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Soft Tissue Reconstruction of the Lower Limb

35

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35.1 Introduction

This chapter aims to review current and past thoughts on reconstruction of the lower limb, discussing in particular the options in terms of soft tissue coverage. This chapter does not aim to review the emergency management of open fractures, or the therapy alternatives to chronic wounds or malignancies of the lower limb, but purely assesses the requirements that should be reviewed on reconstructing a defect of the lower limb. However, this end point needs to be taken into account at the initial evaluation or presentation of the individual as it may spare the patient, and surgeon, multiple procedures and considerable frustration.

A summary of flap options is considered, with literature support, in regard to donor and recipient region, particularly as flap coverage is regarded as the cornerstone of soft tissue coverage of the lower limb.

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35.2 The Lower Limb

The lower extremities of the human body are more commonly known as the human legs, incorporating the foot, the lower or anatomical leg, the thigh, and the hip or gluteal region.

The human lower limb plays a simpler role than that of the upper limb. Whereas the arm allows interaction with the immediate surroundings, the legs' primary goals are support and to allow upright ambulation. Essentially, this means that reconstruction of the leg is less complex than that required in restoring functionality of the upper limb. In terms of reconstruction, the primary goals are based on the preservation of life and limb and the restoration of form and function.

The leg consists of four main regions before attaching to the pelvis. Working proximally, these are the following: the foot, the lower or anatomical leg (from the ankle to knee), the thigh (knee to hip), and the hip or gluteal region. Primarily, the four areas work together to aid balance and support, which, in turn, allow a human to stand and walk.

Evolution has forced the lower limb to gain this distinct feature, and although bipedal gait is not unique to humans, an efficient upright locomotion for long durations is. This adaptation has forced the human leg to become longer and more powerful in comparison with our primate relations, as well as change the way in which the muscles and joints of the leg interact and function [1].

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The ability of the legs to offer support and allow upright ambulation has permitted the adaptation of the upper limb, the arm, to allow precise interaction with the surrounding environment.

35.3 Reconstruction of the Lower Limb: Why Is It Needed?

The lower limb may need to be restored for multiple reasons. Originally, lower limb reconstruction was required as an alternative to amputation, which was the principal treatment for war injuries. Amputation allows for the removal of necrotic tissue and infection, with the aim of saving the victim's life, but can sacrifice potential function and rehabilitation. Since World War I, major developments in applied anatomy, fracture management, wound care, and sterile techniques, as well as the introduction of antibiotics and anesthesia, have allowed surgeons to consider the role of limb salvage, a field which has greatly expanded since its introduction.

The field of reconstruction gained a vast number of options following the improvement of vascular techniques in the 1960s, opening the door to the microvascular reconstruction era.

Nowadays, war injuries still make up a proportion of the number of people who require access to advanced techniques in the field of lower limb salvage and reconstruction. However, the scope of injury mechanisms has been added to with an increasing number of blunt trauma, thanks to urbanization and industry, as well as increased diagnosis of lower limb malignancies and chronic medical conditions, including diabetes and peripheral vascular disease.

Today's goal in lower limb reconstruction has not changed much from those originally cited in the early war victims, with restoration or maintenance of function becoming the essential goal as these injuries became less life-threatening. Function involves the need for a stable skeleton, allowing weight-bearing status, with adequate soft tissue coverage to nourish and protect the underlying bone. "Normal" function of the limb is then more reliant on their rehabilitation of the limb muscles and joints, with proprioception and plantar sensitivity key.

End points of reconstruction are also measured by a return of function to a level required by that individual. Options become dependent on a balance of anatomical, social, and psychological factors. This functionality can be reduced by chronic pain and infection, as well as complications with chronic swelling or wound healing. The aesthetic outcome is also important, but this should never take priority over the limb's ability to function.

35.4 Reconstruction: Easy Options but a Difficult Choice?

The reconstructive ladder should always be addressed when considering closure of wounds. This progresses from secondary healing to primary closure, through the options of grafting to the more complex local, then distant, free tissue flaps (Fig. 35.1), although the "stepwise" assessment to lower limb wounds may not always be the best approach in lower limb reconstruction.

Today, there are options to supplement the reconstructive ladder, with the use of negative-pressure wound therapy, as well as tissue expansion or prefabrication of tissue, before a definitive surgery. In the future, it is hoped that adjuncts in the form of pharmacological therapies and the use of engineered materials for wound coverage will become more significant. Wound coverage requires many aspects of the patient's background and present state to be assessed before making a decision on the most suitable option.

The bed bound patient in their later years with an infected diabetic foot and chronic leg ulcer offers a different challenge to that of the 28-year-old with an open fracture, massive skin loss, and vascular damage following a road traffic incident. Potentially, the latter has a lifetime of earning and dependents as opposed to the former, who may require purely symptomatic relief. The difficult choice then becomes whether you offer both salvage and amputation or either. It may seem obvious that amputation in the younger

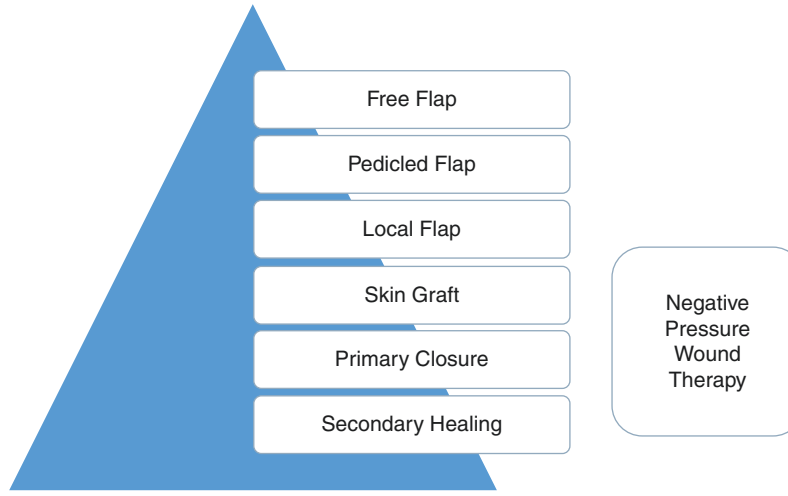

Fig. 35.1 Reconstructive ladder, although the "stepwise" assessment to lower limb wounds may not always be the best approach in lower limb reconstruction

patient is not preferable, but who says that a simple amputation and prosthesis, allowing a quicker return to work and normality, is worse than the potential long-term rehabilitation required with a complex bone and skin coverage procedure?

This highlights the need for lower limb reconstruction to be made on an individual basis and involve a multidisciplinary team. The key aspects being what is missing and what vital structures are exposed as well in consideration of the following points:

35.4.1 Physical Examination of the Wound

This will involve inspecting the wound size and noting the amount of damage and loss to both the skeletal and soft tissue envelope. All devitalized tissue should be removed, and this may have an impact on options for reconstruction particularly if there is degloved tissue. In addition, the vascular supply to both the area and distal regions needs to be assessed. This assessment of the wound is likely to require the input of orthopedic, reconstructive, and vascular professions to varying degrees. The location of the wound also plays a large role in the feasibility of reconstruction options. The surrounding tissue also becomes important in terms of concurrent injuries, such as those in crush injuries, radiation changes following radiotherapy fields in malignancy, chronic infection, or edema-related changes. Once the decision of surgical closure of a wound has been made, appropriate debridement must be undertaken before a final coverage option is chosen.

35.4.2 Patient Assessment [2]

This incorporates the morbidity and mortality risk of undertaking the procedure in the elective patient. Patient age, body mass index, their smoking status, and previous injuries to the limb involved should be noted. Scars crossing regions involving local and distant flap options may rule out these choices. Comorbidities involving cardiac and respiratory disease may discourage a long general anesthetic and potential intensive care stay, as well as having an impact on rehabilitation. Diabetes and peripheral vascular disease, particularly stenosis and atherosclerotic vasculature, will again rule out both donor and wound coverage options. Angiography is often required, particularly in the chronic wound as opposed to the blunt trauma scenario, as a chronic lower limb wound will often heal adequately as long as the area is reasonably perfused, and a nonhealing area due to poor perfusion is unlikely to be successfully grafted. Likewise, nutritional state is strongly influential on both chronic wounds and the healing of the wound coverage options. This will involve dietician support. Pre-injury dementia and ambulation should also be reviewed to

determine rehabilitation and compliance with reconstruction. For the emergency patient, life-threatening injuries take precedence over everything, and the patient will require assessment in a structured way, as defined by the Advanced Trauma Life Support (ATLS) principles [3]. Soft tissue coverage is needed to aid an infection-free fracture union. This should ultimately be completed at the same time as bony fixation if simple or local flap closure is achievable. Free flap reconstruction should be performed on a scheduled trauma list by an experienced, dedicated senior surgical team in a specialist center, preferably within a week of injury [4].

35.4.3 Rehabilitation and Functional End Point

The rehabilitation of the bed bound, chronic wound against the active, acute trauma patient will have a strong influence on what options are used for reconstruction. Is the procedure for symptom relief, functional restoration, or functional improvement? Social status pre-injury and potential rehabilitation options must be assessed. Occupational therapist and physiotherapist inputs emphasize the need for a multidisciplinary team approach to determine the most suitable reconstruction option. Good progress during early rehabilitation can also determine the successful return of normality for the patient.

35.4.4 Patient Expectation: Their Desires and Needs

Exploring the patient's psychological state is equally important. A complication free flap that saves a patient's foot is almost wasted if the patient automatically rejects the rehabilitation phase. The patient's motivation and compliance is critical in the functional end point. The appearance of the reconstruction alongside postoperative pain and swelling is interpreted differently by each individual and will need individual assessment. Likewise, it is important to ensure the patient has a close support system. Offering counseling to those closely involved may aid the patient's recovery. It has been reported that, when offered, a high percentage of patients (93%) would prefer a limb salvage procedure in the traumatic scenario to avoid undergoing amputation [5], and as an option in the chronic wound, reconstruction provides a chance for the patient to remain socially independent and maintain or improve their ambulatory status [6]. This supports patient choice in the reconstructive options, and all options should be discussed in detail by a trained expert to the patient to aid end compliance and balance expectations.

Other factors to be aware of in lower limb reconstruction include, but are not limited by:

1. Cost of care
2. Surgeon's experience
3. Donor site disability
4. Potential complications

Once these areas have been appropriately assessed and individually tailored to the patient, a list of potential surgical options will be made and offered to them.

35.5 Reconstruction Options: The Reconstruction

The reconstructive ladder (Fig. 35.1) offers a list of options in terms of surgical closure of the wound. However, the simplest option is not always the best option. On top of the above preoperative assessment requirements, a failed technique in lower limb reconstruction can have a devastating effect on the patient resulting in further tissue and bone loss, deterioration of comorbidities, and functional deficit with an end point involving amputation. For this reason, the best reconstructive option is often not the easiest choice but the choice that has the highest chance of success. For this reason, free flap tissue transfer is often regarded as the cornerstone of lower limb reconstruction. There has been much debate on the benefits of fasciocutaneous versus muscle based flaps, based on the former being thinner, often less donor comorbidity whereas muscle

flaps may improve the bone healing and reduce the infectious load of the wound. The authors feeling is that the right flap for the right wound should be the route taken with the wound (size/location/deficit), the patient (comorbidity/outcome need/rehabilitation) and the surgeon (capability/instrument availability) being the key aspects of this choice.

35.5.1 Direct Closure and Local Alternatives

Primary and delayed closure, as well as grafting of a wound are well-documented options and should be attempted in both the simple wound, those where expedited recovery is required, or where more complex reconstructive failure would be disastrous.

These options require an adequate blood supply to the wound area and relatively reliable surrounding tissue. Where the blood supply is poor, involves periosteum stripped bone or where there is a requirement of soft tissue depth, the use of reconstructive flaps is generally required. Another option using the nearby soft tissue envelope includes tissue expansion, a choice which negates variance in tissue thickness, texture, and color and offers provision of specialist skin to a region (e.g., hair-bearing). This technique is limited by the reliability of the surrounding tissue but may offer a potential donor site for both direct closure and local flap coverage. Tissue expansion requires time to expand the tissue, so may not be an acute option, and is also known to have a high percent of complications in the lower limb in particular (over 70%).

35.5.2 Flap Reconstruction

Flap reconstruction options can be broken down to local and free flap descriptions. In general flaps can be described based on the blood supply to the flap, the location of the donor site, and the type of tissue being transferred.

The first uses of flap reconstruction initially involved movement of skin around pivot points, with these "local flaps" designed using tissue local to the wound. They will require their blood supply to be intact from the injury, whereas free flaps are based along a distant donor site. Flaps utilize composite tissue blocks and may include the skin, muscle, bone, fascia, and combinations of these.

Local cutaneous flaps can be based along random pattern or axial vascular circulations using the subdermal blood supply. Random pattern cutaneous flaps are limited by the arc of rotation and decreased bacterial resistance, as well as a general rule of a 2:1 ratio between the length and base of the flap used in the lower limb. The discovery of axial pattern flaps, where the flap is perfused by a defined vessel or angiosome, has permitted the use of longer flaps.

Other options for local flaps to aid take have included delayed transfer. An example of this is "the arm carrier" technique, involving abdominal flaps being transferred to a donor site on the arm before final transfer to the leg. This technique is still dependent on the final location wound environment for the take to be successful.

The discovery of random pattern skin flaps led to an investigation into vascular anatomy, and consequently it was found that local flaps could involve muscle, with transposition of either the muscle or a musculocutaneous block supplied by the muscle's dominant vascular pedicle. This finding was further supplemented by the discovery regarding fascial vascular supply and that the deep fascia, with or without skin, also allowed reliable flap creation. In 1981, Ponten [7] noted skin survival in a patient correlated with a single perfused vessel shown on angiography. This led him to raise a calf-based flap including the fascia and sural vessels, prompting a variety of new discoveries in flap options [8].

The use of cutaneous, musculocutaneous, and fasciocutaneous flaps based along specific dominant vascular pedicles has allowed the direct transfer of tissue which is less dependent on the wound bed blood supply. They also introduce new circulation to the area and offer a more reliable and larger wound coverage option. As our understanding continues to develop, it has been noted that both true and "choke" anastomoses

exist between the perforator angiosomes allowing longer flaps to be more successful [9]. The pedicled flaps are restricted by their arc of rotation, something which was greatly increased compared to random pattern skin flaps. The advancement of microscopy, micro-instruments, and sutures has allowed the development of free flap surgery, which essentially involves detaching a known pedicle-based tissue composite unit and transplanting it to the wound area and anastomosing it to a suitable receptor artery and vein in proximity to the wound. Microsurgery has allowed the direct transfer of large tissue units from distant donor sites, allowing wounds to be covered and reconstructed based on flap suitability rather than wound proximity.

Free flap coverage has helped reduce the often bulky pedicled flap seen, particularly in muscle flaps. It also allows direct closure in the majority of the donor regions. A skin graft to this site should only be used if the donor flap is of special significance (superiority in function/shape etc.).

Igari et al. [10] reported end-to-side and end-to-end anastomosis of latissimus dorsi free flaps to the vascular graft on these wounds with 85% flap survival and 100% limb salvage rate. This technique helps with the problem of exposed functional tissues when the wound is debrided.

Free flap reconstruction offers wound coverage but does not improve the distal circulation. However, there are reports of revascularization of critical limb ischemic wounds with free flap coverage being offered as a single procedure with reasonable results [11, 12].

In the traumatic scenario, all open fractures require avascularized soft tissue envelope free of infection to allow appropriate bone healing.

The use of negative-pressure wound therapy (NWPT) can temporarily be used as a substitute for definitive flap coverage [4].

35.5.3 Flap Vascular Anatomy

The blood supply of the raised flap is key to its survival. The classification of flaps can be described by the vascular source. As noted, random pattern flaps have no specific named vessels supplying them, while a recognized artery or group of arteries forms an axial-based flap. The variation in axial blood flow into different muscles is complex, and Mathes and Nahai [13] attempted to subclassify this form of flap vasculature. This classification is well described in reconstructive literature and summarized in Table 35.1. The blood supply to fascial-based flaps has also been classified in Table 35.2.

Table 35.1 Muscle/musculocutaneous flap classification

Type	Pedicle	Example
I	One vascular pedicle	Tensor fascia lata Gastrocnemius
II	One dominant pedicle and minor pedicles	Gracilis Soleus
III	Two dominant pedicles	Gluteus maximus Serratus anterior
IV	Segmental pedicles	Sartorius Extensor halluces longus
V	One dominant and secondary segmental pedicles	Latissimus dorsi Pectoralis major

Table 35.2 Fascia/fasciocutaneous flap classification [14]

Type	A	B	C
	Direct cutaneous pedicle	Septocutaneous pedicle	Musculocutaneous pedicle

35.5.4 Flap Failure and Complications

Flap complications can be wound specific, vary from reconstructive unit to unit, and are dependent on the flap used. They include failure of the flap, involving partial or total necrosis; hematoma and seroma collections (for which the use of postoperative drains is not uncommon); and wound dehiscence and infection. Donor site morbidity should be negligible but could involve a reduction in function, particularly in flaps involving muscle components. In using free flaps, it should be noted that vein grafts are frequently required, and in particular the deeper venous network is targeted for anastomosis due to a predisposition of the superficial system to spasm. The arterial anastomosis if often performed in an end-to-side technique due to vessel mismatch and the very high

chance of a single-vessel perfused limb. A single vessel leg is not a contraindication to free flap limb salvage. Angiography may be useful in planning reconstruction but does not correlate with vessel flow and can miss segmental vessel injury.

Pressure ulcer coverage is particularly complicated, usually due to issues regarding the continuation of pressure at the reconstructed site. One paper quotes flap complications involving ischial, sacral, and trochanteric wounds of 87 complications in 421 (21%), with suture line dehiscence (31%), infection (22%), hematoma (19.5%), partial necrosis (13.7%), and total necrosis (10.3%) noted [15]. This complication needs to be noted in patients where lower limb trauma may predispose to pressure to reconstructed regions either in the rehabilitation period or long-term.

35.5.5 Choice of Reconstruction: The Flap Options [16]

Traditionally the use of local muscle flaps proximally and free flaps distally in the lower limb has been used, although improvement in local flap reliability has allowed their use throughout the limb [17].

35.5.5.1 Local Flap Reconstruction

Random pattern cutaneous flaps can be limited by their vascular input. There are suggestions that the detection of perforators can be made by using thermal imaging to improve the sensitivity of current Doppler and anatomical landmark techniques [18]. In particular, thermal imaging may help locate the "choke" anastomoses which help aid flapper fusion and drainage [19].

"Propeller" flaps are well documented as an option for the majority of coverage in the lower limb, particularly below the knee [20, 21]. The propeller flap is an insular flap mobilized through an axial rotation to cover a defect (like a propeller), with perforator propeller flaps pivoting on a perforating vessel. Most perforator-based flaps can be utilized in a propeller flap idea including the ALT, TFL, and groin flaps, and for the distal limb the peroneal and posterior tibial arteries are commonly used for lateral and medial defects, respectively (Fig. 35.2).

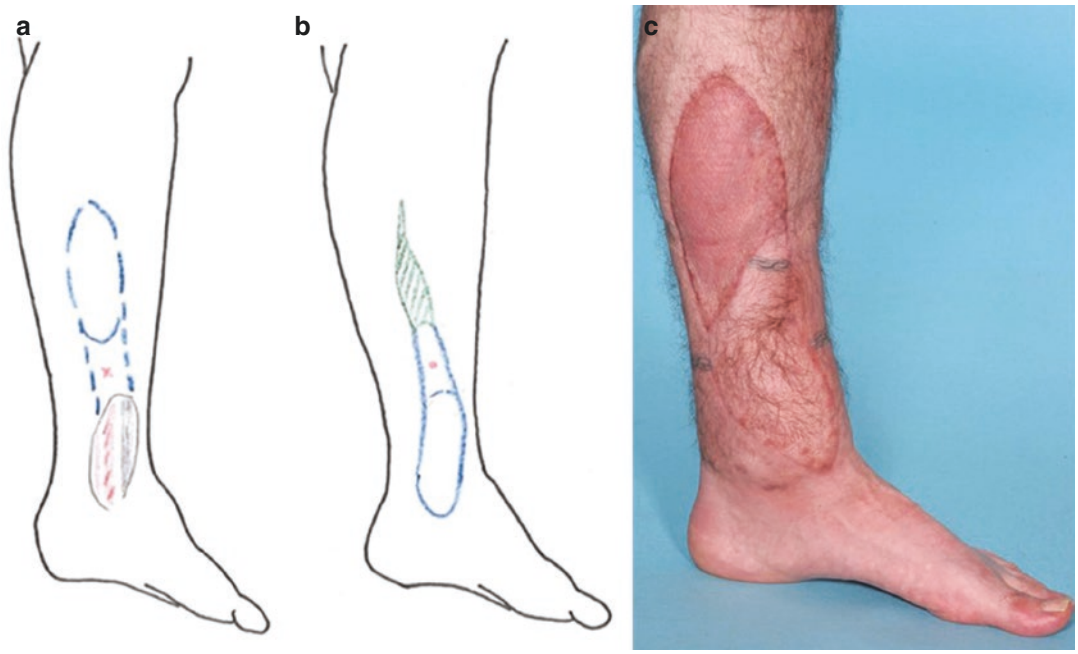

Fig. 35.2 Propeller flap based on posterior tibial artery. (**a**, **b**) Defect and initial design. (**c**) Final position with donor defect grafted. Note movement of tattoo when propeller flap is rotated 180° to cover defect

The posterior tibial artery provides multiple cutaneous perforators but at unpredictable intervals. However, there are three distinct clusters found at predictable distances of 4–9 cm, 13–18 cm, and 21–26 cm proximal to the medial malleolus and are typically of larger caliber than other options more proximal on the limb. When using peroneal artery-based flaps, it should be noted that the blood supply tends to lie posterior to the fibula as opposed to over it when designing the skin paddles. This provides good form and function for elective and traumatic defects, offering an option in forefoot cover [22]. However, a recent literature review reports up to 16% of flaps suffering partial necrosis, with a third of them involving the whole flap [23].

Both the peroneal and anterior tibial artery flaps have small pedicles, around 3 cm, and sub-centimeter diameters, meaning their use as free flaps is limited. They are both type B fasciocutaneous flaps and can be harvested with the superficial peroneal or saphenous nerve for sensate flaps.

V-Y flaps, as described by Blasius in 1848 [24], are another option, particularly around the ankle and lower leg and can provide a sensate flap to the region [25].

Bipedicled flaps are random pattern flaps but, due to two pedicles, their continued viability is improved. They are a flap gaining popularity for closure of lower limb wounds, as is the keystone flap [26, 27].

The ad hoc perforator is a local flap that can be based on any type of perforator. Its concept was first alluded to by Quaba et al. in 1990 [28] and is analogous to the freestyle free flap concept of Wei and Mardini in 2004 [29]. With increased understanding of the cutaneous circulation and a sound knowledge of regional vascular anatomy, the reconstructive surgeon can tailor their approach to the presenting defect without being constrained by a previously described local flap or perforating vessel. Careful Doppler mapping and/or preliminary exploration is performed to identify a perforator adjacent to the defect. The presence of a positive Doppler signal in the territory of the defect allows planning and execution of the flap without the need to dwell on anatomical landmarks or variations [30].

For open fractures of the lower limb, local fasciocutaneous flaps should be used in low-energy tibial fractures (Fig. 35.3). As long as there is no vascular compromise by the initial injury, these can be used, along with free fasciocutaneous flaps, in metaphyseal injuries (particularly around the ankle) [31]. Muscle flaps would be suggested by experimental data in open tibial shaft fractures or where the blood supply is compromised, possibly helping to reduce both the healing time and risk of deep infection [31].

35.5.5.2 Fasciocutaneous Flaps

1. Groin

   The earliest axial-based fasciocutaneous flap, the groin flap, has been used as both a free and pedicled flap (Table 35.3) [32] providing a substantial amount of both tissue and skin. Often needing subsequent debulking and due to the fact that it is a hair-bearing area, this flap can be a poorer aesthetic match compared to other options. The short venous supply to the region also causes an increased risk of flap failure. Often taken using a pedicle approach from the superficial circumflex iliac artery, the groin flap allows up to 20 × 10 cm flaps to be harvested alongside direct closure, and twice this with grafting of the donor site. The groin flap is a type A fasciocutaneous flap.
2. Medial Thigh and Transverse Upper Gracilis

   Typically using the anterior septocutaneous artery and the venae comitantes from the superficial femoral vessels, this flap can be also be raised more anteriorly by using the lateral femoral circumflex artery, where it is more commonly referred to as the anteromedial thigh flap. The saphenous vein can be utilized to aid venous drainage as well as keeping a sensate flap when the medial anterior cutaneous nerve of the thigh is raised; the medial thigh flap is useful both as a free and pedicled flap. The latter will help cover wounds involving the perineum, groin, and thigh up to 10 × 20 cm in size. This skin paddle is now utilized in the transverse upper gracilis (TUG)

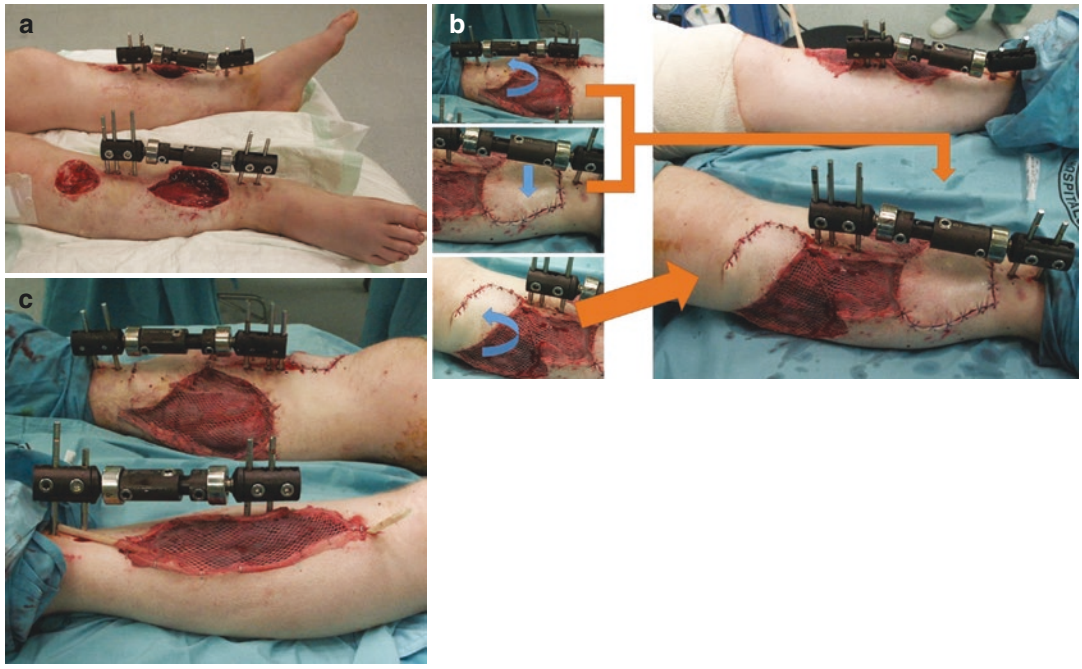

Fig. 35.3 Reconstruction of bilateral leg defects with exposed tibia. (**a**) External fixation in situ for tibial fractures. (**b**) Right leg reconstruction. Bony defects are covered with ad hoc perforator flap laterally and posterior tibial perforator flap medially. Both donor sites grafted. (**c**) Left leg required only skin grafting to defect

Table 35.3 Groin/SCIA flap

Groin/SCIA flap	
Flap attributes	Cutaneous flap taken with fat Can be harvested as free or pedicled flap
Artery	Superficial circumflex iliac artery (external iliac/superficial femoral artery) 1–2 mm
Vein	Cutaneous vein draining into saphenous system
Pedicle	2–5 cm, generally short pedicle but can be extended by more lateral skin paddle
Nerve	N/A

flap which is can be raised solely on medial femoral circumflex artery perforators through the gracilis. The medial thigh flap has a pedicle of 2–4 cm with a 1.5 mm diameter. This is a type B flap.

3. Lateral and Posterior Thigh

   This flap and the posterior thigh flap exploit the profunda femoris perforating branches. Of the four, the first supplying the lateral thigh is used for proximal regions including the trochanteric and ischial areas, and the third the posterior thigh [33]. The lateral thigh flap may also be harvested to include the lateral femoral cutaneous nerve as it can in the ALT flap. The lateral thigh flap often has a pedicle of 5–6 cm with a diameter between 1 and 2 mm. This is a fasciocutaneous type B flap.

4. Anterolateral Thigh (ALT) (Table 35.4)

   Taken from the descending branch of the lateral femoral circumflex artery and thanks to an extended pedicle, the ALT is typically used as a free flap. A 7 × 20 cm skin paddle can be raised with a tight closure of the donor site. A type B and C fasciocutaneous flap, it is well used in head and neck reconstruction and allows a relatively slim flap for upper limb reconstruction. Its use as a pedicled flap or propeller flap is also useful for defects across the groin to knee regions (Fig. 35.4).

5. Sural

   The sural artery allows probably the longest pedicled fasciocutaneous or fascial flap (Table 35.5). Also, with the ability of being

Table 35.4 ALT flap

Anterolateral thigh flap (ALT)	
Flap attributes	Skin, fat and fascial flap, pedicled or free flap options Skin paddle can be up to 12 × 25 cm Can be harvested as only an adipofascial or fascial flap Vastus lateralis muscle can also be included in the flap dissection if required
Artery	Descending branch of lateral femoral circumflex artery (profunda femoral trunk) 1.5–3 mm
Vein	Slightly larger than artery, draining into profunda femoral vein junction
Pedicle	~7 cm, dependent on perforator entry to flap and position skin paddle
Nerve	Lateral femoral cutaneous nerve of the thigh can be harvested with this flap, entering at proximal part of design

reversed, this flap can cover defects around the knee, anterior and posterior and upper third of the leg, as well as proximal foot defects. A type A fasciocutaneous flap skin flaps of up to 12 x 20 cm can be raised. The Medial Sural Artery Perforator (MSAP) flap has slowly gained popularisation. This utilises the same pedicle that the medial gastrocnemius flap would normally be harvested on so does mean sacrificing this muscle flap, but the fasciocutaneous flap dissection allows a long pedicle with often little disruption to the underlying muscle function.

6. Saphenous

Coverage of the knee can be achieved by raising this flap using the saphenous artery and venae comitantes. This is a continuation of the descending genicular branch of the superficial femoral artery. The saphenous artery perforator flap is supplied by septocutaneous perforators supplying the medial thigh skin above the knee. The saphenous artery also supplies another region of the skin anterior and medial aspects of the leg below the knee and originates from the descending genicular artery. A line from the anterior superior iliac spine to the medial epicondyle of the tibia approximates to the sartorius muscle, the key landmark in finding the vascular pedicle. The cutaneous branches are found between 3 and 10 cm from the saphenous artery origin which lies toward the adductor canal. It has been reported in varying degrees that the saphenous artery joins the dorsalis pedis artery in the foot allowing distal leg and foot coverage. With a more difficult dissection than those listed above due to increased vascular anatomy variance, this flap can also be reversed and includes an osteofasciocutaneous option (from the medial femoral condyle) using the articular branches of the genicular artery. Skin paddles 7 × 20 cm are typical, with a section of sartorius occasionally taken with the raised tissue to aid flap survival rates. The pedicle can be 5–15 cm length with a diameter of 1–2 mm. This is a type A flap (Fig. 35.5).

35.5.5.3 Muscle and Musculocutaneous Flaps

1. Gluteus Maximus

Being the largest muscle of the body and having both two dominant and two minor pedicles, this allows for a high degree of versatility. Reliable coverage of the buttock, hip, perineal, and upper thigh regions is achievable. Raised either from the lateral femoral circumflex artery to allow posterior thigh coverage in a reversed technique, or off of one of the gluteal arteries (superior or inferior) with the muscle split preserving function [34] and tissue to cover either anterior or posterior defects. These can incorporate either only muscle or muscle and skin coverage options.

(a) The superior and inferior gluteal perforator flaps (SGAP/IGAP) have replaced gluteal muscle flaps as they reduce buttock morbidity and are a mainstay of breast reconstruction. They allow a fasciocutaneous flap to be raised from the superior or inferior gluteal arteries with a 3–4 mm artery caliber, often larger veins, and pedicles up to 7 cm, with the IGAP-based flap also known as the posterior or gluteal thigh flap and not to be mixed up with Song's description of a posterior thigh flap raised on the third perforating branch of the profunda femoris artery [35]. This

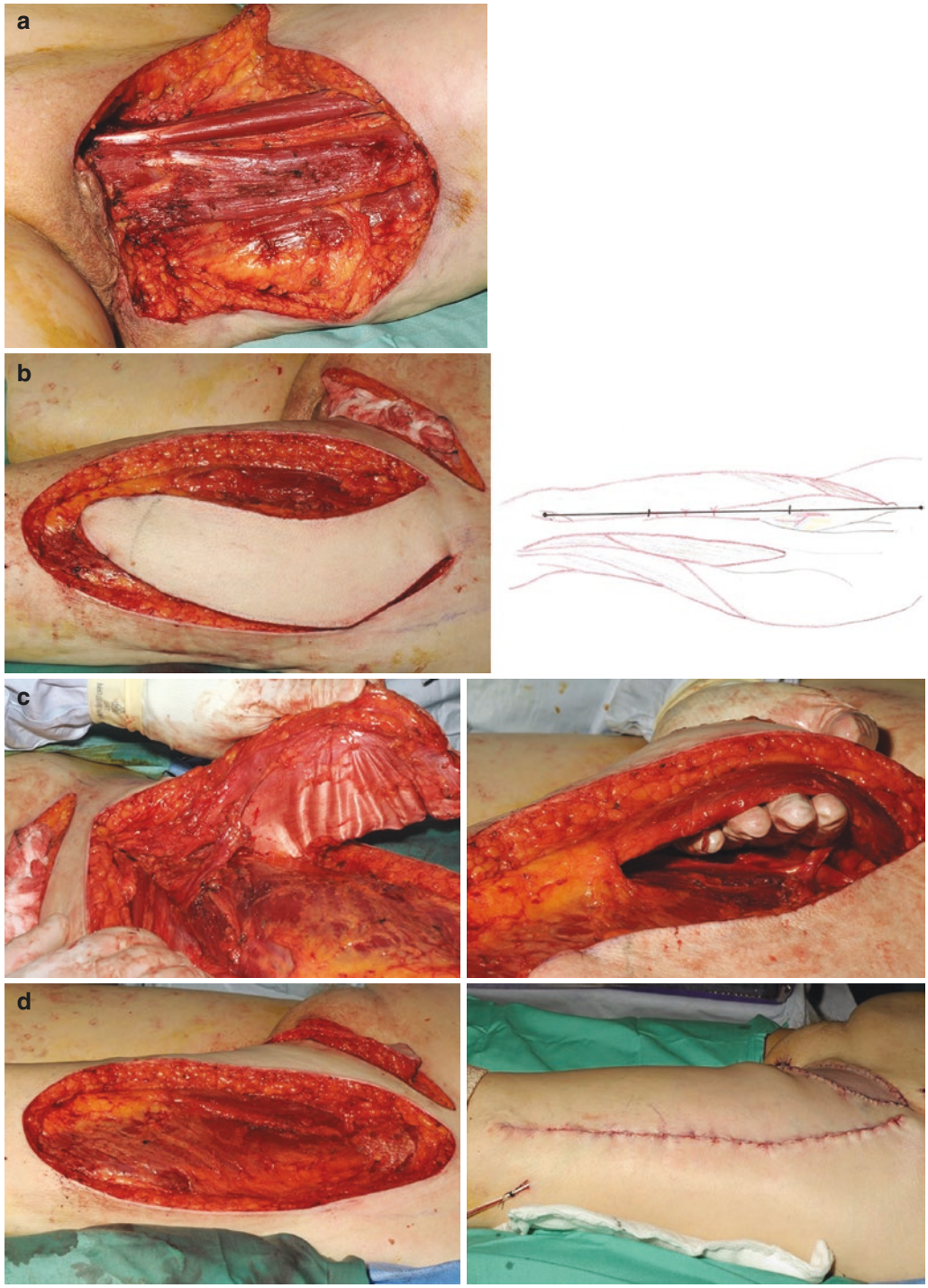

Fig. 35.4 Anterolateral thigh (ALT) flap for medial groin defect. This case highlights a large defect in the medial groin of the left leg following excision of a fungating tumor. (**a**) Defect medial thigh. (**b**) Left and right: pedicled ALT raised on same leg, surface markers for perforators is the middle third along a line drawn between the anterior superior iliac spine and the lateral edge of the patella. (**c**) Left: raised ALT flap showing perforator pedicle. Right: tunnel for ALT to be passed through to reach medial thigh defect. (**d**) Left: lateral thigh donor site defect. Right: this can generally be closed directly up to 8 cm. (**e**) Left and right: sutured pedicled ALT flap with donor site drain in situ. (**f**) Results at 4 weeks

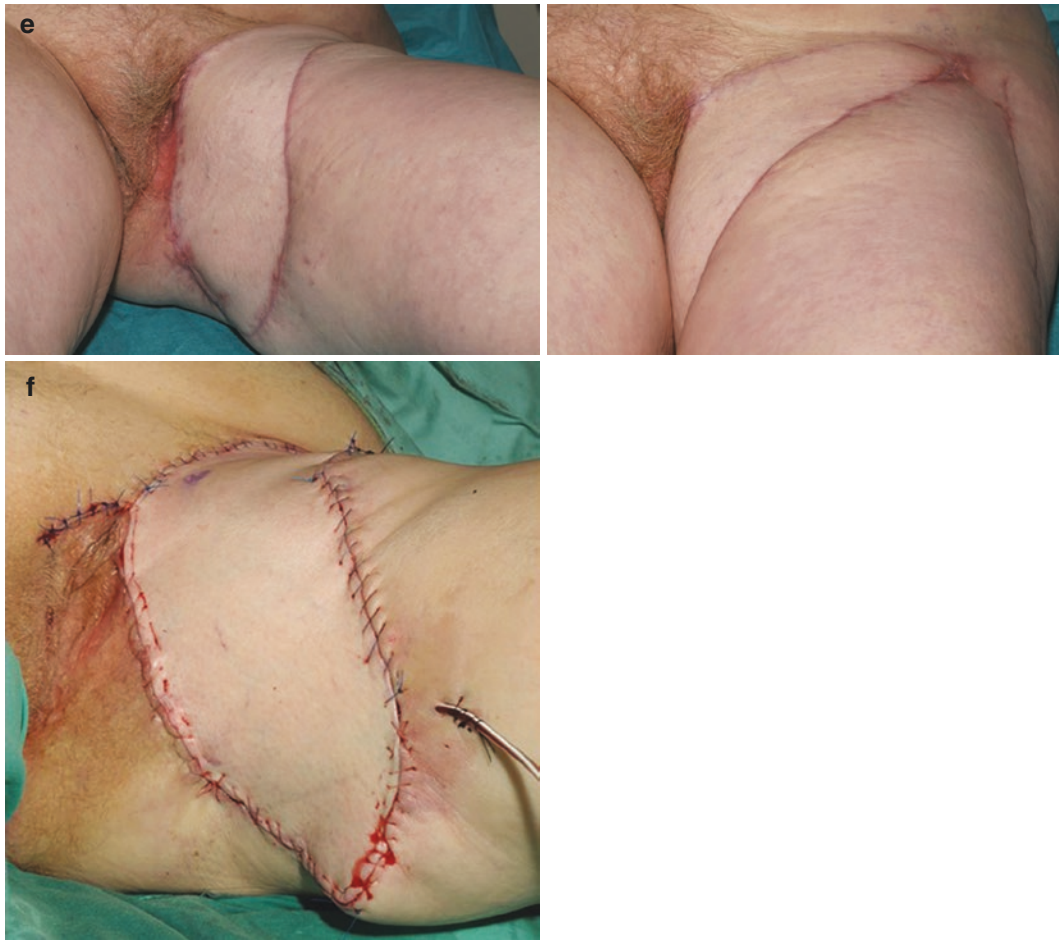

Fig. 35.4 (continued)

Table 35.5 Sural flap

Sural	
Flap attributes	Fasciocutaneous ± muscle
Artery	Perforating branches of medial sural artery as well as perforators from the peroneal artery, 1–2 mm diameter
Vein	Similar size to artery
Pedicle	3 mm, although reverse sural flap 10–15 cm
Nerve	Sural nerve can be harvested

latter flap gives a 5–10 cm pedicle with an artery diameter less than 2 mm. The posterior cutaneous nerve of the thigh can be harvested with the flap.

2. Tensor Fascia Lata (TFL)

Useful as a pedicled or free flap, the thin muscle belly and long fascial extension allow this flap to be used in a multitude of scenarios, as well as it being an expendable muscle unit in the majority (Table 35.6). Able to reach the umbilical region, perineum, ischium, and groin, it can incorporate skin to cover defects in the proximal lower limb, as well as iliac bone for osteomusculocutaneous coverage. When planning its dissection, the TFL flap is designed along a line between the anterior superior iliac spine and the lateral femoral condyle. The lateral femoral cutaneous nerve can be harvested to provide a sensate flap and enters the region of the flap just above the greater trochanter between the gluteus medius and gluteus minimus muscles (Fig. 35.6).

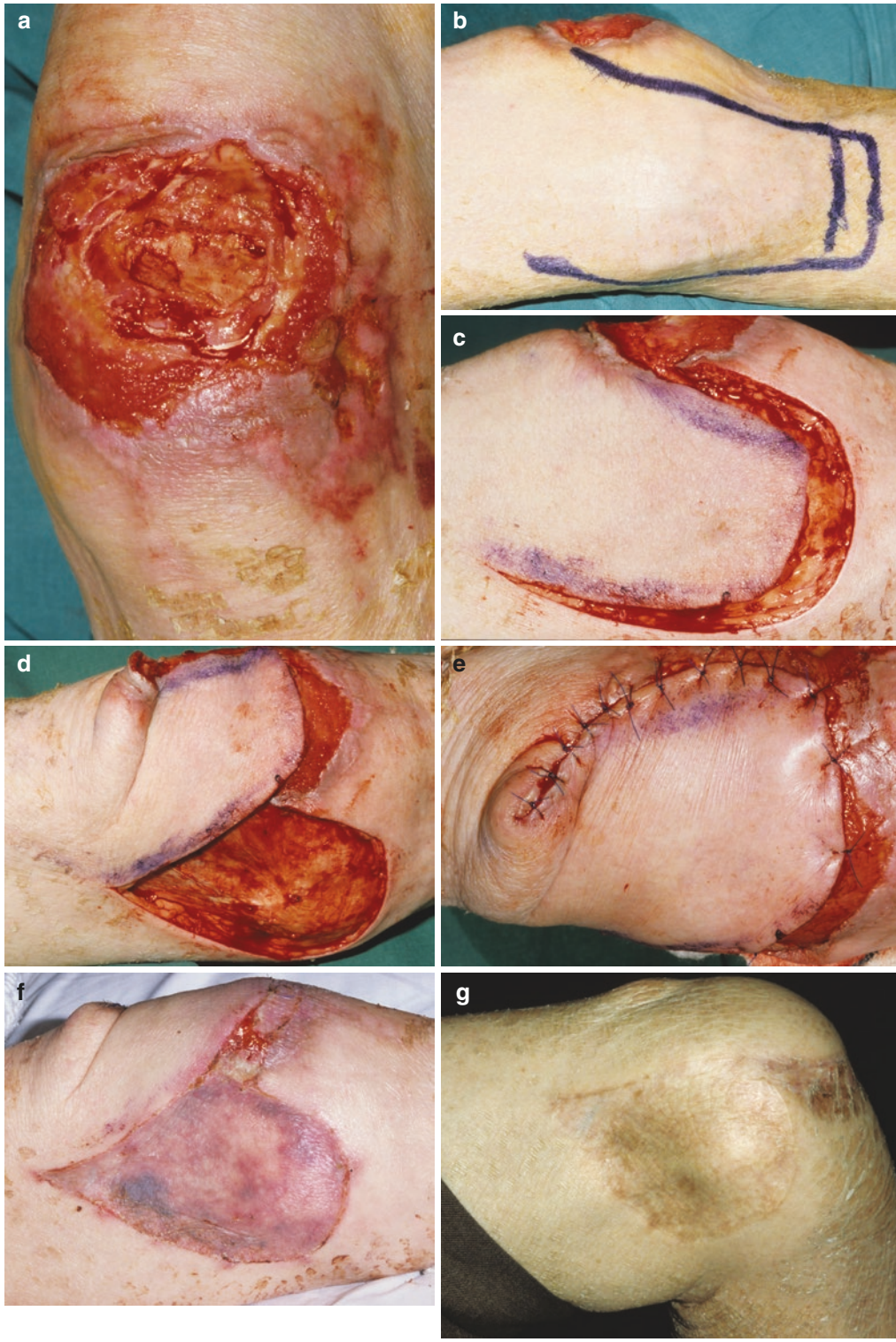

Fig. 35.5 (**a**) Cement burn to knee exposing patella and extensor apparatus of knee. (**b**) Saphenous flap drawn. (**c**) Saphenous flap dissected. (**d**) Transposed fasciocutaneous flap over exposed knee structures. (**e**) Transposed flap. (**f**) Three weeks postoperative. (**g**) Six months postoperative showing healed flap and sheet skin graft to donor site medially

Table 35.6 TFL flap

Tensor fascia lata (TFL)	
Flap attributes	Muscle flap but can be harvested with skin paddle Beware lateral knee instability in the athletic patient
Artery	Ascending, or Transverse, branch of lateral femoral circumflex artery (profunda femoral trunk) 1–3 mm
Vein	Vein travels with artery draining into lateral femoral circumflex
Pedicle	5–10 cm
Nerve	Lateral cutaneous nerve of the thigh

3. Gracilis

A pedicled and innervated gracilis flap is useful in perineal and ischial coverage, but its relative lack of functional deficit on removal means its use as a free flap for the lower leg cannot be underestimated (Table 35.7). It allows purely lower limb anesthesia, and its small size does not cause gross contour changes in its final location. It is raised from its main pedicle, the terminal branch of the medial circumflex femoral artery. Although a consistent flap (the muscle lies posterior and

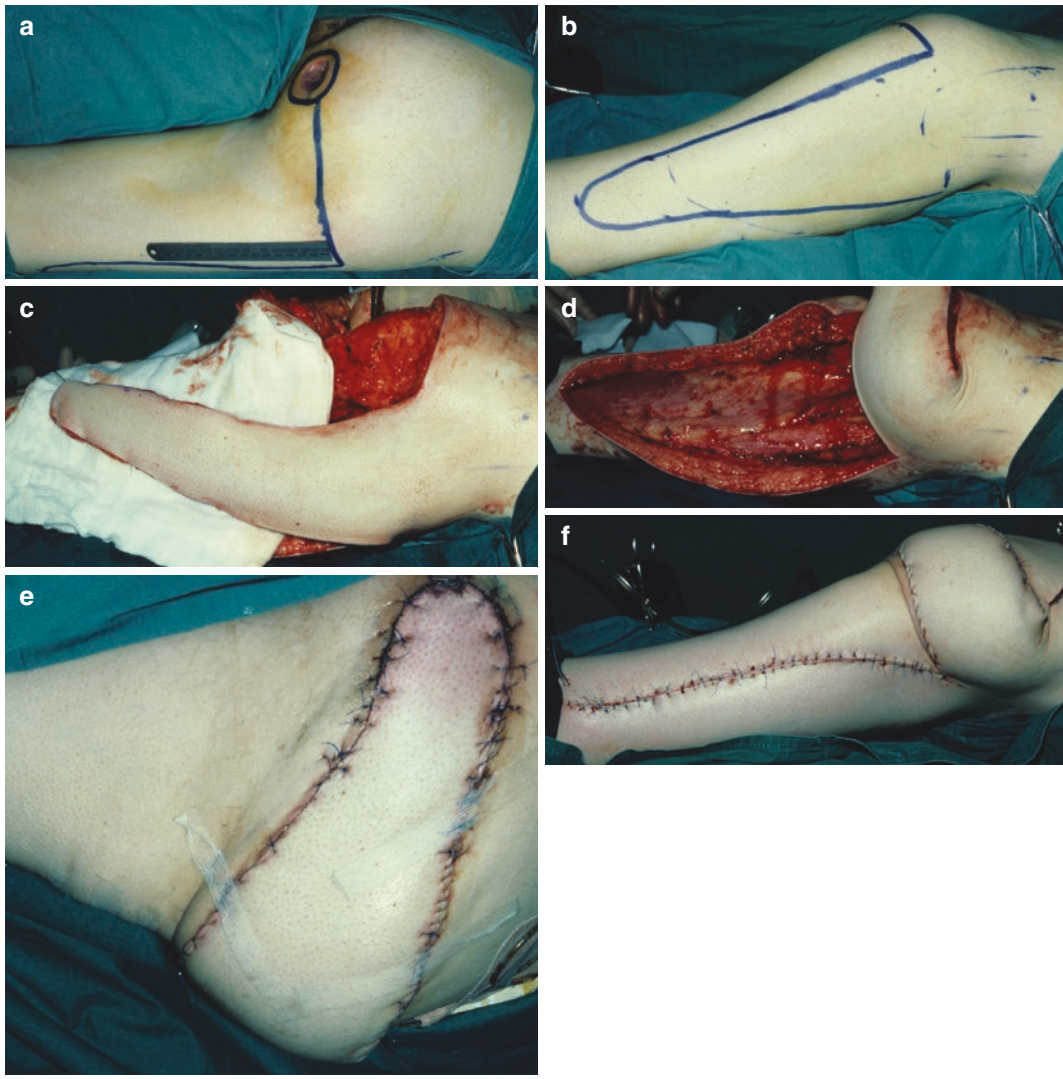

Fig. 35.6 (**a**) Ischial pressure sore. (**b**) Marking for pedicled tensor fascia lata muscle flap. Pedicle found at proximal and middle third junction along an axis drawn from anterior superior iliac spine and the lateral femoral condyle. (**c**) Raised pedicled TFL. (**d**) Transposition to defect showing donor site wound. (**e**) Wound coverage by transposition of pedicled flap. (**f**) Closure of donor wound.

Table 35.7 Gracilis flap

Gracilis	
Flap attributes	Muscle flap Generally can cover 6 × 20 cm wounds Can be harvested as a transverse upper gracilis (TUG) flap to include a skin paddle of up to 11 × 25 cm
Artery	Gracilis vessels from medial femoral circumflex system (profunda femoral artery branch) 1–2 mm
Vein	Two venae, can be bigger than artery
Pedicle	6 cm
Nerve	Nerve to gracilis (branch of obturator nerve)

Table 35.8 Soleus flap

Soleus	
Flap attributes	Muscle flap Both muscle bellies can be utilized but generally medial belly is preferred as reliable blood supply, and hemisoleus maintains foot plantar flexion strength
Artery	Medial half has reliable medial perforators from posterior tibial artery 1.5–2 mm diameter
Vein	Similar size to artery
Pedicle	5–15 cm, generally used as a pedicled flap but can be proximally or distally based
Nerve	N/A

Table 35.9 Gastrocnemius flap

Gastrocnemius	
Flap attributes	Muscle flap Both muscle bellies can be utilized but generally medial belly is used (larger)
Artery	Medial sural artery (medial belly) from popliteal artery 1–4 mm
Vein	Two venae or medial sural vein draining into the popliteal vein (medial belly)
Pedicle	2–5 cm (short) generally used as a local pedicled muscle flap but can be used free
Nerve	Medial and lateral branches to muscle bellies from posterior tibial nerve

parallel to a line drawn between the pubic tubercle and the medial tibial epicondyle) it can often be too small to cover more severe defects of the lower limb, with any skin paddle unreliable when using the more distal skin of the thigh. For this reason, it is often useful to locate the distal insertion of the gracilis tendon before finalizing the design of a musculocutaneous flap if this is required.

4. Soleus

   Used for defects of the middle third of the lower leg, soleus can be split to form a hemisoleus flap, thanks to its dual pedicle supply and bipennate morphology (Table 35.8) [36]. Due to an increased risk of substantial ankle flexion weakness alongside loss of lower limb venous return through the muscle, its use has been criticized [37].

5. Gastrocnemius

   The two origins of this muscle allow separate muscle or musculocutaneous flaps to be raised on separate pedicles, along the lateral or medial sural arteries (Table 35.9). The cutaneous defect can be unsightly, so it is preferred to harvest as a muscle flap and cover this with a skin graft. Useful for distal femur, proximal tibia, and knee coverage, it may be advanced minimally to allow coverage over the Achilles tendon or rotated to the mid-tibia, thanks to the anastomosis across the muscular raphe. The gastrocnemius flap can only be employed in scenarios where soleus is intact

as rehabilitation and walking are dependent on ankle plantar flexion (Fig. 35.7).

6. Vastus Lateralis

   This muscle provides a musculocutaneous flap which offers no great deficit in ambulation which is not afforded by flaps raised using the rectus femoris muscle for hip and proximal thigh defects. The latter is now more utilized for abdominal defects, and the ALT flap supersedes both, using the same pedicle without the donor site morbidity of taking the muscles below.

35.5.5.4 Other Flap Choices

Free osteocutaneous flap options include, but are not limited to:

1. Iliac osteocutaneous flap (Table 35.10)
2. Vascularized rib transfer ± serratus anterior and/or latissimus dorsi
3. Fibula osteocutaneous flap (Table 35.11)
4. Radial osteocutaneous flap (Table 35.12)

35.5.6 Choice of Reconstruction: By Anatomical Location of Injury

Anatomical zone of injury is a key determinant of introducing a more reliable pedicled flap to a wound bed. Many authors have stated success with varying options, and different flaps may be more suitable in a particular surgeons' expertise and personal experience.

The gluteal and the thigh regions, due to their option as free flap choice, allow great versatility in regard to local flaps. V-Y, bipedicled, and keystone flaps are all utilized with good outcomes (Fig. 35.8) (Table 35.13) [38].

The upper thigh and groin have a multitude of options. Most of the thigh flaps described before will reach groin to knee as pedicled options, with the sartorius being a further muscle or musculocutaneous option. The sartorius muscle is often "switched" to reduce dead space and protect the femoral vessels when performing groin or inguinal node-basin dissections.

Due to the unique anatomy of the knee, reconstruction of the soft tissues has been attempted with both local and free flaps, the choice being more dependent on surrounding tissue availability and the amount of bony and soft tissue injury [39].

The knee and popliteal region can be covered by pedicled gastrocnemius, or proximally based hemisoleus, flaps [40, 41]. Significant tissue coverage here is important as loss of the extensor mechanism of the knee can have long-term functional consequences. Improved microsurgery has allowed the use of the genicular arteries as recipient vessels, minimizing trauma to the popliteal and femoral vessels [42].

The tibia provides a unique challenge as it not sheathed in a muscle coat, for instance, the femur, and consequently inhabits a poorly vascularized environment predisposing it to nonunion and infection. The tibialis anterior muscle, if not involved in the zone of injury, can be split to cover small defects over the bone. This can be done in a bipedicled turn-over or "book flap" design. As well as in traumatic injuries, it should be noted that sarcomas involving the tibial bone are not uncommon.

The proximal tibia is usually well placed to be covered with local flaps involving the gastrocnemius or soleus muscles.

The mid-tibia defect can also be covered using a combination medial hemisoleus and gastrocnemius flap, with an aim of maintaining the Achilles tendon and posterior tibial vessels to allow ankle plantar flexion post reconstruction [43] with the author also describing his use of the medial hemisoleus [44] and the reversed hemisoleus flap for distal tibia defects [45].

The middle and distal tibia defect has also been salvaged using the osteocutaneous fibula flap with good results, both as a pedicled and free flap [46–48]. The fibula's blood supply allows the bone to be hinged using osteotomy sites as needed, providing the periosteum is kept intact as the source of blood supply. This can be double-barreled for reconstruction of long-bone defects, such as the femur. Another option is distraction osteogenesis. After removing the dead or infected bone of the tibia, achieving closure of the soft tissue, which is made easier with the shortened limb, the bone can be then distracted, often by as

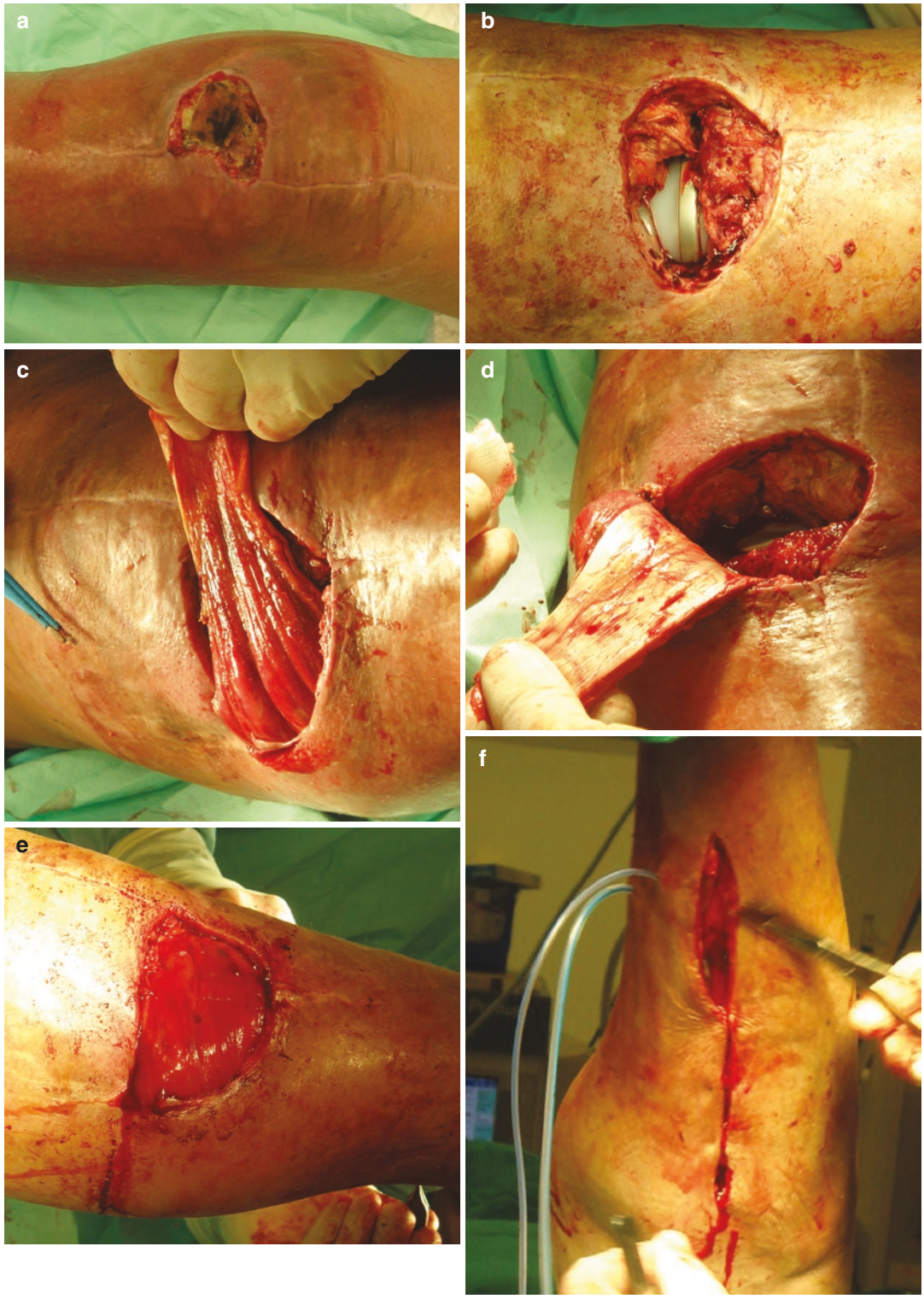

Fig. 35.7 (**a**) Defect to anterior knee region following chronic wound after total knee replacement. (**b**) Following debridement of necrotic tissue. (**c**) Pedicled gastrocnemius muscle flap brought from donor to tunnel and pulled through defect – anterior view. (**d**) Posterior flap view. (**e**) Muscle flap in situ – graft required as only muscle flap harvested. (**f**) Closure of donor site with donor site drain in situ

Table 35.10 Iliac osteocutaneous/DCIA flap

Iliac osteocutaneous/DCIA	
Flap attributes	Iliac crest bone with adjacent periosteum, iliacus muscle ± skin paddle Useful if fibula option is not viable Skin flap can be bulky
Artery	Deep circumflex iliac artery, branch of the external iliac artery 1.5–3 mm caliber vessel
Vein	Usually larger than artery, draining into the saphenous vein
Pedicle	4–7 cm, dependent on harvest technique of bone and or skin paddle and whether common DCIA/SCIA trunk
Nerve	N/A

Table 35.11 Fibula osteocutaneous flap

Fibula osteocutaneous	
Flap attributes	Fibula none with adjacent periosteum and muscle cuff ± skin paddle Take with muscle to improve skin flap reliability of up to 5 cm width to allow primary closure Preserve lower 6 cm of fibula for ankle function. Up to 25 cm bone able to be harvested Peroneal nerve in close proximity to proximal region of bone Check intact vascular arch supply to lower limb
Artery	Peroneal artery 1–4 mm caliber vessel
Vein	Two venae, draining into posterior tibial veins
Pedicle	Generally short length but can be increased up to 12 cm by using distal bone and dissecting free of proximal fibula
Nerve	Lateral sural nerve can be harvested

Table 35.12 Radial (osteocutaneous) forearm flap

Radial (osteocutaneous) forearm	
Flap attributes	Fasciocutaneous ± tendon (palmaris longus) ± bone (radius) Skin paddle up to 10 × 40 cm Check intact vascular arch supply to hand
Artery	Radial artery with perforators 2–4 mm
Vein	Subcutaneous venous system +/− cephalic vein Venae comitantes of the radial artery are also harvested but can be too small
Pedicle	12 cm
Nerve	Superficial radial nerve or antebrachial nerves (medial/lateral) can be harvested

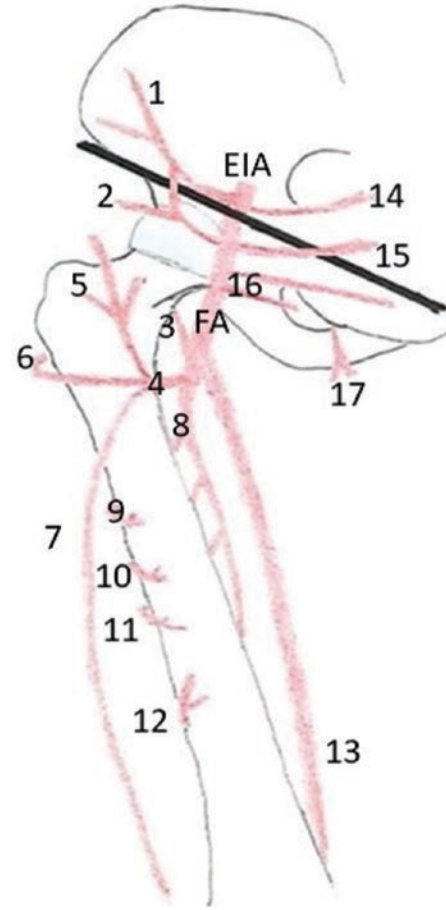

Fig. 35.8 Arterial axis of the groin region showing arterial pedicles for the main pedicle and free flap options in this region

Table 35.13 A variety of flaps

Artery	Perforating branch (typically)	Flap
Deep circumflex iliac artery		Iliac osteocutaneous flap
Superficial circumflex iliac Artery		Groin flap
Medial femoral circumflex Artery		Gracilis and TUG flap (Medial thigh/ groin flap)
Lateral femoral circumflex Artery	Ascending	Tensor fascia lata flap
	Transverse	
	Descending	Anterolateral thigh flap Rectus femoris/ vastus lateralis

Table 35.13 (continued)

Artery	Perforating branch (typically)	Flap
Profunda femoris (deep femoral) artery	1	Supplies adductor magnus muscle and overlying skin
	2	Lateral thigh flap
	3	Posterior thigh flap (as described by Song [37])
	4 (terminal)	
Superficial femoral artery		Supplies skin paddle over region of anterior thigh
		Sartorius

much as 1 mm a day, using external fixation. This can help to re-lengthen the bone over time which stretches the soft tissue envelope over it and reducing long-term disability.

This region can also be reached by fasciocutaneous flaps based on the peroneal and posterior tibial artery perforators described previously (Figs. 35.9 and 35.10).

The lower third of the lower leg, heel, and hind foot are technically challenging areas to cover. Options include the reverse saphenous and sural flaps. The saphenous flap provides a reliable and versatile option for the medial and anterior lower leg, as well as the hind foot and malleolar region [49]. The sural fasciocutaneous flap has successfully been used to reconstruct tissue loss in these areas. The distally based sural flap is safe, reliable, and operatively quick to perform negating free flap reconstruction [50]. However, this flap may be limited by the size of the defect, reasonably covering an area up to 10 cm square. Other flaps utilized in this region include the lateral calcaneal artery flap which can be transposed to cover the Achilles tendon which can be raised as a fasciocutaneous flap to cover defects up to 4 cm in size. Otherwise, local muscle coverage of the Achilles and malleolar region is fairly limited.

The plantar region of the foot has two distinct zones in terms of function: the weight-bearing and the non-weight-bearing. The former is made up of the heel, the head of the metatarsals, and the lateral strip adjoining them. The toe pulps to a lesser degree are used in some positions of gait for weight-bearing too. Evolution has allowed these areas to become hyper-keratinized, and there is increased strong fibrous attachments to the skin to deal with the repeated trauma. This needs to be taken into account when replacing defects of this region as thin grafts may do well in the non-weight-bearing zones but stand little chance when reconstructing the heavier loaded sites, and a repeatedly ulcerated reconstruction may be of poorer use to a patient than an amputation if the heel and weight-bearing zones have been degloved.

Heel defects in particular present a difficult area to reconstruct, being the main weight-bearing zone. Delayed reverse sural flaps have been used successfully in cases of distal tibial and calcaneal fracture with neurofasciocutaneous coverage, improving long-term function and rehabilitation. The perforator supply to the sural-based flap allows numerous options, allowing reduced donor morbidity [51].

The dorsal foot and ankle are likewise difficult to reconstruct due to a functional lack of tissue around this site. Distal-based lateral supramalleolar adipofascial flaps have been described, providing less bulky flaps although requiring grafting of the transposed flap and covering only small defects [52]. They may also reduce long-term ulcer formation due to some retained sensation compared to the majority of free flap reconstructions.

The anterior tibial artery provides an adipofascial flap suitable for coverage over the malleolar regions [53]. Generally, if the paratenon is present, a skin graft is adequate, although there can be long-term pain and wound issues similar to those seen in dorsalis pedis donor site surgery. The temporoparietal fascial flap is a particularly unbulky flap which is ideal for this region.

Foot coverage has been successfully performed using sural artery, lateral calcaneal artery-based, extensor digitorum brevis muscle rotation, and abductor hallucis muscle rotation flaps (Table 35.14) [54–56]. The latter is of use in smaller defects of the medial foot, but does not reach the medial malleolus. It is based on the medial plantar artery and nerve. The lateral plan-

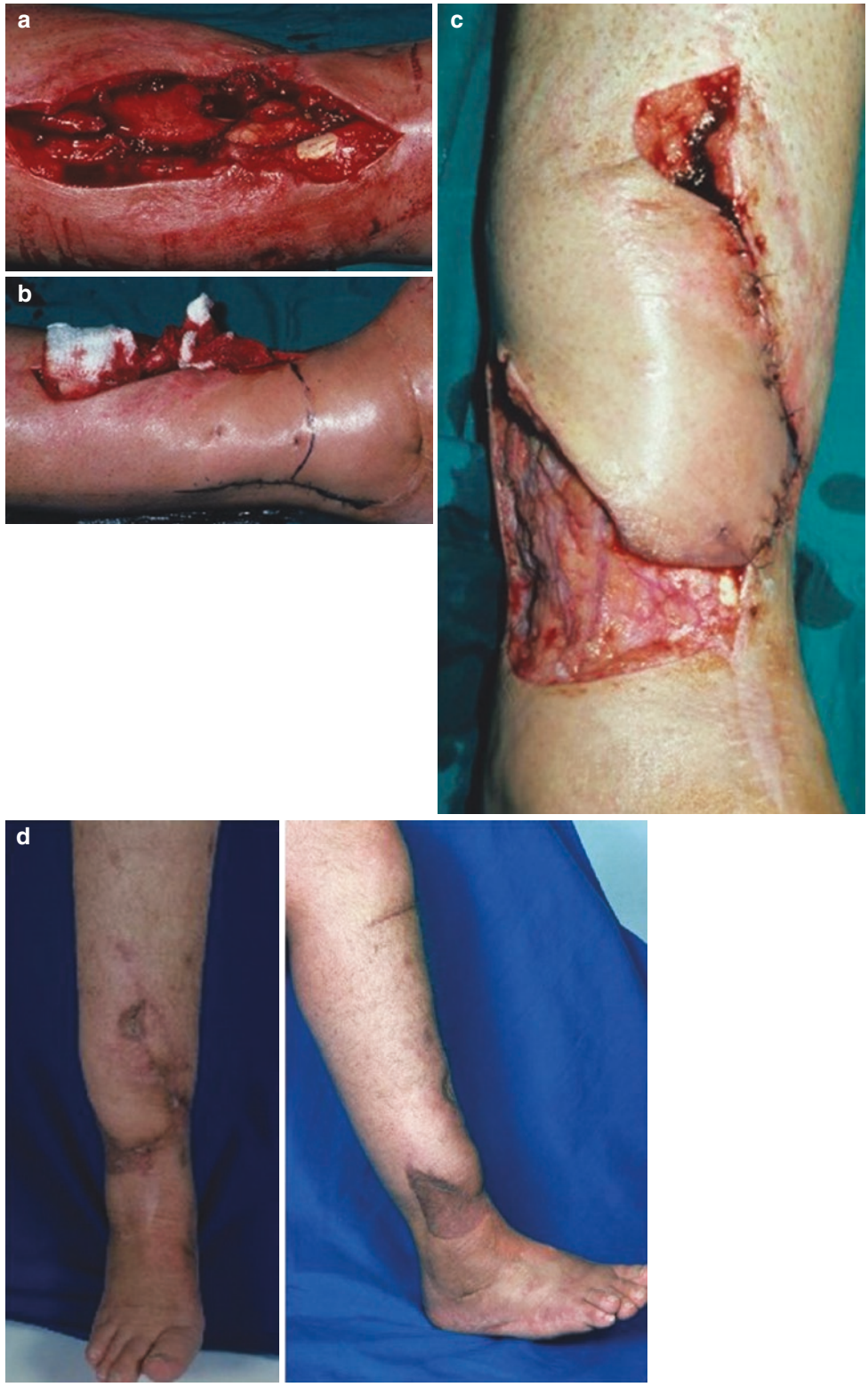

Fig. 35.9 (**a**) Anterior open tibial fracture with tissue loss. (**b**) Peroneal (fibular) artery fasciocutaneous perforator marked. (**c**) Pedicled perforator flap mobilized over open fracture and donor defect reconstructed with skin graft. (**d**) Left and right: patient at 3 months

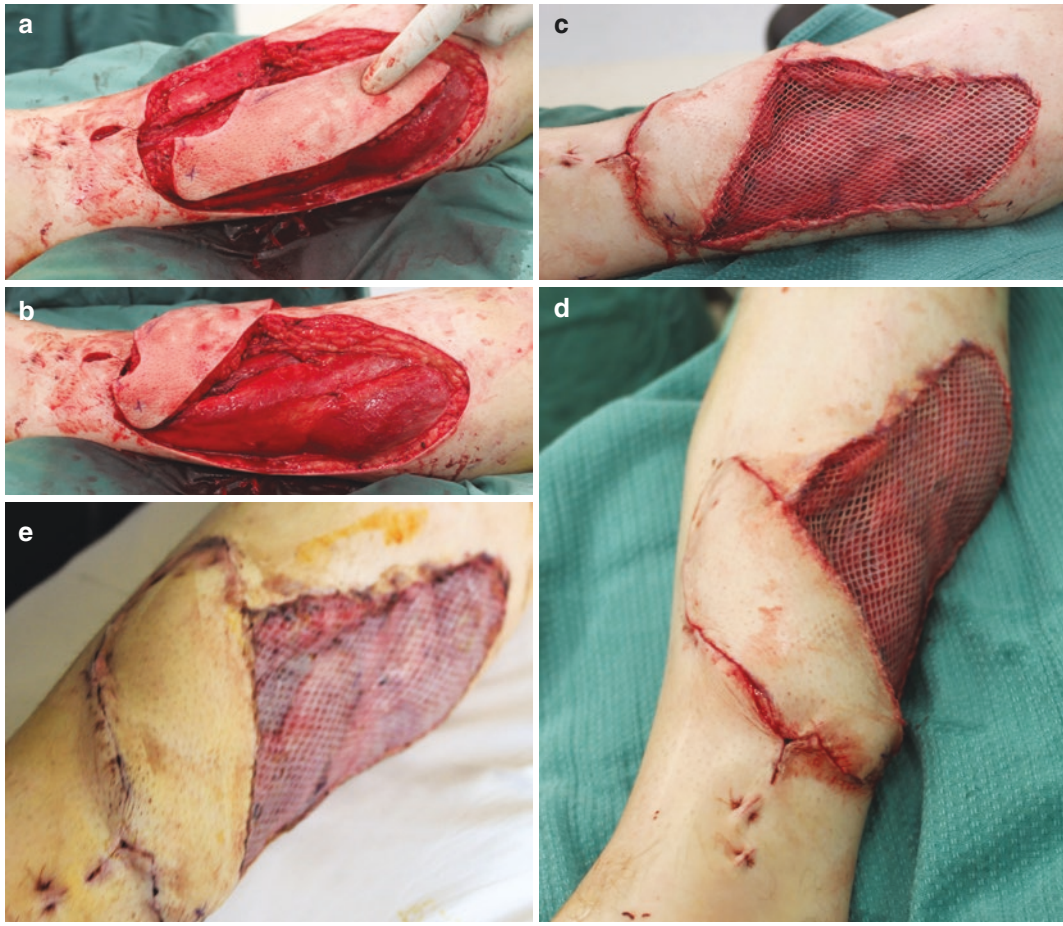

Fig. 35.10 (**a**) Open tibial fracture with dissected posterior tibial perforator flap (perforator marked). Note expansion of donor wound to be taken into account for donor site closure. (**b**) Fasciocutaneous perforator flap "propellered" laterally to cover bony defect. (**c**–**d**) Flap sited and donor site grafted. (**e**) Outcome at 2 weeks with healed flap and healing graft

Table 35.14 EDB flap

Extensor digitorum brevis	
Flap attributes	Muscle flap, generally used as pedicled flap 4 × 5 cm Use of medial slip of EDB also known as extensor hallucis brevis
Artery	Lateral tarsal artery and branch to extensor hallucis brevis, both branches of dorsalis pedis
Vein	Venae associated with dorsalis pedis or anterior tibial artery
Pedicle	Short, can be extended by following anterior tibial arteriovenous system
Nerve	Deep peroneal nerve (branch of)

tar artery supplies the abductor digiti minimi which can be utilized to cover smaller lateral foot and heel defects.

Dorsalis pedis-based flaps are also often considered in the foot including variations arising from the first dorsal and plantar metatarsal arteries (Table 35.15) [57]. The dorsalis pedis offers a thin pliable free flap but unfortunately does result in a greater donor site morbidity. It has also been commented as absent in up to 15% of the population! A strong palpable pulse may be the only examination required; otherwise, angiography

Table 35.15 Dorsalis pedis flap

Dorsalis pedis	
Flap attributes	Fasciocutaneous flap Donor site morbidity as most defects need grafting and blood supply to foot diminished
Artery	Dorsalis pedis artery (leave the deep plantar arch to supply the foot) 1–2 mm
Vein	Subcutaneous venous system (superficial dorsal arch) draining into greater saphenous vein medially and lesser saphenous vein laterally
Pedicle	Up to 3 cm
Nerve	Superficial peroneal nerve

Table 35.16 Medial plantar flap

Medial plantar	
Flap attributes	Fasciocutaneous flap Up to 2 cm width can be closed directly
Artery	Medial plantar artery 1–2 mm
Vein	Subcutaneous venous system draining into saphenous vein Medial plantar vein often harvested but very small
Pedicle	Up to 3 cm
Nerve	Cutaneous sensory branch from posterior tibial nerve

may be beneficial. It should be noted that the defect created does in the mainstay need grafting and due to the location and reduced blood supply can result in tender chronic wound breakdowns. This a type B fasciocutaneous flap.

Medial plantar flaps, both described as free or pedicled, have also been reported as successful in plantar forefoot repair, giving a sensate region which is preferential in the weight-bearing regions (Table 35.16) [58]. These are a variant on the abductor hallucis brevis flap utilizing the same pedicle as required with or without muscle.

Free flaps are generally a reliable option, with a specialist team, for both traumatic and nontraumatic defects. The serratus anterior, latissimus dorsi, lateral arm, rectus abdominis (including the deep and inferior epigastric perforator flaps), and parascapular free flaps can be added to those described above, with good results in both adult and child populations [59, 60].

It has been noted that the latissimus dorsi and rectus abdominis muscle flaps offer a more reliable flap option with the less microsurgically experienced team than the anterolateral thigh flap (Table 35.17) [61]. It should be noted that these can be quite bulky flaps when transferred. The former has lost some of it's 'workhorse flap' function as thinner flaps have been utilised as well as the increased surgical time in rolling the patient for access to the back and the shoulder dysfunction post-operatievely which can impede the use of crutches in rehabilitation of the lower limb trauma.

Table 35.17 LD flap

Latissimus dorsi (LD) (Fig. 35.11)	
Flap attributes	Muscle flap, can be harvested with skin paddle Can cover defects up to 20 × 40 cm size Used as a free flap for lower limb reconstruction
Artery	Thoracodorsal artery, from subscapular artery 1–3 mm
Vein	Venae comitantes, similar size to artery
Pedicle	From 5 to 15 cm
Nerve	Thoracodorsal nerve

In scenarios where a large defect is unable to be covered by a pedicled flap and there is contraindication to a free flap (included only one intact vascular axis), particularly in the heel region, the cross-leg flap has been used with reasonable results, using the medial saphenous flap with a mean division time of 27 days (Fig. 35.12) [62]. The cross-leg flaps have also been described [63], as well as scenarios where free flaps have been taken from one amputated limb to cover severe tissue loss of an intact lower limb.

Another option for the severely injured limb is the fillet flap. Utilizing the "spare parts" concept, tissue of non-salvageable limbs including amputations can be used to reconstruct complex defects of other regions of the body [64]. The use of fillet flaps works on the basis of axial pattern flaps of composite tissues. Pedicled and free flap options are dependent on the viable tissue but can minimize the need for more proximal amputation or help to lengthen limb, as well as reducing further donor site formation. Examples of these would include using toe or foot tissue to reconstruct foot and ankle defects, the fillet foot flap harvesting the entire soft tissue envelope of the foot raised on the dual pedi-

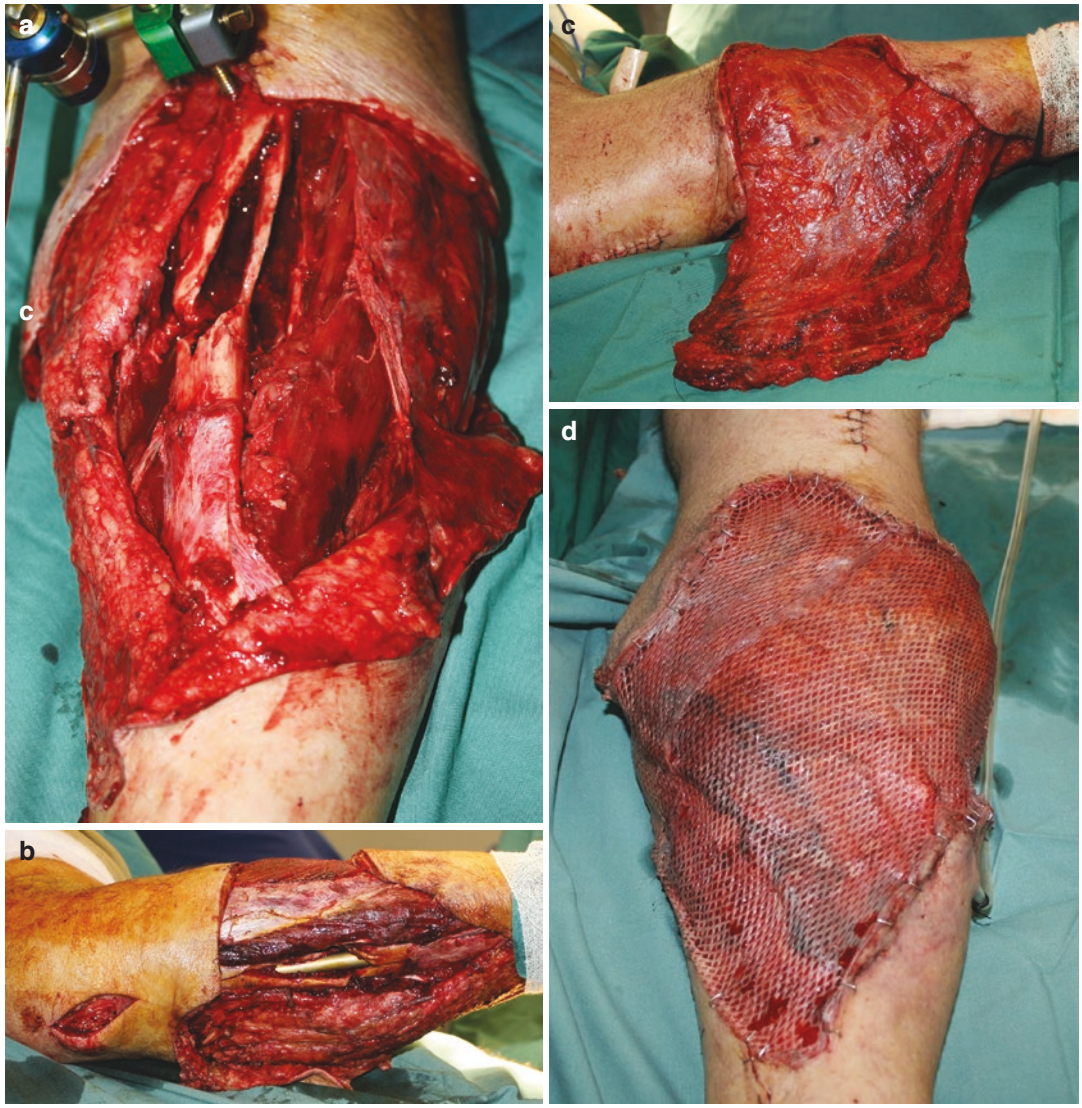

Fig. 35.11 Massive soft tissue defect to open tibia following road traffic incident. (**a**) Defect after first debridement and external fixator in situ. (**b**) Debrided wound with tibial pin in situ. (**c**) Free latissimus dorsi muscle flap harvested from back and side-to-side anastomosis to posterior tibial artery. (**d**) Meshed graft to muscle flap

cle of the dorsal pedis and posterior tibial arteries. These need to both be included if plantar and dorsal skin is utilized as the deep plantar branch does not often allow adequate perfusion to both regions if only one vessel is anastomosed. This can also be sensate using the sensory portions of the tibial nerve. It is harvested at a deeper plane to the dorsalis pedis artery flap to allow a greater padding (often of the amputation stump) and easier dissection – often the extensor tendons being included and the plantar aspect being dissected straight off the bone. If the fillet flap is not a viable option, skin grafts can also be taken from the tissue as necessary.

35.5.7 Choice of Reconstruction: Alternatives and Adjuncts to a FLAP

35.5.7.1 Amputation

Amputation is an option for both traumatic and chronic wounds of the lower limb with them being generally taken electively at foot, ankle,

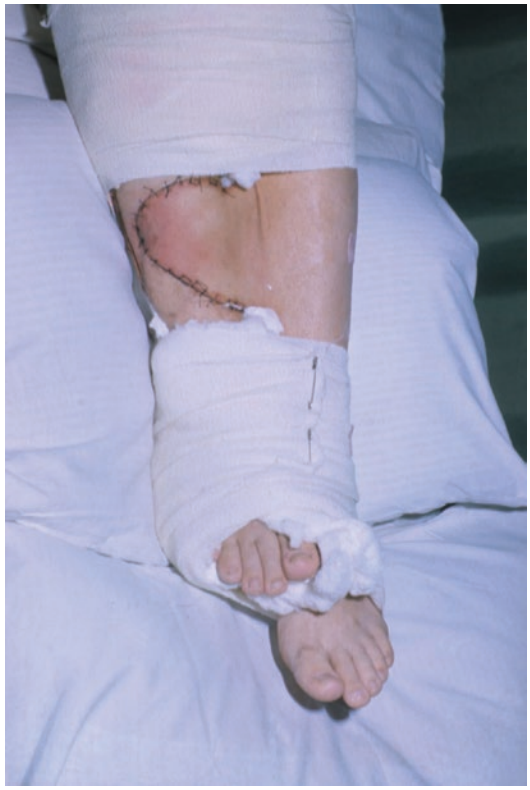

Fig. 35.12 Cross-leg flap for right leg defect prior to flap division

below-knee, or above-knee levels. Current consensus is for a trans-tibial or transfemoral level with the former thought to involve less physical effort and a superior quality of life.

There are few absolute indications for a primary lower limb amputation, them being a total leg amputation or sciatic nerve transection in an adult or the presence of irretrievable devascularization. An avascular limb with warm ischaemia time longer than 4-6 hours, two muscle compartment involvement with segmental muscle loss or segmental bone loss greater than one-third of the tibial length are also indicative of poor salvagability and primary amputation considered.

Other relative contraindications have been discussed but would include life-threatening multi-trauma, an insensate or degloved plantar foot, the crushed foot, extensive loss or multiple bone and joint disruptions, and multilevel injuries. The insensate sole often predisposed amputation but it is now thought to be an often common clinical finding and may often be in relation to neuropraxia and if a query exists the nerve should be explored. Transection of the tibial nerve may weigh towards amputation and continuity away from amputation. The very poor potential rehabilitation patient also requires specific consideration, and it has been shown that there is a failure for elective elderly patients (>55 years of age) to regain baseline function after 6 months, particularly in patients having a higher amputation level, poor baseline cognitive function, and high comorbidity including diabetes or advanced peripheral vascular disease [65]. Any decision for primary amputation should be preferably made by two consultant surgeons with patient and family involvement when able.

Similarly, above-knee amputation has been seen to have a larger impact on war victims compared with below-knee and through-knee amputation and requires greater energy expenditure to later mobilize [4, 66]. The normal below-knee amputation level is 6 cm below the knee joint, but any below-knee tissue may be of benefit as compared to a standard above-knee amputation if the choice is there.

Early distal amputation may also help minimize the need for major limb amputation as a definitive therapy [67], particularly after misguided reconstruction attempts which include significant morbidity [68, 69].

Hertel et al. [70] compared amputation versus patients undergoing complex microvascular reconstruction. They found an increased number of interventions (8 vs 3.5, $p < 0.009$) and rehabilitation time (30 vs 12 months, $p < 0.009$) in the reconstructed group, although this group retained their profession (81 vs 46%, $p < 0.025$) and required a less costly and lifelong invalidity pension (16 vs 54%, $p < 0.02$). There was no great difference in the cost of different interventions. Indications for amputation remain those having a fully severed limb or posterior tibial nerve (loss of foot plantar sensation), with a poor pre-injury health history, > 8 cm segmental tibial loss, or a limb ischemia time greater than 6 hours [4, 70].

There have been multiple attempts at guiding the choice of salvage and reconstruction versus

primary amputation with the use of injury severity scoring systems. These include the following: the Mangled Extremity Severity Score (MESS) (Table 35.18) [71], the Predictive Salvage Index (PSI) [72], the Hanover Fracture Scale 1998 (HFS-98) [73], the Limb Salvage Index (LSI) [74], and the Nerve injury, Ischemia, Soft tissue injury, Skeletal injury, Shock, Age system (NISSSA) [75]. These have all been evaluated in their use to describe a recommended threshold for primary amputation in the adult trauma population. As with the majority of predictive index scores, they all have limitations, being difficult to apply, failing to define functional versus viability of limb as successful, or to predict those that fail in salvage techniques and result in delayed amputation.

The importance of the allied health professionals should not be underestimated. Contracture formation can severely hamper future mobility, and so physiotherapy and the avoidance of joint contractures are imperative. Likewise, in both the reconstructed and amputated limb, the transition from inactivity to a rehabilitated patient can be a long road, and therapy should be introduced at an early stage, even when the patient is still bed bound. Occupational and physical therapists will work together on improving, maintaining, and optimizing the patient's rehabilitation.

The amputation stump is prone to chronic pain and wound breakdown, often due to a combination of poorer blood supply and sensory input, changed pressure points, and residual limb swelling. These problems can be exasperated by a poorly fitting or maintained prosthesis, and so regular orthotic or prosthetic input is important. Prosthetics are generally well tolerated in the proactive patient and often allow a return to a high quality of functionality.

35.5.7.2 Negative-Pressure Wound Therapy

This has been documented for its use in wound coverage until definitive therapy is decided or indicated [5], as well as helping to reduce the size of wound, allowing free flap reconstruction of the massive lower limb wound, or the passage down

Table 35.18 Mangled Extremity Severity Score (MESS); this score was designed as a standard for deciding upon whether to salvage an extremity or whether to amputate, and its use has expanded

Tissue injury	Characteristics	Details	Points
1	Low energy	Stab wound, simple closed fracture, small caliber firearm	1
2	Medium energy	Multiple/open fractures, dislocation, moderate crush injury	2
3	High energy	High caliber/velocity firearm, shotgun	3
4	Massive crush	Logging, railroad, oil rig accidents	4
Shock group			
1	Normotension	BP stable	0
2	Transient hypotension	BP unstable but responding to resuscitation	1
3	Prolonged hypotension	SBP <90 mmHg and responding to resuscitation only when in theatre	2
Ischemia group			
1	None	Pulse without signs of ischemia	0
2	Mild	Diminished pulse without signs of ischemia	1
3	Moderate	No pulse on Doppler, prolonged capillary refill, paresthesia, diminished motor activity	2
4	Advanced	Pulseless	3
Age group			
1	<30 years		0
2	30–50 years		1
3	>50 years		2

It considers four aspects of the injury: degree of soft tissue/skeletal injury, ischemia of the limb, the degree of hemodynamic shock, and the age of the patient. A MESS score of 7 or more indicates the need for amputation [71]

the reconstructive ladder to the point of foregoing the need of even a local flap, something improving investigations and surgical technique is also allowing [76]. NPWT helps to reduce dessication of the wound tissues, promotes wound granulation and provides a dressing for the wound. It should not be used as a substitute for prompt debridement and lavage of the wound, but post-debridement their use can be useful in patients with significant comorbidity or with antibiotic-impregnated cement beads where segmental bone loss of established infection exists. Also, improving investigations and surgical technique can limit the need for free flap reconstruction.

NPWT is also of benefit in its use after amputation and wound line dehiscence for healing the wound [60] and in aiding flap success [77].

35.5.8 The Future, Replantation, and Regeneration

Upper limb allotransplantation has already been performed in many units across the world. The long-term immunosuppression and length of transplant survival are still to be quantified, and we are still away from lower limb cases, which is a feasible but little practiced option. The main reason being that opposed to the diverse function and interaction required in the upper limb, prosthetics alternatives are widely acceptable. Achieving a sensate, painless limb with a stable stance and a functional gait is probably out with most units limits and as with replantation, transplants may well cause a protracted and frustrating for all those concerned. With the improvement of microvascular techniques, replantation of amputated lower limbs may become a reliable option with improved results in the future.

There is ongoing research into regenerative potential of the limb, knowing that some areas of the human body have this potential (e.g., finger pulp or liver). Certain lower vertebrate species (salamander and newt genomes) are able to regenerate an amputated limb including nerve, skin, and bone structures. However, stem cell research and tissue engineering are likely to produce a quicker alternative in both finding an answer to and a quicker time frame in aiding human limb reconstruction.

Stem cell research is likely to be the main future of regenerative medicine, and its application is already being performed with success in laboratory work. Adipose-derived stem cells have been shown to have a potential therapeutic potential in tissue repair, restoring muscle function and increasing perfusion in mouse-modeled peripheral artery disease [78].

Fat grafting has also been utilized as an adjunct to help improve healing in chronic wounds, although these would not be used in the acute trauma setting. Mesenchymal stem cells have been biologically augmented with sutures and have demonstrated increased biomechanical and failure strength in repair of rat Achilles tendons [79].

There are promising signs in the use of autologous platelet-rich plasma in patients with chronic wounds of the lower limb, including those secondary to critical limb ischemia. However, there is not enough data to support its treatment recommendations for tendon and muscle injuries at present [80].

Scaffolds are being developed to provide a three-dimensional framework mimicking the natural environment for specific cell types, in particular research looking at skin, bone, and cartilage cell types.

Dermal substitutes, or acellular dermal matrices, are gaining popularity in aiding soft tissue coverage with good results reported. Usually in association with skin grafting, these templates are used to help cover areas where, otherwise, skin grafts would fail (lack of paratenon and periosteum) and in conjunction with NPWT. They are also becoming useful as a wound closure option in the emergency situation, particularly in war injuries, both allowing delayed, or negating, flap reconstruction [81, 82]. At present, they are supported in the use of aiding healing or soft tissue coverage of the lower limb, although current data is limited [83]. Their future use may well be determined by their long term ability to allow better pliabilty over areas where movement is needed in particular coverage of joints.

Biomolecular studies into the growth of both the upper and lower limb are sure to highlight

potential areas for future research. These are likely to involve the hedgehog and wingless-type (WNT) signaling pathways and fibroblast and insulin-like growth factors involved in limb development.

35.6 Conclusions

Lower limb surgery, in particular reconstruction, is important to restore and maintain both balance and ambulation. Loss of the lower limb is a possible outcome in trauma, malignancy treatment, diabetes, peripheral vascular disease, and neuropathy. After appropriate debridement, reconstruction of any wound has a significant impact on the patient and their family. The salvage of the limb is preferred to amputation, reportedly being more cost-effective over the patient's lifetime [84].

Soft tissue coverage must be wound and area specific, involving the patient and a multidisciplinary approach as the unmotivated, poor pre-injury ambulatory patient with multiple comorbidities is likely to have poorer outcomes.

The reconstruction ladder offers options, and the improvement in both pedicled and free flap microsurgery has made these the mainstay of therapy options. The choice of coverage should be determined by reliability, rather than ease of a procedure, and should be the least disabling with the future likely to provide pharmaceutical and engineered adjuncts to help reach these aims.

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Correction to: The Regulatory Landscape of Cell- and Tissue-Based Regenerative Medicine: Current Challenges and Emerging Issues

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In the original version of Chapter 22 (pp. 229–243), Fig. 22.1 and Fig. 22.3 were inadvertently interchanged. The captions were correct. The figures are now paired with the correct captions.

The original version of this chapter has been revised.

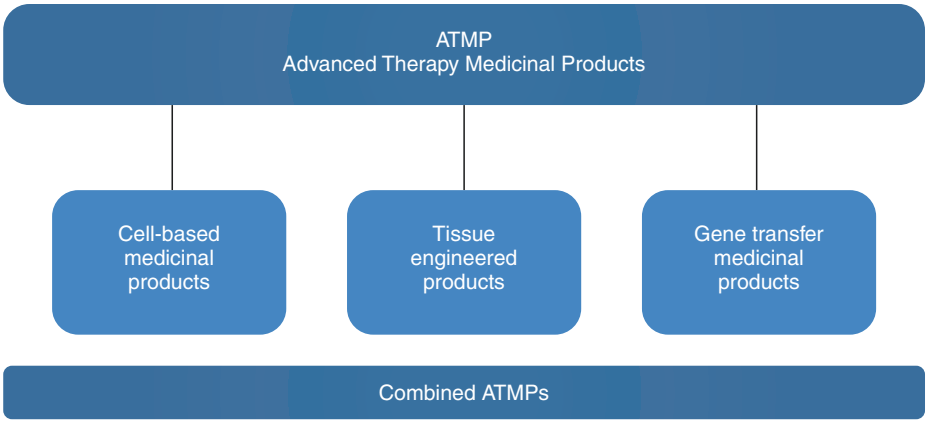

Fig. 22.1 Classification of ATMPs according to EMA. (Adapted from Paul-Ehrlich-Institut Booklet [8])

The updated online version of this chapter can be found at https://doi.org/10.1007/978-3-030-19958-6_22

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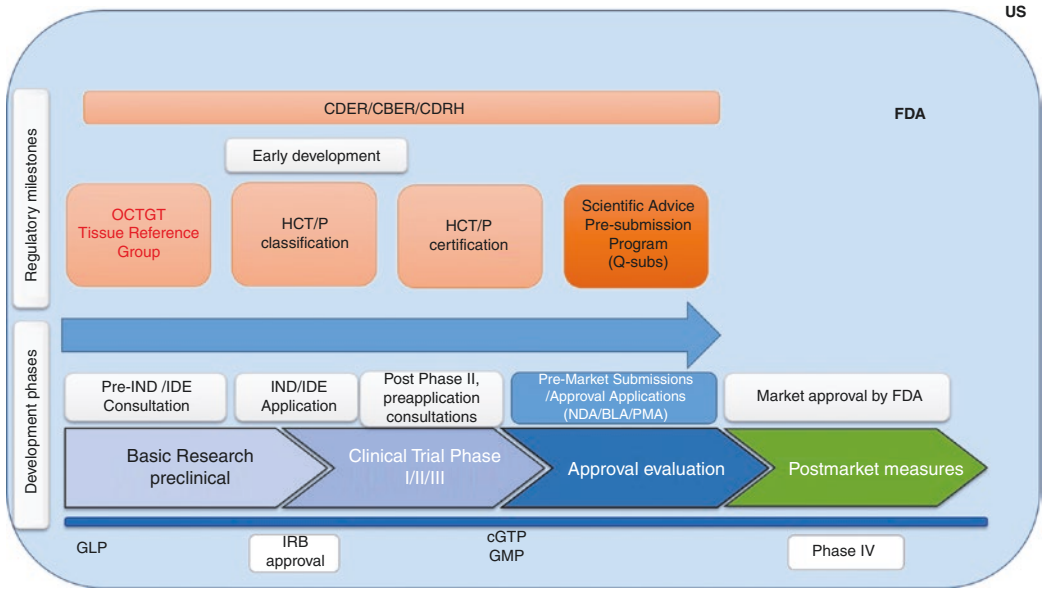

Fig. 22.3 Regulatory pathways and development phases for RM products in the USA. (Adapted from Sakai et al., 2017 [23] and FDA Regulations). CDER: Center for Drug Evaluation and Research, CBER: Center for Biologics Evaluation and Research, CDRH: Center for Devices and Radiological Health, IND: Investigative New Drug, IDE: Investigational Device Exemption, NDA: New Drug Application, BLA: Biologics Licence Application, PMA: Premarket Authorization, OCTGT: Office of Cellular, Tissue and Gene Therapies classification

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