Nishant Chakravorty ·
Praphulla Chandra Shukla *Editors*

Regenerative Medicine

Emerging Techniques to Translation Approaches

Springer

Regenerative Medicine

Nishant Chakravorty •
Praphulla Chandra Shukla
Editors

Regenerative Medicine

Emerging Techniques to Translation Approaches

Springer

Editors
Nishant Chakravorty
School of Medical Science and Technology
Indian Institute of Technology Kharagpur
Kharagpur, West Bengal, India

Praphulla Chandra Shukla
School of Medical Science and Technology
Indian Institute of Technology Kharagpur
Kharagpur, West Bengal, India

ISBN 978-981-19-6007-9 ISBN 978-981-19-6008-6 (eBook)
https://doi.org/10.1007/978-981-19-6008-6

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

Regenerative Medicine is often described as the art and science of integrating the skills of tissue engineering and molecular biology for regeneration and repair of diseased and damaged organs. This branch of medical science therefore provides us with opportunities to develop therapeutic solutions for a wide range of clinical conditions without resorting to transplantation techniques.

The field of regenerative medicine relies on its three pillars—stem cells, biomaterials, and principles of tissue engineering. With the rapid evolution of scientific technology, new translational opportunities are being created every now and then, and therefore it is essential for researchers and med-tech companies across the globe to keep pace with these new developments. This will help us develop newer methods and create better standards of practice in this area. Hence, it is important for us to immerse in learning and understanding the existing and upcoming concepts in stem cells, materials, and tissue engineering.

This book brings together a collection of chapters that discuss the various aspects of Regenerative Medicine—from the basics to the technological advancements in the field, and is expected to generate new ideas in the minds of young researchers in the field of regeneration research.

Kharagpur, West Bengal, India

Nishant Chakravorty
Praphulla Chandra Shukla

Acknowledgements

The Editors would like to express their heartfelt gratitude and sincere thanks to all the authors and experts for agreeing to share their knowledge and expertise.

We wish to thank all the members of the Regenerative Medicine lab and Cardiovascular Research lab at the School of Medical Science and Technology, IIT Kharagpur, for their help and support in compiling the book.

We would also like to take this opportunity to thank our loving and adorable families for their love, affection, and support.

Contents

Editors and Contributors

About the Editors

Nishant Chakravorty leads the Regenerative Medicine lab as a faculty member at the School of Medical Science and Technology, Indian Institute of Technology Kharagpur (India). He has earlier served as a Clinical Specialist with Philips India Ltd (2014–2015). Dr. Chakravorty is a physician-scientist by training (MBBS, M. Med. Sci. & Tech.) with a PhD from Queensland University of Technology (QUT), Australia (2014). His research interests involve utilization of the concepts of cellular and molecular biology, biomaterials, and tissue engineering to study human pathophysiological conditions. He has several international research publications, book chapters, and conference presentations to his credit. He is an editorial board member of a few journals and also serves as a reviewer for several international journals. Dr. Chakravorty is a selected Member of India's one and only young science academy, Indian National Young Academy of Sciences (INYAS), and is an elected Member of the Royal Society of Biology, UK. He is also the recipient of several awards, notably Outstanding Doctoral Thesis Award—Executive Dean's Commendation Award for 2014 (Science and Engineering Faculty, QUT, Australia) and Institute Silver Medal (IIT Kharagpur 2010). He is a Life Member of Indian Society of Hematology and Blood Transfusion, Bengal Society of Haematology (BSH), India, and Eastern Haematology Group, India.

Praphulla Chandra Shukla is a faculty of School of Medical Science and Technology, IIT Kharagpur. He was earlier associated with Charité Medical University Berlin, Germany (2014–2016), BITS Pilani, India (2012–2014), Whitaker Cardiovascular Institute, Boston University, Boston (2011–2012), and University of Toronto (2006–2011). His research interests are myocardial damage and regeneration, endothelial cells transformation, atherosclerosis, cardiomyocyte cell cycle, and epigenetics. He has been selected for various fellowships and been the finalist for Young Investigator Awards from American College of Cardiology, American Heart Association. He has published more than 20 international peer-reviewed papers. He is also an active member of various societies such as German Cardiac Society, Canadian and American Heart Association as well as American College of Cardiology.

Contributors

Long Bai Key Laboratory for Ultrafine Materials of Ministry of Education, East China University of Science and Technology, Shanghai, China

Abhinaba Banerjee Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India
Department of Chemical Engineering, Indian Institute of Technology Madras, Chennai, India

Satarupa Banerjee School of Bio Science and Technology, Vellore Institute of Technology (VIT), Vellore, Vellore, Tamil Nadu, India

Anwesha Barik Department of Pharmaceutical Technology, School of Medical Sciences, Adamas University, Barasat, West Bengal, India

Ananya Barui Centre for Healthcare Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur, Howrah, West Bengal, India

Martin Batstone Herston Biofabrication Institute, Metro North Hospital and Health Service, Brisbane, QLD, Australia

Anindita Bhattacharya School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Sushmita Bist Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India

Khalil N. Bitar Cellfbio LLC, Richmond, VA, USA

Rituparna Chakrabarti School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Nishant Chakravorty School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Jyotirmoy Chatterjee Indian Institute of Technology Kharagpur, Kharagpur, India

Prabhash Dadhich Cellfbio LLC, Richmond, VA, USA

Brateen Datta NanoBiosensors and Biodevices Lab, School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India

Parry Dey School of Bio Science and Technology, Vellore Institute of Technology (VIT), Vellore, Vellore, Tamil Nadu, India

Santanu Dhara Biomaterials and Tissue Engineering Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Krishna Dixit Biomaterials and Tissue Engineering Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Sushmitha Duddu School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Gorachand Dutta NanoBiosensors and Biodevices Lab, School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India

Manoj Kumar Eradath Princeton Neuroscience Institute, Princeton University, Princeton, NJ, USA

Manaswini Gattupalli School of Bio Science and Technology, Vellore Institute of Technology (VIT), Vellore, Vellore, Tamil Nadu, India

Biswajoy Ghosh Indian Institute of Technology Kharagpur, Kharagpur, India
UiT—The Arctic University of Norway, Tromsø, Norway

Ruiqiang Hang Shanxi Key Laboratory of Biomedical Metal Materials, College of Materials Science and Engineering, Taiyuan University of Technology, Taiyuan, China

Anita Hansda Immunology and Inflammation Research Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Saso Ivanovski The University of Queensland, School of Dentistry, Centre for Oral Regeneration, Reconstruction and Rehabilitation (COR3), Herston, QLD, Australia

Sruthi Rayadurgam Jayaprakash Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India

Anisha Kabir Centre for Healthcare Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur, Howrah, West Bengal, India

Moumita Das Kirtania Department of Pharmaceutical Technology, School of Medical Sciences, Adamas University, Barasat, West Bengal, India

Parveen Kumar Department of Pharmaceutical Sciences, Shri Ram College of Pharmacy, Karnal, Haryana, India

Mukti Mandal NanoBiosensors and Biodevices Lab, School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India

Debasish Mishra School of Bio Science and Technology, Vellore Institute of Technology (VIT), Vellore, Vellore, Tamil Nadu, India

Gayatri Mukherjee Immunology and Inflammation Research Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Sayan Mukherjee Biomaterials and Tissue Engineering Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Francesca Oieni Menzies Health Institute Queensland, Griffith University, Southport, QLD, Australia
Clem Jones Centre for Neurobiology and Stem Cell Research, Griffith University, Southport, QLD, Australia
School of Pharmacy and Medical Sciences, Griffith University, Southport, QLD, Australia

Riya Ben Patel School of Bio Science and Technology, Vellore Institute of Technology (VIT), Vellore, Vellore, Tamil Nadu, India

Indira Priyadarshani Patra Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India

Shanmugam Poovizhi School of Bio Science and Technology, Vellore Institute of Technology (VIT), Vellore, Vellore, Tamil Nadu, India

Shantanu Pradhan Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India

Harshavardhan Renikunta Department of Cardiology, Charité Universitätsmedizin Berlin, Berlin, Germany

Ronak Reshamwala Menzies Health Institute Queensland, Griffith University, Southport, QLD, Australia
Clem Jones Centre for Neurobiology and Stem Cell Research, Griffith University, Southport, QLD, Australia
School of Pharmacy and Medical Sciences, Griffith University, Southport, QLD, Australia

Anirban Roy Department of Chemical Engineering, Birla Institute of Technology & Science, Pilani, K K Birla Goa Campus, Goa, India

Anwita Sarkar Centre for Healthcare Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur, Howrah, West Bengal, India

Megha Shah Menzies Health Institute Queensland, Griffith University, Southport, QLD, Australia
Clem Jones Centre for Neurobiology and Stem Cell Research, Griffith University, Southport, QLD, Australia
School of Pharmacy and Medical Sciences, Griffith University, Southport, QLD, Australia

Jai Shukla NanoBiosensors and Biodevices Lab, School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India

Praphulla Chandra Shukla School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India

Kshitiz Singh Queen's University, Kingston, ON, Canada

Rajat Sureka Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India

Cedryck Vaquette The University of Queensland, School of Dentistry, Centre for Oral Regeneration, Reconstruction and Rehabilitation (COR3), Herston, QLD, Australia
Herston Biofabrication Institute, Metro North Hospital and Health Service, Brisbane, QLD, Australia

Yin Xiao Centre of Biomedical Technology, Queensland University of Technology, Brisbane, QLD, Australia
Australia-China Centre for Tissue Engineering and Regenerative Medicine, Queensland University of Technology, Brisbane, QLD, Australia

Xiaohong Yao Shanxi Key Laboratory of Biomedical Metal Materials, College of Materials Science and Engineering, Taiyuan University of Technology, Taiyuan, China

Yuyu Zhao Shanxi Key Laboratory of Biomedical Metal Materials, College of Materials Science and Engineering, Taiyuan University of Technology, Taiyuan, China

Regeneration and Tissue Microenvironment

1

Sushmitha Duddu, Anindita Bhattacharya, Rituparna Chakrabarti, Nishant Chakravorty, and Praphulla Chandra Shukla

1.1 Introduction

Human beings are complex multicellular organisms made up of trillions of cells. Although these cells share the same genetic material (with a few exceptions such as red blood cells [RBCs], gametes), yet a cardiac cell is different from a bone cell and a liver cell is different from a kidney cell. This mystery of cell fate determination, which is believed to be a complex process driven by epigenetic mechanisms modulating gene expression and thus cellular phenotypes, has intrigued scientists for ages.

Over the years, cumulative evidence suggests that the tissue microenvironment, which comprises a dynamic population of cellular and non-cellular components, is a key modulator of cellular differentiation. Comprised primarily of three components—surrounding nearby cells, insoluble extracellular matrix (ECM), and soluble signaling molecules—the microenvironment is a complex and synergistic architecture that varies from tissue to tissue (Sachs et al. 2017). Multicomponent microenvironmental system of stem cells, collectively referred to as stem cell niches, is well recognized for its role in tissue maintenance and regeneration (Lumelsky 2021). In adult tissues, these stem cells generally remain quiescent under homeostasis, but are induced to self-renew and differentiate into specific cell lineages for replenishment of functional tissues compromised or lost due to disease, injury, or aging. However, the regenerative potential of different body tissues varies greatly. For instance, tissues of the skin, liver, and oral and intestinal mucosa have high regenerative capacity, while certain other tissues such as those of the heart, pancreas, and teeth hardly regenerate at all (Iismaa et al. 2018).

S. Duddu · A. Bhattacharya · R. Chakrabarti · N. Chakravorty · P. C. Shukla (✉)
School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India
e-mail: pcshukla@smst.iitkgp.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_1

Modulating the cellular microenvironment is a fundamental aspect of tissue engineering and regeneration, which encompasses an emerging field of medicine with the goal of replacing, engineering, or regenerating human cells, tissues, or organs to restore or reestablish normal function. With the successful isolation and characterization of pluripotent embryonic stem cells, the discrete factors required for differentiation to various cellular lineages of the body were precisely identified (Williams et al. 2012). More importantly, the discovery that cellular differentiation is not strictly a unidirectional phenomenon, and that cells could be "reprogrammed" to become pluripotent, has opened up new avenues for research in the field of regenerative medicine (Williams et al. 2012; Gurdon et al. 1958; Takahashi et al. 2007).

While a comprehensive review of all the related information concerning cell fate determination is beyond the scope of discussion, this book chapter will particularly focus on the different aspects of the tissue microenvironment and its involvement in cellular differentiation and regeneration, and highlight how this knowledge could be utilized for tissue engineering and regenerative medicine.

1.2 Role of Tissue Microenvironment

As discussed previously, certain tissues of the body have a higher capacity of cellular proliferation, and thus regeneration, than others. In this context, tissues can be subdivided into three types:

1. **Continuously dividing tissues:** These tissues comprise highly proliferative cells that replace dead or sloughed-off cells. For example, hematopoietic tissues and epithelial tissues of skin, gastrointestinal tract, and salivary gland.
2. **Quiescent tissues**: These tissues comprise cells that generally remain in a non-dividing state, but may enter the cell cycle and divide in response to certain stimuli, for example, tissue injury, when needed. Examples include mesenchymal cells like smooth muscle cells and fibroblasts; parenchymal cells of kidney, liver, and pancreas; endothelial cells; and lymphocytes.
3. **Non-dividing tissues:** These tissues comprise cells that have permanently left the cell cycle, and thus are unable to divide any further. For example, cardiac and skeletal muscle tissues.

This variation of proliferative and regenerative capacity of tissues is attributed to the diverse nature of tissue-specific microenvironments. Over the years, scientists have become more aware of the complexity of the cellular microenvironment and its critical involvement in regulating cell behavior. In this section, the evidences of the major components of the microenvironment influencing cellular differentiation and tissue regeneration are briefly discussed.

1.2.1 Cells

In vivo, a particular cell is in vicinity with various other cell types, including platelets, fibroblasts, immune cells, and stem cells, present in the tissue microenvironment, which play a crucial role in regeneration.

1.2.1.1 Stem Cells

Bone-marrow-derived stem cells, comprising hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), are capable of differentiating into multiple cell lineages, owing to their pluripotent nature. These cells are majorly involved in replenishment of lost or injured tissue (Grove et al. 2004).

1.2.1.2 Fibroblasts

These cells play a crucial role in wound regeneration by modulating critical processes like fibrin clot dissolution, new ECM and collagen formation, and wound contraction (Bainbridge 2013). Of note, fibroblasts have also been demonstrated to transdifferentiate into other cell types. For instance, these cells have been reported to play a role in neovascularization at the site of cardiac injury by undergoing mesenchymal–endothelial transition leading to the generation of de novo endothelial cells mediated via p53 and VECAD co-expression (Ubil et al. 2014). Besides, another study showed that activation of certain genes, such as Lmx1a, FoxA2, Lmx1b, and Otx2, leads to differentiation of transplanted fibroblasts into neurons (Torper et al. 2013).

1.2.1.3 Immune Cells

These cells play a critical role in tissue repair and regeneration. Macrophages (M1) are the main regulator of inflammatory response, and are involved in phagocytosis of cellular debris, pathogens, and apoptotic cells, and also in the proliferation and movement of other immune cells at the site of tissue injury, resulting in a hypoxic microenvironment (Wynn and Vannella 2016). M2 macrophages, on the other hand, secrete a variety of growth factors, including platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor alpha (VEGF-α), that promote angiogenesis and initiate cell proliferation, differentiation, vascularization, and return to normoxia (Das et al. 2015). In addition, a few reports suggest the ability of macrophages to undergo transdifferentiation. For instance, a study showed that two-thirds of the fibroblasts present at the site of tissue injury were derived from macrophages (Sinha et al. 2018). Moreover, in neoplastic microenvironment, for example, multiple myeloma, macrophages have been found to transdifferentiate into functional endothelial cells, indicating their capability in modulating angiogenesis (Ribatti and Vacca 2018).

1.2.1.4 Platelets

The main function of these cells is to accumulate at the site of injury and bring about hemostasis. Interestingly, emerging evidences indicate a role of platelets in angiogenesis and tissue remodeling. These cells promote angiogenesis at the site of injury

by furnishing pro-angiogenic growth factors like VEGF, PDGF, IGF-1, fibroblast growth factor (FGF), hepatocyte growth factor (HGF), epidermal growth factor (EGF), and angiopoietin. Besides, platelets apparently influence phenotype switching of infiltrating inflammatory cells and resident tissue cells toward the tissue cell lineage. For instance, it was demonstrated that during cardiac regeneration following cardiac injury, platelets modulate the tissue microenvironment by releasing extracellular vesicles comprising various miRNAs (miR-199, miR-126, miR-29), which regulates the fate of cardiac progenitor cells. Besides, these cells also have been reported to regulate hepatocyte proliferation by producing interleukin-6 (IL-6) and VEGF through sphingosine-1-phosphate, and also by releasing HGF, VEGF, and insulin-like growth factor-1 following interaction with Kupffer cells.

1.2.2 Extracellular Matrix

The extracellular matrix (ECM) is a multifaceted acellular tissue that is an intricate network of organic and inorganic molecules that are meticulously organized. ECM is crucially involved in cellular signaling, which controls the fundamental characteristic and behavior of cells such as migration, proliferation, differentiation, adhesion, and apoptosis. ECM is a dynamic network that keeps modifying itself as per the pathophysiological demands of the body. Several cellular proteins and matrix degradation enzymes aid in maintaining this dynamism (Karamanos et al. 2021; Hastings et al. 2019). The ECM contains several different types of biomolecules like proteins (collagen, elastin, fibronectin, etc.), glycosaminoglycans (GAGs), and proteoglycans.

1.2.2.1 Collagen

Collagen is a triple-helical polypeptide molecule that provides strength and resistance to the tissue architecture as well as structural support to the cells. Twenty-six distinct types of collagen molecules have been identified based on their chain assembly. Some of these can form fibrils (like types I, II, III), some can form networks (collagen IV), while other types include "fibril-associated collagens with interruptions in their triple helices" (FACITs; e.g., types IX, XII), short chains (e.g., types VIII, X), and others (Gelse et al. 2003; Ricard-Blum 2011; Karsenty and Park 2009).

Collagen is the most abundant protein in the human body, constituting about 30% of the total protein mass. They are mainly deposited in the ECM, which provides structural support to various connective tissues like bones, ligaments, skin, and tendons. Along with maintaining structural integrity, collagen is also known for communication with cells by storing and delivering several growth factors and cytokines that further help in the development and tissue repair (Sorushanova et al. 2019). Synthetic collagen is potentially utilized to generate various tissue engineering products that are used worldwide for tissue and organ regeneration. Type I collagen is extensively used for skin grafting and bone tissue engineering due

to its ability to provide an environment suitable for stem cell adhesion and proliferation (Di Lullo et al. 2002). Clinically, collagen-based scaffolds, hydrogels, microspheres, and films are reported to be successfully used in regenerating wide range of tissue types (Ferreira et al. 2012; Shpichka et al. 2019).

1.2.2.2 Fibronectin

Fibronectin is another protein commonly found in ECM. It mainly binds to more than ten types of integrins (receptor proteins on cell membranes) that are further involved in several biological processes. Fibronectin exists as a dimer molecule in the cell membrane composed of two identical subunits bound covalently near the C-terminal by a pair of disulfide bonds. Each monomer is made up of three different subunits, types I, II, and III, and studies have reported the presence of these subunits in other molecules suggesting that the evolvement of fibronectin is through exon shuffling (Pankov and Yamada 2002; Dalton and Lemmon 2021).

Fibronectin is predominantly synthesized by hepatocytes and also produced by a variety of cells such as myocytes, chondrocytes, and fibroblasts. It is crucially involved in the intra- and extracellular communication of the cell, thus promoting cell adhesion, cell basement-membrane attachment, clot stabilization, embryogenesis, fibroblast migration, and many more (Singh et al. 2010; Proctor 1987). Recently, studies have reported therapeutic role of fibronectin in cutaneous wound healing and grafting. Fibronectin majorly provides microenvironment to control infection at wound site and promotes re-epithelialization and eventually offering a proper tensile strength for the skin repair during wound healing (Brotchie and Wakefield 1990; Patten and Wang 2021). In addition, it is also well explored as diagnostic biomarker in solid tumor types including ovarian, prostate, and breast cancers and also for viral infections (Tas et al. 2016; Spada et al. 2021).

1.2.2.3 Glycosaminoglycans (GAGs)

GAGs are also known as mucopolysaccharides and are polysaccharide molecules made up of repeating disaccharide units. They are negatively charged long and linear polymers. The disaccharide units are usually composed of uronic acid and an amino sugar (keratan is an exception—it contains galactose instead of uronic acid). They are primarily thought to be instrumental in maintaining cellular hydration and scaffolding role. Recent evidences also suggest that GAGs play an important role in guiding cellular signaling and thus in properties like adhesion, growth, proliferation, differentiation, and tissue healing. Some of the common GAGs of physiological significance include hyaluronic acid (in synovial fluid, loose connective tissue); dermatan sulfate (skin, blood vasculature, heart valves); chondroitin sulfate (cartilage, bone); heparin (mast cells) and heparin sulfate (basement membranes, cell surfaces); and keratan sulfate (bone, cartilage).

GAGs are well studied for their involvement in the pathophysiology of various infections by microbes, viruses, parasites, and fungi. The pathogens use GAGs as a shield against the immune attacks thereby promoting their entry and proliferation in the host system (Aquino and Park 2016). As mentioned earlier, GAGs are involved in many cellular signaling and physiological processes; GAGs are well explored for

their clinical significance. Hyaluronic acid is best known for its hydration capacity, which is clinically used for lubrication of synovial joints and wound-healing process (Mende et al. 2016). Lately, studies have reported the use of hyaluronic acid in the hydrogels, which overcomes the limitation of organic solvents that cause toxicity (Sideris et al. 2016). Similarly, the other types like dermatan sulfate and chondroitin sulfate are clinically proven for their critical role in bone tissue regeneration and their anti-inflammatory capacity (Salbach-Hirsch et al. 2021).

1.2.2.4 Proteoglycans

Proteoglycans, expressed on the cell surface and in the ECM, are comprised of a core protein covalently linked to several GAG chains (Weyers and Linhardt 2013). These large glycoproteins play important structural and regulatory roles in the ECM, while being actively involved in signaling cascades governing tissue growth and development (Chen et al. 2021). Such mechanically, chemically, and biologically relevant characteristics make proteoglycans a critical component of tissue engineering scaffolds (Ferdous and Grande-Allen 2007). Some of the commonly known proteoglycans include aggrecan, decorin, fibromodulin, versican, perlecan, neurocan, brevican, and lumican. Proteoglycans are abundantly present in the cartilage tissue and mainly involve in cartilage development and regeneration. Proteoglycans along with other GAGs provide suitable microenvironment for the differentiation and proliferation of various stem cells. This characteristic is utilized to synthesize GAG-based hydrogel, scaffolds, and nano- and microparticles to enhance the anti-inflammatory and osteogenesis potentials (Chen et al. 2021; Walimbe and Panitch 2020).

1.2.3 Soluble Signaling Molecules

Growth factors are soluble secreted proteins that have the ability to influence a range of cellular processes that are critical for tissue regeneration such as growth, differentiation, migration, cellular metabolism, and apoptosis. These factors modulate cellular function through specific binding to its corresponding receptors, and in turn delivering a particular signal to the target cell population. The common growth factors and their main functions are listed in Table 1.1.

1.3 Regeneration

The impact of the endogenous stem cells in tissue regeneration influences human health by repairing tissues and organs after injury. In most of the organisms, long-term survival requires the capacity to regenerate tissue morphology and function, even though the degree and ability to regenerate varies among different species and also depends on the developmental stage. In case of mammals, the ability of scar-free healing and regeneration is mostly confined to the early stages of life and gradually lost with the progression of the development accounting to the decreased

Table 1.1 Major functions of common growth factors

Growth factor	Function	References
BMP-2	Osteogenesis; bone regeneration	Fu et al. (2008)
BMP-7	Osteogenesis; bone regeneration; also regulates neural progenitor cell proliferation	Calori et al. (2009)
EGF	Involved in epithelial cell proliferation and differentiation	Schultz and Morck (2021); Fernández-Montequín et al. (2009)
FGF-2	Angiogenic factor; also involved in periodontal regeneration	Seghezzi et al. (1998); Li et al. (2017)
GCSF	Mobilization of bone-marrow-derived stem cells	Theiss et al. (2010)
GDF-5	Involved in skeletal and joint development	Koch et al. (2010)
HGF	Angiogenesis; also involved in epithelial cell proliferation and morphogenesis	Morishita et al. (2011)
KGF	Involved in epithelial differentiation, migration, and morphogenesis	Ray (2005)
IGF-1	Regulates neuronal growth and myelination	Wang et al. (2018)
PDGF	Acts as chemotactic and mitogenic factor	Jayakumar et al. (2011)
TGF b-1	Involved in differentiation of bone-forming cells; regulates cellular metabolism in cartilage	Park et al. (2008)
VEGF	Angiogenesis; acts as a potent endothelial cell mitogen	Stefanini et al. (2008)

BMP bone morphogenetic protein; *EGF* epidermal growth factor; *FGF* fibroblast growth factor; *GCSF* granulocyte colony-stimulating factor; *GDF* growth differentiation factor; *HGF* hepatocyte growth factor; *IGF* insulin-like growth factor; *KGF* keratinocyte growth factor; *PDGF* platelet-derived growth factor; *TGF* transforming growth factor; *VEGF* vascular endothelial growth factor

regenerative capacities of the endogenous stem cells. After injury, tissue regeneration is achieved by the endogenous stem cells residing in the local environment or the niche and also recruited from the circulation or by undergoing transdifferentiation (Xia et al. 2018). The mechanism of regeneration varies among the different tissues with hardly any self-renewal activities in the heart and the central nervous system, liver and lungs undergoing very slow cell turnover, and dynamic renewal in case of skin and blood. The response of the immune system toward regeneration takes place through the innate immunity that strongly affects the injury repair process. Immune system participates in restoring the tissue homeostasis by clearing the cellular debris, remodeling the extracellular matrix (ECM), and producing multiple cytokines and growth factors. Likewise, influx of macrophages to the injury site is a very crucial stage of tissue repair (Lucas et al. 2010). In spite of these facts, the relationship between tissue regeneration and immune system is complex. It can play both positive and negative roles depending on the stage of life, organ, and tissue (Xia et al. 2018). Owing to the development of both the innate and adaptive immune responses, the prospective scar-free repair of the mice heart is slowly lost between embryonic day 1 (E1) and E7 (Porrello et al. 2011). During repairing of the tissue after injury, the endogenous stem cells or the resident

de-differentiated cells crosstalk with the immune system, the extracellular matrix, soluble growth factors, and the cell signals (Wulff et al. 2012). Communication and intercellular signaling between immune cells, endogenous stem cells, and the mesenchymal cells indicates the initiation of the regeneration-specific processes. The key requirements in case of the tissue and organ regeneration in amphibians and fish are the reprogramming of cells into stem cells at the site of injury, endogenous stem cells recruitment from the blood, and direct differentiation of the residual endogenous stem cells (Hui et al. 2017).

Endogenous stem cells have the capability to self-renew and differentiate into specific cell types and are tissue-specific adult stem cells. For restoring the tissue function after tissue injury, activation of the endogenous stem cells from the quiescent state is required. During self-regeneration of the injured adult tissue, sufficiently high numbers of endogenous stem cells and progenitor cells are required from multiple tissues to the site of injury. This migration mostly happens through the ECM. Thus, it is very crucial for the stem cells to home in the damaged area during tissue regeneration and thus also be considered for the stem-cell-based therapies (Driskill and Pan 2021). Healing of deep skin wounds is thought to be accomplished by the stem cells that are residing in the adipose tissue and dermis of the human skin; since the adipose tissue is exposed in the wound bed that is margined by the dermal tissue (Kroeze et al. 2009), hence optimal chemokine and cytokine gradients are necessary for the stem cells' homing—the area that needs to be explored for treatments of deep burns and skin wounds.

Regeneration of the ocular lens, derived from the surface ectoderm, has been reported in the non-mammalian vertebrates (Barbosa-Sabanero et al. 2012). In humans, after congenital cataract removal in infants, disorganized regrowth of doughnut-like lens tissues has been observed. Lens epithelial stem/progenitor cells (LECs) that are positive for the proteins like paired box, Pax-6, and polycomb complex protein, Bmi-1, are present throughout the life and are important for the lens regeneration (Lin et al. 2016). To preserve the endogenous LECs and the microenvironment for functional lens regeneration, the cataract can be surgically removed. Still, the mechanism of lens regeneration in humans is yet to be explored in detail at the molecular level (Lin et al. 2016).

The liver is composed of two main epithelial cell types—the hepatocytes and the cholangiocytes. Liver also has a very high degree of regeneration. Hepatocytes are the main players of the metabolic activities and the cholangiocytes are the channels for the transport of bile to the stomach. In case of injury or in response to a loss of considerable amount of the liver parenchyma, the endogenous hepatocytes and the cholangiocytes get activated from the quiescent stage to repair the damaged part (Kaur et al. 2015). These cells have a very low turnover rate in a healthy liver, but in acute injury they proliferate and differentiate through many divisions. Liver also homes facultative stem cells, residing near the portal region of the hepatic lobule in the Canal of Herring. On activation, these cells proliferate and differentiate first into bile duct cells and hepatocytes and then into functional mature hepatocytes. The proliferation of hepatocytes and cholangiocytes gets impaired in chronic liver

diseases and a backup mechanism of regeneration is initiated by the liver progenitor cells that differentiate into hepatocytes and cholangiocytes (Chen et al. 2017).

Heart failure being the reason of high morbidity and mortality is pathophysiologically associated with the basic inability of the adult heart to regenerate the lost or damaged myocardium (Laflamme and Murry 2011). Turnover of the myocytes is limited in the adult heart and also insufficient to restore cardiac tissue integrity and contractility. Cardiac regeneration has been described in detail in the later chapter.

1.4 Conclusion

In this chapter, we studied how various aspects of the microenvironment are critical for regulation of cell behavior. The identification and understanding of the exact roles of the different players in a tissue microenvironment is of central importance for the development of successful regenerative medicine therapy for the treatment of complex wounds and pathologies. Evidences from series of studies attempting to mimic the microenvironment using bioengineered scaffolds and growth factor therapy have yielded promising results. Future studies to unravel the minimal and exact set of biophysical and biochemical cues required to modulate specific signaling pathways guiding cell behavior are expected to result in major advancement in the field of regenerative medicine, leading to improved therapeutic outcomes.

References

Aquino RS, Park PW (2016) Glycosaminoglycans and infection. Front Biosci (Landmark Ed) 21(6):1260–1277

Bainbridge P (2013) Wound healing and the role of fibroblasts. J Wound Care 22(8):407–412. https://doi.org/10.12968/jowc.2013.22.8.407

Barbosa-Sabanero K, Hoffmann A, Judge C, Lightcap N, Tsonis PA, Del Rio-Tsonis K (2012) Lens and retina regeneration: new perspectives from model organisms. Biochem J 447(3):321–334

Brotchie H, Wakefield D (1990) FIBRONECTIN: STRUCTURE, FUNCTION AND SIGNIFICANCE IN WOUND HEALING. Australas J Dermatol 31(1):47–56

Calori GM, Donati D, Di Bella C, Tagliabue L (2009) Bone morphogenetic proteins and tissue engineering: future directions. Injury 40:S67–S76

Chen J, Chen L, Zern MA, Theise ND, Diehl AM, Liu P et al (2017) The diversity and plasticity of adult hepatic progenitor cells and their niche. Liver Int 37(9):1260–1271

Chen J, Sun T, You Y, Wu B, Wang X, Wu J (2021) Proteoglycans and glycosaminoglycans in stem cell homeostasis and bone tissue regeneration. Front Cell Dev Biol 9

Dalton CJ, Lemmon CA (2021) Fibronectin: molecular structure, fibrillar structure and mechanochemical signaling. Cell 10(9):2443

Das A, Sinha M, Datta S, Abas M, Chaffee S, Sen CK et al (2015) Monocyte and macrophage plasticity in tissue repair and regeneration. Am J Pathol 185(10):2596

Di Lullo GA, Sweeney SM, Körkkö J, Ala-Kokko L, San Antonio JD (2002) Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. J Biol Chem 277(6):4223–4231

Driskill JH, Pan D (2021) The hippo pathway in liver homeostasis and pathophysiology. Annu Rev Pathol 16:299

Ferdous Z, Grande-Allen KJ (2007) Utility and control of proteoglycans in tissue engineering. Tissue Eng 13(8):1893–1904

Fernández-Montequín JI, Betancourt BY, Leyva-Gonzalez G, Mola EL, Galán-Naranjo K, Ramírez-Navas M et al (2009) Intralesional administration of epidermal growth factor-based formulation (Heberprot-P) in chronic diabetic foot ulcer: treatment up to complete wound closure. Int Wound J 6(1):67–72. https://pubmed.ncbi.nlm.nih.gov/19291119/

Ferreira AM, Gentile P, Chiono V, Ciardelli G (2012) Collagen for bone tissue regeneration. Acta Biomater 8(9):3191–3200

Fu YC, Nie H, Ho ML, Wang CK, Wang CH (2008) Optimized bone regeneration based on sustained release from three-dimensional fibrous PLGA/HAp composite scaffolds loaded with BMP-2. Biotechnol Bioeng 99(4):996–1006. https://doi.org/10.1002/bit.21648

Gelse K, Pöschl E, Aigner T (2003) Collagens—structure, function, and biosynthesis. Adv Drug Deliv Rev 55(12):1531–1546

Grove JE, Bruscia E, Krause DS (2004) Plasticity of bone marrow–derived stem cells. Stem Cells 22(7):1127–1127

Gurdon JB, Elsdale TR, Fischberg M (1958) Sexually mature individuals of Xenopus Laevis from the transplantation of single somatic nuclei. Nature 182(4627):64–65

Hastings JF, Skhinas JN, Fey D, Croucher DR, Cox TR (2019) The extracellular matrix as a key regulator of intracellular signalling networks. Br J Pharmacol 176(1):82

Hui SP, Sheng DZ, Sugimoto K, Gonzalez-Rajal A, Nakagawa S, Hesselson D et al (2017) Zebrafish regulatory T cells mediate organ-specific regenerative programs. Dev Cell 43(6): 659–672.e5

Iismaa SE, Kaidonis X, Nicks AM, Bogush N, Kikuchi K, Naqvi N et al (2018) Comparative regenerative mechanisms across different mammalian tissues. NPJ Regen Med 3(1):1–20. https://doi.org/10.1038/s41536-018-0044-5

Jayakumar A, Rajababu P, Rohini S, Butchibabu K, Naveen A, Reddy PK et al (2011) Multi-centre, randomized clinical trial on the efficacy and safety of recombinant human platelet-derived growth factor with β-tricalcium phosphate in human intra-osseous periodontal defects. J Clin Periodontol 38(2):163–172. https://doi.org/10.1111/j.1600-051X.2010.01639.x

Karamanos NK, Theocharis AD, Piperigkou Z, Manou D, Passi A, Skandalis SS et al (2021) A guide to the composition and functions of the extracellular matrix. FEBS J 288(24):6850–6912

Karsenty G, Park R-W (2009) Regulation of type I collagen genes expression. Int Rev Immunol 12(4):177–185

Kaur S, Siddiqui H, Bhat MH (2015) Hepatic progenitor cells in action: liver regeneration or fibrosis? Am J Pathol 185(9):2342–2350

Koch FP, Becker J, Terheyden H, Capsius B, Wagner W (2010) A prospective, randomized pilot study on the safety and efficacy of recombinant human growth and differentiation factor-5 coated onto β-tricalcium phosphate for sinus lift augmentation. Clin Oral Implants Res 21(11): 1301–1308. https://doi.org/10.1111/j.1600-0501.2010.01949.x

Kroeze KL, Jurgens WJ, Doulabi BZ, Van Milligen FJ, Scheper RJ, Gibbs S (2009) Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. J Invest Dermatol 129(6):1569–1581

Laflamme MA, Murry CE (2011) Heart regeneration. Nature 473(7347):326–335

Lin H, Ouyang H, Zhu J, Huang S, Liu Z, Chen S et al (2016) Lens regeneration using endogenous stem cells with gain of visual function. Nature 531(7594):323

Li F, Yu F, Xu X, Li C, Huang D, Zhou X, Ye L, Zheng L (2017) Evaluation of recombinant human FGF-2 and PDGF-BB in periodontal regeneration: a systematic review and meta-analysis. Sci Rep 7(1):65

Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Müller W et al (2010) Differential roles of macrophages in diverse phases of skin repair. J Immunol 184(7):3964–3977

Lumelsky N (2021) Creating a pro-regenerative tissue microenvironment: local control is the key. Front Bioeng Biotechnol 9:1–5
Mende M, Bednarek C, Wawryszyn M, Sauter P, Biskup MB, Schepers U et al (2016) Chemical synthesis of glycosaminoglycans. Chem Rev 116(14):8193–8255
Morishita R, Makino H, Aoki M, Hashiya N, Yamasaki K, Azuma J et al (2011) Phase I/IIa clinical trial of therapeutic angiogenesis using hepatocyte growth factor gene transfer to treat critical limb ischemia. Arterioscler Thromb Vasc Biol 31(3):713–720. https://pubmed.ncbi.nlm.nih.gov/21183732/
Pankov R, Yamada KM (2002) Fibronectin at a glance. J Cell Sci 115(20):3861–3863
Park JS, Park K, Woo DG, Yang HN, Chung HM, Park KH (2008) PLGA microsphere construct coated with TGF-β 3 loaded nanoparticles for neocartilage formation. Biomacromolecules 9(8): 2162–2169. https://doi.org/10.1021/bm800251x
Patten J, Wang K (2021) Fibronectin in development and wound healing. Adv Drug Deliv Rev 170: 353–368
Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN et al (2011) Transient regenerative potential of the neonatal mouse heart. Science 331(6020):1078–1080
Proctor RA (1987) Fibronectin: a brief overview of its structure, function, and physiology. Rev Infect Dis 9(Supplement_4):S317–S321
Ray P (2005) Protection of epithelial cells by keratincoyte growth factor signaling. Proc Am Thorac Soc 2(3):221
Ribatti D, Vacca A (2018) New insights in anti-angiogenesis in multiple myeloma. Int J Mol Sci 19(7)
Ricard-Blum S (2011) Hynes and Kenneth Yamada Additional Perspectives on Extracellular Matrix Biology available at Cite this article as. Cold Spring Harb Perspect Biol 3:4978–4979
Sachs PC, Mollica PA, Bruno RD. Tissue specific microenvironments: a key tool for tissue engineering and regenerative medicine. J Biol Eng 2017;11(1):1–11
Salbach-Hirsch J, Rauner M, Hofbauer C, Hofbauer LC (2021) New insights into the role of glycosaminoglycans in the endosteal bone microenvironment. Biol Chem 402(11):1415–1425
Schultz CL, Morck DW (2021) Contact lenses as a drug delivery device for epidermal growth factor in the treatment of ocular wounds. Clin Exp Optom 93(2):61–65. https://doi.org/10.1111/j.1444-0938.2010.00459.x
Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P (1998) Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. J Cell Biol 141(7):1659–1673
Shpichka A, Butnaru D, Bezrukov EA, Sukhanov RB, Atala A, Burdukovskii V et al (2019) Skin tissue regeneration for burn injury. Stem Cell Res Ther 10(1):1–16
Sideris E, Griffin DR, Ding Y, Li S, Weaver WM, Di Carlo D et al (2016) Particle hydrogels based on hyaluronic acid building blocks. ACS Biomater Sci Eng 2(11):2034–2041
Singh P, Carraher C, Schwarzbauer JE (2010) Assembly of fibronectin extracellular matrix. Annu Rev Cell Dev Biol 26:397–419
Sinha M, Sen CK, Singh K, Das A, Ghatak S, Rhea B et al (2018) Direct conversion of injury-site myeloid cells to fibroblast-like cells of granulation tissue. Nat Commun 9(1):1–19. https://www.nature.com/articles/s41467-018-03208-w
Sorushanova A, Delgado LM, Wu Z, Shologu N, Kshirsagar A, Raghunath R et al (2019) The collagen suprafamily: from biosynthesis to advanced biomaterial development. Adv Mater 31(1):1801651
Spada S, Tocci A, Di Modugno F, Nisticò P (2021) Fibronectin as a multiregulatory molecule crucial in tumor matrisome: from structural and functional features to clinical practice in oncology. J Exp Clin Cancer Res 40(1):1–14
Stefanini MO, Wu FTH, Mac Gabhann F, Popel AS (2008) A compartment model of VEGF distribution in blood, healthy and diseased tissues. BMC Syst Biol 2:77. https://pubmed.ncbi.nlm.nih.gov/18713470/

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872

Tas F, Bilgin E, Karabulut S, Tastekin D, Duranyildiz D (2016) Levels of serum fibronectin as a biomarker in gastric cancer patients: correlation with clinical diagnosis and outcome. Mol Clin Oncol 4(4):655

Theiss HD, Brenner C, Engelmann MG, Zaruba MM, Huber B, Henschel V et al (2010) Safety and efficacy of SITAgliptin plus GRanulocyte-colony-stimulating factor in patients suffering from Acute Myocardial Infarction (SITAGRAMI-Trial)—rationale, design and first interim analysis. Int J Cardiol 145(2):282–284. https://pubmed.ncbi.nlm.nih.gov/20051299/

Torper O, Pfisterer U, Wolf DA, Pereira M, Lau S, Jakobsson J et al (2013) Generation of induced neurons via direct conversion in vivo. Proc Natl Acad Sci U S A 110(17):7038–7043

Ubil E, Duan J, Pillai ICL, Rosa-Garrido M, Wu Y, Bargiacchi F, et al. Mesenchymal–endothelial transition contributes to cardiac neovascularization. Nat 2014;514(7524):585–590. https://www.nature.com/articles/nature13839

Walimbe T, Panitch A (2020) Proteoglycans in biomedicine: resurgence of an underexploited class of ECM molecules. Front Pharmacol 10:1661

Wang L, Yang M, Jin M, Wu Y, Zheng T, Gu S et al (2018) Transplant of insulin-like growth factor-1 expressing bone marrow stem cells improves functional regeneration of injured rat uterus by NF-κB pathway. J Cell Mol Med 22(5):2815–2825. https://pubmed.ncbi.nlm.nih.gov/29516621/

Weyers A, Linhardt RJ (2013) Neoproteoglycans in tissue engineering. FEBS J 280(10):2511–2522

Williams LA, Davis-Dusenbery BN, Eggan KC (2012) SnapShot: directed differentiation of pluripotent stem cells. Cell 149(5):1174–1174.e1. https://doi.org/10.1016/j.cell.2012.05.015

Wulff BC, Parent AE, Meleski MA, Dipietro LA, Schrementi ME, Wilgus TA (2012) Mast cells contribute to scar formation during fetal wound healing. J Invest Dermatol 132(2):458–465

Wynn TA, Vannella KM (2016) Macrophages in tissue repair, regeneration, and fibrosis. Immunity 44(3):450

Xia H, Li X, Gao W, Fu X, Fang RH, Zhang L et al (2018) Tissue repair and regeneration with endogenous stem cells. Nat Rev Mater 3(7):174–193

Non-stem Cell Mediated Tissue Regeneration and Repair

2

Ronak Reshamwala, Francesca Oieni, and Megha Shah

2.1 Introduction

While the concept of tissue regeneration in clinical context is relatively modern, the earliest hints to the mankind's knowledge about the organ regeneration can be found in the ancient Greek mythology. There are references to hepatic regeneration in the tales of Prometheus's punishment for stealing the fire for mankind by Zeus. It is astonishing that our ancestors were aware of not only the liver's ability to regenerate, but also that they had described this ability to be endlessly repetitive. This can be also interpreted that without the specific knowledge of the stem cells, our forefathers knew of certain body parts' potential to completely regrow and endlessly self-renew themselves. It was only a matter of time that this dormant knowledge emerged within the modern medicine as regenerative medicine.

Regenerative medicine is a relatively new branch of medicine that focuses on regrowing, repairing, or replacing the cells, organs, or tissues affected by injury or diseases in order to restore the structure and reestablish the lost function (Anonymous 2021). In addition to the common knowledge regarding the use of stem cells, regenerative medicine also includes several other approaches such as tissue engineering and artificially creating the internal organs. Tissue repairs can also be achieved using somatic cells other than the stem cells, such as precursor cells, restricted progenitor cells, and, in some cases, the fully differentiated mature cells.

R. Reshamwala (✉) · F. Oieni · M. Shah
Menzies Health Institute Queensland, Griffith University, Southport, QLD, Australia

Clem Jones Centre for Neurobiology and Stem Cell Research, Griffith University, Southport, QLD, Australia

School of Pharmacy and Medical Sciences, Griffith University, Southport, QLD, Australia
e-mail: r.reshamwala@griffith.edu.au

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_2

In this chapter, we will provide an overview of the regenerative approaches that are not dealing with the use of stem cells.

2.1.1 Regeneration and Repair: Are They the Same?

The terms "regeneration" and "repair" are both used to describe healing following pathologies of all kinds, in broad context. According to Krafts et al., the term "regeneration" indicates a type of healing where the new growth completely and totally restores the damaged tissues to their original, pre-pathology state. This process is not accessible to certain tissues or following certain types of injuries where the healing must take place by connective tissue laydown to take the place of the parts of tissue that are lost to the injury. This is generally referred to as scarring, or replacement (Krafts 2010; Reinke and Sorg 2012).

However, the term "repair" generally includes parts of both regeneration and replacement. Few instances of healing are achieved by complete regeneration under natural circumstances, such as minor skin wounds or abrasions. Most commonly, the healing is naturally conducted by the process of repair, where regeneration and scarring are both observed in part (Krafts 2010).

Though seemingly minor, this subtle difference is fundamentally important for regenerative medicine. The location and extent of scar formation determine the ultimate functionality of tissue or organ after the healing is completed, since scar formation generally occurs at the expense of normal tissue function. Significantly large injuries generally result in extensive scar formation. Thus, scar is the hallmark of most natural repair processes, and generally indicates some loss of function (Atala et al. 2010). In other words, the most important quality of tissue regeneration is the absence of scarring, and, after any injury, it is the balance between scarring and regeneration that indicates the effectiveness of the natural repair processes.

To this end, the purpose of regenerative medicine is to essentially tip this balance in favor of regeneration and away from scarring in order to maximize the functional regain following a reparative intervention.

2.1.2 Stem Cells and Tissue Repair

Several types of tissues regularly undergo healing by regeneration in the human body. The best example of this is the epithelial tissue. Skin, mucosa, and glands are some of the examples of the epithelia. The reason behind epithelial tissue's ability to regenerate is its natural state of a continuously dividing tissue. Tissues such as this are more capable of cellular proliferation, which is an important factor that determines the tissue's natural tendency toward regeneration or scarring while healing from an injury.

Accordingly, tissues can be classified in these three categories: (1) continuously dividing, (2) quiescent, and (3) non-dividing (Abbas and Aster 2015). Continuously dividing tissues can regenerate due to their large reserves of stem cells. The stem

cells undergo asymmetric division by producing a differentiated daughter cell and an undifferentiated daughter cell, thereby maintaining its ability to continually self-renew and simultaneously sustaining rapid proliferation. The quiescent tissues have limited capability of regeneration. The cells in these tissues only proliferate under the effects of an injury, and otherwise remain in a non-dividing state. Mesenchymal cells, endothelial cells, and parenchymal cells of certain viscera such as liver, kidney, and pancreas are considered quiescent cells. However, some highly specialized cells are terminally differentiated and exist permanently outside their cell cycle, such as neurons, cardiac muscles, and skeletal muscles. Therefore, they cannot proliferate at all, and an injury to these tissues always heals by scarring (Abbas and Aster 2015; Krafts 2010).

Thus, the presence of stem cells is viewed as critical for regeneration and, therefore, of key interest in the research into regenerative approaches. Several different approaches using stem cells are under exploration and have been used with varying degrees of success (Simkin and Seifert 2018).

2.1.3 Stem Cells vs. Non-stem Cells

Stem cells, although critically important for regeneration, are not the only cells capable of executing repairs. Several types of non-stem cells are also able to repair and regenerate the injured tissues by using various different mechanisms.

2.1.3.1 Stem Cells

Stem cells are defined as the cells that possess these two fundamental properties (Schofield 1983): (1) the ability of perpetual self-renewal, and (2) the ability of differentiation. In other words, stem cells are able to maintain their own population and proliferate indefinitely and are able to generate cells in a specific functional state, different from their own.

Based on their differentiation potential (or stemness), stem cells may be classified into different types in the decreasing order of stemness: totipotent, pluripotent, multipotent, oligopotent, and unipotent. Similarly, based on their developmental stages, stem cells can be additionally classified into: embryonic, fetal, infant, umbilical cord blood, and adult stem cells (Kalra and Tomar 2014) (Zakrzewski et al. 2019).

As explained in Fig. 2.1, the stem cells undergo asymmetric cell divisions to self-renew and produce the stem cell next on the stemness continuum. Zygotes are essentially totipotent stem cells that eventually differentiate into all the cell types of that organism as well as extraembryonic structures. Pluripotent stem cells are the cells of the three germ layers, which in time develop into the entire organism. The next stem cells on the differentiation spectrum are the multipotent stem cells that can differentiate into specific cell lineages—such as the hemopoietic stem cells. The multipotent stem cells further differentiate into oligopotent stem cells that can give rise to several interrelated cell types within a cell lineage. Myeloid and lymphoid progenitor cells, differentiating from the hemopoietic stem cells, are examples of

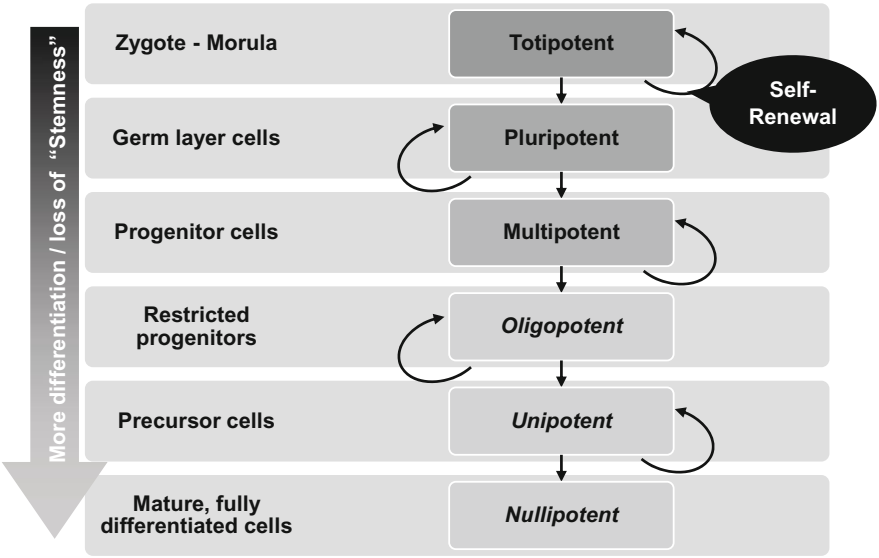


Fig. 2.1 The hierarchy on the differentiation spectrum: The stem cell with highest differentiation potential is the totipotent stem cell. The other cells in a decreasing order of stemness are pluripotent, multipotent, and oligopotent stem cells, followed by unipotent cells that only possess the ability of self-renewal but cannot differentiate in any other cell type than the one to which they are committed. Finally, the nullipotent cells are the fully differentiated mature cells of the body that can neither self-renew nor differentiate in other cell types and are lost at the end of their life cycle

oligopotent stem cells. Finally, the oligopotent progenitor cells differentiate into unipotent precursor cells. These precursor cells are committed to a differentiation into a single cell type such as erythroid precursor cells (differentiate into erythrocytes—which are nullipotent cells), osteoblasts (differentiate into osteocytes) and basal layer cells of epidermis (differentiate into keratinocytes of epidermis) (Zakrzewski et al. 2019).

Given the importance of the differentiation spectrum of the stem cells, two major classes of stem cells are scientifically and experimentally most relevant—pluripotent stem cells, and multipotent stem cells (Biehl and Russell 2009). For the purpose of this chapter, therefore, we will consider the cells downstream from the multipotent stem cells as non-stem cells, namely the oligopotent (restricted progenitors) and unipotent (precursor) cells along with the fully matured nullipotent cells.

During developmental stages, the totipotent cells are found up to the formation of morula. From the blastocyst stage onward, the pluripotent stem cells forming inner cell mass and, eventually, the three germ cell layers are known as embryonic stem cells. The adult or somatic stem cells are limited to their own specific stem cell niche where they co-exist with other non-stem cells (Li and Xie 2005). Some well-known examples of adult stem cells are: hemopoietic stem cells in the bone marrow, epidermal stem cells in the skin, limbal stem cells at the sclero-corneal junction (Pellegrini and De Luca 2014), etc.

2.1.3.2 Non-stem Cells

Contrary to the pluripotent and multipotent stem cells, progenitor cells and precursor cells have some ability for self-renewal but have a very limited differential potential and are usually oligopotent or unipotent, acting primarily as proliferative cells (Seaberg and van der Kooy 2003; Weiss et al. 1996; Potten and Loeffler 1990). Some examples of such proliferative non-stem cells included the satellite cells in the muscles, neural progenitor cells, osteoblasts, chondroblasts, pancreatic progenitor cells (PPCs), angioblasts, lymphoblasts, and many more. Due to their fixed lineage commitment and rapidly proliferative tendencies, they have a predetermined differentiation fate, and they can be readily cultured and expanded. These features have made them very attractive candidates for therapeutic use in regenerative medicine (Zakrzewski et al. 2019).

2.1.4 Regenerative Medicine Beyond the Stem Cells

As discussed before, the process of regeneration and repair involves regrowth and/or replacement of the injured tissues. Since tissues include different types of cells, and acellular components like extracellular matrices (ECMs), regeneration of tissues must also include regrowth and/or replacement of both the cellular and acellular components. Therefore, in addition to the use of stem cells, this also includes tissue engineering and biomaterials for scaffolding; functional stimulation of the host cells with growth factors, natural compounds, or other synthetic pharmaceutical agents; and limited progenitor cells, precursor cells, or fully matured somatic cells. The nullipotent somatic cells have also been used experimentally, to push them back on the differentiation spectrum to create induced pluripotent stem cells (iPSCs), which has opened up several interesting possibilities for the future of regenerative medicine (Takahashi and Yamanaka 2006).

The non-stem cells occupy a strategically crucial place in the contemporary medicine for several other reasons as well.

- Identifying and isolating non-stem cells is easier compared to stem cells. Adult stem cells have highly specific niches, and not all the niches have been thoroughly identified or studied for different types of stem cells. Similarly, culturing and expanding the non-stem cells is also relatively simpler. Unlike the stem cells, maintaining their stemness during the culture period is not a requirement for them.
- Non-stem cell based therapeutic options have been long since studied and approved for clinical uses in therapies. In general, more information and data are available for the non-stem cells.
- Embryonic stem cells and induced pluripotent stem cells have shown great promise for their potential clinical application; however, further exploration has also revealed several concerns for health and safety (Rohban and Pieber 2017). There are several possible side effects (tumorigenesis, undesired differentiation, etc.) of stem cell therapies that must be considered and addressed.

- A major concern with stem cell therapies is their tumorigenic potential, which represents the single most impediment for any clinical applications (Herberts et al. 2011; Li et al. 2006; Werbowetski-Ogilvie et al. 2009). A number of similarities between stem cells and cancer cells makes them likely to undergo malignant transformation in vivo, namely their unrestricted replication potential and self-renewal ability, and capacity to escape apoptosis and to evade effects of inhibitory growth regulators (Li et al. 2006). Any potential therapy using pluripotent stem cells must be characterized using a teratoma formation study in order to be considered safe or otherwise (Nussbaum et al. 2007).
- Upon transplantation in vivo, stem cells have also been found to evoke an immune response (Nussbaum et al. 2007). They have also been shown to have immunomodulatory effects (Herberts et al. 2011). These effects stand to complicate any preventative measure clinically employed to prevent the graft rejection response.
- Other than the iPSCs, the only known sources for pluripotent stem cells are embryonic stem cells, which has been the focal point of ethical and moral debates for decades. Concerns regarding the effects of harvesting cells from a live embryo have also led several countries to ban or disapprove any research or clinical application using embryonic stem cells. Despite recent evidence attempting to alleviate these concerns, many countries continue to disapprove stem cell therapies due to political, religious, and cultural considerations (Rohban and Pieber 2017; Lo and Parham 2009). Additionally, different countries implement different regulations and ethical guidelines for the research and clinical applications using embryonic stem cells, which further complicates the possibility of global application of therapies using stem cells.

  In spite of everything, our knowledge of the stem cells, their niches, their genetic and epigenetic regulations, and their differentiation tendencies, and our ability to assert some control over their fate have improved leaps and bounds in the last 15 years, since the first reports of the iPSCs. Despite the seemingly unlimited potential, however, we still have a lot more to learn about the stem cells to realize the promise of the stem cells in clinically viable, widespread, and affordable therapies that are compatible with our current technological limitations.

2.2 Types of Non-stem Cell Mediated Regeneration and Repair

Regeneration and repairs can be achieved using several different cell types, acellular components of the tissue such as extracellular matrix (ECM), stimulatory biomolecules such as growth factors, or conditioned media. In some cases, partially or completely donated tissues are also used in regenerative medicine (Orlando et al. 2011; Atala 2006; Hirayama et al. 2013). This section gives examples and detailed explanations of many such instances that are clinically relevant, currently under use as therapeutic options, or are being investigated in a preclinical setting (see Fig. 2.2 for an overview). The pros and cons of the same are outlined in Table 2.1.

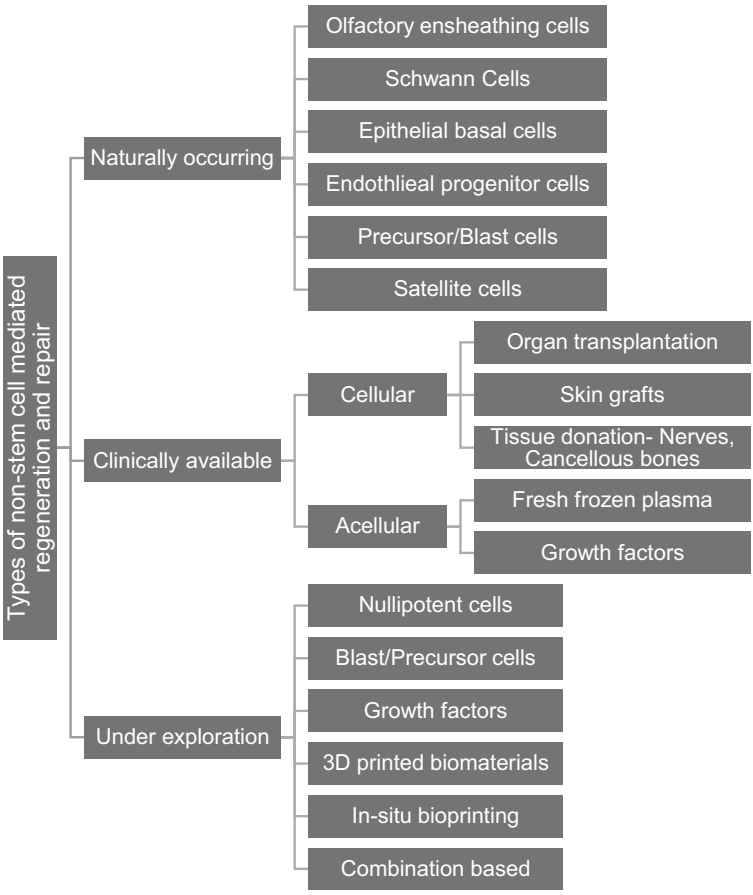


Fig. 2.2 Non-stem cell mediated regeneration and repairs: an overview

Table 2.1 Pros and cons of using non-stem cells for tissue repair

Pros	Cons
• Easy to identify, locate, and isolate from the body • Easy to culture and expand in vitro • No tumorigenic potential • Cell differentiation not required • No ethical or moral considerations	• Cannot be differentiated into different cell types • Cannot be expanded or cultured indefinitely • Limited/specific applications for each cell type

2.2.1 Naturally Occurring

First, we will look at the naturally occurring regeneration in the body where stem cells are not involved. Some unipotent precursor cells are also included here. We will specifically discuss the olfactory ensheathing cells (OECs) and Schwann cells (SCs)

in more detail as these cells are fully differentiated nullipotent cells that help regenerate cells other than their own cell type, namely the neuronal cells. The other nulli- or uni-potent cells either give rise to the same cell types as their own, or help create the extracellular matrix (ECM) around them to help repair sizable defects in tissues.

2.2.1.1 Olfactory Ensheathing Cells (OECs)

Olfactory ensheathing cells (also known as olfactory glia) are the supporting glial cells of the olfactory system, where their primary function is to support and ensheathe the axons of the olfactory nerve (cranial nerve I) extending between olfactory mucosa at the roof of the nasal cavity and olfactory bulb within the anterior cranial fossa. Interestingly, they were misidentified at first as Schwann cells of the olfactory nerve at the end of the nineteenth century (Golgi 1875; Blanes 1898). It was almost a century later when their characteristics similar to astrocytes (expression of glial fibrillary acidic protein) were uncovered (Barber and Lindsay 1982), which led to their identification as a separate and unique cell type (Higginson and Barnett 2011). This was a very important discovery, because the olfactory nervous system is unique in that the olfactory neurons are subject to damage by regular wear and tear and, hence, continuously undergo regeneration; even after extensive injuries, the lost sense of smell can be regained (Graziadei and Graziadei 1979, 1980, 1985). OECs are considered crucial for the regenerative capacity of the olfactory nervous system (Barton et al. 2017; Ekberg et al. 2012; Ekberg and St John 2014; Nazareth et al. 2015; Reshamwala et al. 2019).

Over the years, the exact mechanism by which the OECs help regenerate the olfactory neurons was investigated in depth. The olfactory neurons extend from the olfactory mucosa till the olfactory bulb, and OECs accompany them throughout their traversing zone, while maintaining a close contact with these axons (Doucette 1989, 1990). The OECs ensheathe the axons with a non-myelinating phenotype, where OECs are wrapped around large bundles of the axons, thereby creating a long tunnel around the axon fascicles (Doucette 1990). OECs guide and augment the axons, and support them structurally as well as secrete neurotrophic substances, growth factors, and basement membrane components (Barton et al. 2017; Barnett and Riddell 2004; Bartolomei and Greer 2000; Ramón-Cueto and Avila 1998; Roet and Verhaagen 2014). As the axons traverse from the mucosa to the bulb, they undergo several stages of rearrangements where they are functionally sorted by fasciculation, de-fasciculation, and re-fasciculation by the OECs based on the neurons' odorant receptor profile (Doucette 1989, 1990).

As the progenitor cells in the olfactory mucosa constantly give rise to new neuronal bodies, the axons start growing again toward the olfactory bulb, under the OECs' guidance. OECs also play a crucial role as the main phagocytic cells of the olfactory system to remove the axonal cell debris generated by the damaged or dead neurons (Leung et al. 2008; Wewetzer et al. 2005; Zhang et al. 2014; Su et al. 2013; Nazareth et al. 2015). Such specialized abilities of OECs, in addition to structural remodeling (Roet and Verhaagen 2014), enable them to play this critical role in axon regeneration. This is also why OEC transplantation has been tested as a

potential treatment for spinal cord injury (SCI) repair in animals and humans with promising but highly variable outcomes (Reshamwala et al. 2019).

While OECs bear striking resemblance with Schwann cells in some aspects such as developmental origin, or functional and morphological characteristics, they show a very important and striking difference in tumorigenesis, where they appear less prone to cancerous transformation than the Schwann cells (and by extension, also stem cells) (Murtaza et al. 2019).

2.2.1.2 Schwann Cells

These cells get their name from their discoverer Theodore Schwann, who is also the co-founder of the cell theory. He described the cells wrapped around the axons, which later became known as the Schwann cells (SCs) (Bhatheja and Field 2006).

SCs are the main supporting cells of the peripheral nervous system (PNS), where they myelinate the peripheral nerve axons. They are also important for neuronal survival, signal transmission, and structural organization of the peripheral nervous system. They are also crucial for the damaged or regrowing axons; the SCs help guide and augment the growth of regrowing axons (Riethmacher et al. 1997). SCs also secrete desert hedgehog factor (dhh), which is essential for the formation of a protective barrier for the PNS, known as the peri-neural sheath (Jessen and Mirsky 2005).

SCs have an incredible ability of switching phenotypes in case of an injury to the axons, which plays a key role in the regeneration of PNS axons. This ability is also known as Schwann cell plasticity (Boerboom et al. 2017; Carr and Johnston 2017; Jessen and Mirsky 2005, 2016, 2019). Even in adults, SCs can revert to a reparative phenotype to regenerate the injured nerve (Jessen and Mirsky 2019) (Min et al. 2021). This is how the SCs can help restore an injured nerve in a limited capacity.

After an injury to the peripheral nerves, a complex series of event ensues, known as the "Wallerian Degeneration," which in turn facilitates axonal regeneration (Coleman and Freeman 2010). SCs play an important role in this process, where they initially begin the breakdown of myelin distal to the injury (Lutz et al. 2017; Gomez-Sanchez et al. 2015; Li et al. 2020), and help degrade the axons by modifying the axonal cytoskeleton (Vaquié et al. 2019). They also produce chemotactic cytokines to attract macrophages and rapidly clean up the injury zone (Martini et al. 1990; Stratton et al. 2018; Zigmond and Echevarria 2019). Later, the SCs secrete neurotrophic factors and extracellular matrix proteins to stimulate axonal regrowth, and proliferate and form band-like structures to guide the regrowing axons (Jessen and Mirsky 2016; Ma et al. 2016b). Finally, SCs switch to myelinating phenotype when they detect regenerated axons in the proximity and secrete macrophage inhibitory substances to repel the macrophages and complete the maturation process of newly repaired peripheral nerve (Fry et al. 2007; Jessen and Mirsky 2016; Min et al. 2021).

Due to their reparative potential, Schwann cells have also been tried to repair spinal cord injury (Kamada et al. 2005; Bachelin et al. 2005; Kohama et al. 2001).

2.2.1.3 Epithelial Basal Cells

These are unipotent, proliferative cells that do not differentiate into any other cell types but have a high replicative and self-renewal potential. These cells reside in the basal layer of different epithelia. Epidermis of skin, glandular tissues, and mucosal epithelia of the gastrointestinal tract as well as respiratory tract are some common examples of such cells where they continuously and constantly proliferate to meet the high turnover demand of these tissues. The epithelial linings are completely renewed every 3–5 days in the intestinal tissues, for example. A similar phenomenon is also observed in the corneal lining, and the cells from the sclero-corneal junction (limbus) are found to be responsible for the constant corneal turnover (Pellegrini and De Luca 2014).

2.2.1.4 Endothelial Progenitor Cells

Similar to the epithelial basal cells, the endothelial progenitor cells (EPCs) help repair the endothelium to maintain the endothelial integrity (Dong and Goldschmidt-Clermont 2007). Although they are called progenitor cells, they are essentially unipotent. They were first isolated a little over two decades ago (Asahara et al. 1997). The possibility of cells that can induce angiogenesis in adults is of great interest for potential development of treatments for several clinical conditions. This has garnered a lot of attention and sparked intense debates regarding the root of their ability to create new endothelial tissues in adults (Barber and Iruela-Arispe 2006).

Their primary regenerative mechanism of action is relatively simple, where they proliferate and migrate to the injury site, secrete angiogenic cytokines, and give rise to matured vascular endothelium (Nissen et al. 2006; Rafii and Lyden 2003; Mukai et al. 2008; Sandhu et al. 2017). Several studies have also investigated the role of EPC in angiogenesis around the coronary heart diseases (Qiu et al. 2018; Xu et al. 2014).

2.2.1.5 Precursor or "-Blast" Cells

Cells with the suffix "-blast" are generally the precursor (unipotent) cells with large proliferative reserves and play an active role in development as well as repairs following injury. Some well-known examples of these cells are fibroblasts, osteoblasts, chondroblasts, etc. Erythroblasts are the precursors that develop into red blood cells, and lymphoblasts mature into B- and T-lymphocytes (Awong and Zuniga-Pflucker 2011; Carotta 2008). Fibroblasts are well known for their role in wound healing and scar formation. While their natural activities are targeted more toward scarring than regeneration, they have been the focus of research for their regenerative potential (Abbas and Aster 2015). Osteoblasts and chondroblasts give rise to the ECM for bones (osteoid formation) and cartilage, respectively. They both are derived from the oligopotent progenitor cells of periosteum (De Bari et al. 2006; Ringe et al. 2008; Sakaguchi et al. 2005; Stich et al. 2008).

2.2.1.6 Satellite Cells

These precursor cells are found in muscles. Under normal circumstances they are found in dormant or quiescent state, and they only get activated following an injury.

These cells were first identified in 1961. They are the only known cells responsible for skeletal muscle repairs in adults (Bischoff 1994; Morgan and Partridge 2003).

The satellite cells are found underneath the basal laminae of the muscle fibers, distinctly separate from the myocytes. They are found to have limited self-renewal potential and their numbers also decline with age of the person (Bischoff 1994). Other than the injury, satellite cells can be artificially induced by hepatocyte growth factor—HGF (Tatsumi et al. 1998).

2.2.2 Clinically Available

Although regenerative medicine is a relatively new branch of medicine, there are several regenerative approaches already clinically available. Other than the use of stem cells, ECM, healthy tissue transplantation, growth factor mediated stimulation, etc. can be used in clinical settings to achieve this.

2.2.2.1 Cellular Approaches

Clinical approaches using cellular components are regularly used for patient management, although they may be somewhat more subtle forms of regenerative approaches.

Tissue grafting or transplantation can be considered a regenerative approach. Full or partial thickness skin grafting has clinically been used for decades, which replaces the damaged or lost skin (commonly due to burns, trauma, or surgical removal) with healthy skin taken from elsewhere on the patient's own body for an autologous transplantation.

Tissue donation in the form of peripheral nerve bridge is done to repair an injured motor/mix peripheral nerve in some cases. Similarly, spinal nerve transfer surgeries are also performed to repurpose the less critical, intact spinal nerves to take over the functions of injured, more critical spinal nerves. Tissue regeneration is an important aspect for these treatment approaches to be successful.

Similarly, cartilages or cancellous bones are sometimes harvested to repair and regenerate injured or diseased joint or bones. Cancellous bone transplantations are also used for corrective or reconstructive orthopedic surgeries following removal of a cancerous bone, or to repair a severely injured or shattered long bone.

In transfusion medicine, blood cells are regularly transfused in patients to replace the lost red bloods cells or platelets as a life-saving measure. While the transfused products are not regenerating a body part, this still satisfies the "replacement" aspect of the tissue repair.

2.2.2.2 Acellular Approaches

Some acellular approaches using ECM, cell products, and growth factors have been approved for clinical use in limited capacity, whereas several more are under investigation or awaiting approval.

The fresh frozen plasma (FFP) is one of the most commonly used acellular approaches in transfusion medicine. The FFP is a very effective treatment to replace

blood proteins, especially in cases of coagulopathies, where the patient's blood is unable to clot on its own.

Certain growth factors show great potential for clinical use. Granulocyte-macrophage colony stimulating factor (GM-CSF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) are some of the more promising candidates; however, only GM-CSF and PDGF are currently approved for use in clinical setting, and the others are still under investigation (Barrientos et al. 2014). Both GM-CSF and PDGF can be used in treatment of different skin conditions such as pressure ulcers, skin wounds, venous ulcers, diabetic ulcers, and certain other non-healing wounds (Barrientos et al. 2014).

2.2.3 Under Exploration

Several approaches using the natural properties of the cells and acellular tissue components are being investigated in preclinical or clinical setting for their potential translation into approved clinical therapies for tissue regeneration. Here, we will briefly discuss some of the approaches using cellular and acellular approaches. The list described here not exhaustive; however, significantly important areas of current research have been covered.

2.2.3.1 Cellular Approaches

Cells showing natural abilities for tissue regeneration are obvious choice for expanding their roles or their scope of regeneration to apply it to other tissues or organs. Cell transplantation based treatment options have shown huge promise in recent years as candidates for regenerative therapies.

Nullipotent Cells

As discussed before, nullipotent cells such as OECs and SCs possess natural ability for nerve regeneration. Some other cell types are also emerging as promising regenerative options. Thus, they have become the focus enormous amounts of research in the recent years.

Olfactory ensheathing cells: OECs have been tried in preclinical and clinical settings for their potential capacity for treating spinal cord injuries (SCIs) and peripheral nerve injuries (PNIs). In the late 1990s, the OECs were first tried in rodent SCI models (Ramon-Cueto and Nieto-Sampedro 1994; Ramon-Cueto et al. 2000). In 2002, they were proven safe in a phase I clinical trial for transplantation in an injured spinal cord (Féron et al. 2005). Another animal trial in a canine model showed their efficacy as autologous transplants (Granger et al. 2012). Their efficacy was shown in a later joint English–Polish clinical trial, albeit in limited capacity (Tabakow et al. 2014). Despite showing great promise in terms of safety, feasibility, and efficacy, the results were somewhat inconsistent and unpredictable across the preclinical and clinical trials (Reshamwala et al. 2019, 2020a; Reshamwala 2020). Hence, now the research focus has shifted somewhat to making the outcomes more consistent (Reshamwala et al. 2020b; Reshamwala 2020), possibly using them in

combination with other cell types (including stem cells) (Reshamwala et al. 2019; Cloutier et al. 2016; Gomes et al. 2016, 2018), as well as using scaffolding and tissue engineering approaches to enhance survival and integration of OECs in the injury site (Kang et al. 2015; Wang et al. 2006; Deumens et al. 2013; Badhiwala et al. 2018; Reshamwala 2020). OECs in the form of nerve bridges are also used in clinical settings with some success (Tabakow et al. 2014). OECs have been tried in preclinical experiments as possible treatment for PNI as well (You et al. 2011; Penna et al. 2012).

Schwann cells: SCs, similarly, have also been tried as potential treatments for SCI in preclinical and clinical settings. Given their proficiency for PNI repairs, they have shown promise for SCI treatments as well. Several preclinical studies have investigated SCs with or without OECs or other cell types (Li et al. 2012; Sun et al. 2013; Nategh et al. 2016; Zhang et al. 2017). The SCs have also been tried in clinical settings with some success as well (Chen et al. 2014; Anderson et al. 2017; Gant et al. 2021). Similarly, several studies have reported SCs being a promising candidate for PNI repairs (Sullivan et al. 2016; Büttner et al. 2018; Liu et al. 2020; Modrak et al. 2020; Nocera and Jacob 2020).

Satellite glia: Additionally, satellite glial cells have also been reported recently as having some success in PNI repair (Avraham et al. 2020).

Pancreatic islet cells: The cells in the islet of Langerhans in the pancreas are responsible for production and secretion of insulin hormone. Transplantation of fully matured islet cells is being explored to restore normal endocrine function of the pancreas (Halban et al. 2010). If functionally normal islet cells can be derived in vitro, and then can be transplanted in vivo, more predictable and consistent outcomes can be achieved. The approach is still in very early stages and has not been proven safe or effective in a clinical setting yet.

Hepatocytes: Organ matching and availability is always the greatest bottleneck factor in any organ transplantation approach. Hence, to overcome this challenge for patients needing a liver transplant, an approach using mature hepatocytes is being investigated as an alternative (Donato et al. 2008). In this approach, hepatocytes are acquired by freeing them from their host tissue matrix using enzymatic digestion process; this, however, presents a significant challenge since cell–ECM interactions are critical to maintain the cellular functions and homeostasis. This approach also is significantly away from its clinical fruition at this stage (Hamilton et al. 2001).

Osteocytes: Recent research into the function of osteocytes within a matured bone has revealed that they play an active role in production of biomechanical signaling, internal regulation of bone resorption/formation, remodeling, and possibly even bone regeneration (Cao et al. 2020). The suggested role of osteocytes in bone regeneration is a novel suggestion, and the idea that osteocytes may be able to enhance bone regeneration is very important and groundbreaking, which opens a whole new paradigm of research. Osteocytes by themselves, or with other stem cells, can potentially revolutionize the field of osteogenesis if these properties can be wielded therapeutically.

Osteocytes have now been shown to play a role in fracture repairs, osteolysis, as well as matrix regeneration. Pathological demineralization of bones, as seen in

osteoporosis, hyperparathyroidism, etc., can also be mediated via osteocytic osteolysis (Robling and Bonewald 2020). Since calcium metabolism in the bones is finely balanced bilateral process, osteocytes can also repair the demineralized bone matrix by replacing it (Tsourdi et al. 2018).

Oral mucosa: The oral mucosal and submucosal grafting is emerging as a novel approach for the treatment of esophageal strictures following submucosal dissections for the treatments of esophageal carcinomas (Isomoto et al. 2013; Ohki and Yamamoto 2020). Strictures are a very common complication of esophageal trauma or tumor resection surgeries (Ono et al. 2009). The usual preventative measure for the strictures, the endoscopic balloon dilation (Ezoe et al. 2011), also carries a high risk of perforation in severe cases (Sato et al. 2013). Hence, a safer regenerative approach was devised for clinical prevention or correction of esophageal strictures (Ohki et al. 2012). The approach has been proven safe and is undergoing further clinical testing for its clinical application, as well as its possible translation to an ulcer treatment (Ohki and Yamamoto 2020).

Blast or Precursor Cells

Endothelial progenitor cells: The role of EPCs in angiogenesis has been discussed earlier in this chapter. These properties of EPCs are being studied in preclinical and clinical settings to develop therapeutic interventions using the EPCs. EPCs can be easily harvested from bone marrow, which makes them a preferred candidate for experimental explorations (Bianconi et al. 2018). In rodent models, EPCs have improved blood perfusion after intracardiac injections (mice) in the previously ischemic limbs (Kalka et al. 2000). EPCs have also shown efficacy in reducing post-infarction scarring of the left ventricle and enhancing cardiac function (Kawamoto et al. 2001; Kocher et al. 2001). The EPCs represent a promising new hope for patients suffering from cardiovascular pathologies such as coronary artery diseases, stroke, myocardial infarction, atherosclerosis, hypoxia, and other ischemic diseases. More than 160 clinical trials are ongoing to investigate the EPCs' efficacy in the same (Kaushik and Das 2019).

Fibroblasts: The natural role of fibroblasts is predominantly in the scar formation following an injury. Fibroblasts and their secreted collagen-based matrix have also been found to play a key role in the proliferation and correct morphological arrangement of the keratinocytes, and, thereby, the regeneration of the epidermis (El-Ghalbzouri et al. 2002). However, several avenues of research now focus on how to use fibroblasts for space-filling modalities, cosmetic interventions, and also as accompanying cells with other fully matured or stem cells. Due to their primary role in ECM secretion, they are highly valuable in interventions to treat very large defects that are likely to leave significant disfigurement or scarring if they healed naturally, or at all. Thus, the fibroblasts can be autologously harvested from dermis with a minimally invasive procedure and can be expanded in vitro. These fibroblasts have been successfully injected to correct minor facial deformities (Zhao et al. 2008).

Pancreatic progenitor cells (PPCs): PPCs are a subject of great clinical interest due to their potential as a curative or replacement treatment for diabetes mellitus (type I) (Ku 2008). Of all the known progenitor cells, they are perhaps studied the

most (Awong and Zuniga-Pflucker 2011). In the recent years, extensive studies have been conducted to characterize and streamline the potential therapy using the PPCs in vitro and in vivo (Mihara et al. 2017; Zou et al. 2017; Gratte et al. 2018; Carroll et al. 2019; Quijano et al. 2019).

2.2.3.2 Acellular Approaches

Recent years have seen the rise and rapid growth of tissue engineering, 3D printing, and bioprinting technologies. This has led to several groundbreaking advancements in the field of regenerative medicine. Here, we will discuss some of the prominent approaches that do not make the direct use of stem or non-stem cells, rather these approaches use either cell products such as growth factors and other signaling biomolecules, or secreted ECM (whole or components), alone or in combination with the aforementioned technologies.

Growth factors: In our body, repair and regeneration are governed by several different growth factors, cytokines and differentiation and transcription factors, of which the fibroblast growth factors (FGFs) are considered the "master regulators" of tissue genesis, regeneration, and homeostasis (Tanner and Grose 2016; Ornitz and Itoh 2015; Beenken and Mohammadi 2009). Several studies investigate the role of FGFs in tissue regeneration and their interactions with other signaling molecules (Maddaluno et al. 2017). Over 20 different subtypes of FGFs have been identified in lower animals with their specific functions over the years, including spinal cord regeneration in zebrafish and several other tissue regeneration in mice such as nerve, bones, muscle, skin, digit, liver, and heart (Maddaluno et al. 2017). The FGFs' role in skin repair (Goodarzi et al. 2018), periodontal tissue repair (Fujihara et al. 2019), and muscle tissue regeneration (Huang et al. 2019) has been studied in the humans over the recent years. Some other examples of growth factors that are being explored for tissue regeneration are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), tissue growth factor (TGF), epidermal growth factor (EGF), etc.

3D-printed biomaterials: The largest and most common application of 3D printing is seen in the field of bone regeneration, specifically following bone resection for cancer removal (Ma et al. 2016a). The 3D printing technology allows for incorporation of several cardinal requirements for bone regeneration following a cancer occurrence. The material needs to be osteo-inductive with ability to regenerate bone and repair the defects and should also prevent possible recurrence of the cancer at the same time (Wang et al. 2016; Yang et al. 2016; Cheng et al. 2014). 3D-printed bioceramics have shown great promise in terms of tissue compatibility, integration ability, and biodegradability, as well as ability to carry anticancer agents to prevent recurrence in rabbit models (Ma et al. 2016a). The material has also been used for transplanting embedded bone mesenchymal stem cells for complete bone regeneration (Bettinger et al. 2009; Liu et al. 2013; Sedó et al. 2013).

Similarly, recently silicon resin derived scaffolds have also been shown useful in killing human osteosarcoma cells under photothermal effect and promoting bone regrowth in preclinical animal trials (Fu et al. 2020).

In situ bioprinting: This technology uses viable "bio-inks" containing cells and other cellular products or biomaterials and can be useful in regenerating/recreating functional tissues in situ (Murphy and Atala 2014; Tasnim et al. 2018). This technology has been successfully used in animal models to print several complex constructs for the bone, nerves, cartilages, cornea, and cardiovascular system (Xia et al. 2019; De Ruijter et al. 2019; Romanazzo et al. 2018; Park et al. 2017). Bioprinting has also been proven safe and effective for printing viable functional tissues in vitro as well as in vivo (Hespel et al. 2014). Despite the promising outcomes, the clinical translation of this technology is hindered by ethical concerns, regulatory issues, as well as technological constraints (Li 2014; Kelly 2017; Ahangar et al. 2019).

2.2.3.3 Combination Based

Combination of the cellular and acellular approaches can be very useful in certain conditions. Some recent approaches have investigated the possibilities for using a composite approach with cells and ECM or other matrices to attempt regenerating different tissues.

An innovative replacement for partial thickness skin grafting was developed using keratinocytes and fibroblasts cultured from skin biopsy, which were then placed in a dermal scaffold made with autologous clotted plasma in two severe burns patients in a clinical trial (Llames et al. 2004). This bioengineered autologous skin transplant showed promising results following the grafting as well as on long-term follow-ups.

Another such novel approach for artificially developing bioengineered human skin has been recently established using keratinocytes and dermal fibroblasts in a 3D scaffold made with fibrin–agarose combination. This graft was successfully tested in preclinical animal trials and translated to be tested on 12 burns patients in a clinical trial (Egea-Guerrero et al. 2019).

2.3 Conclusion

The most important take-home message from this chapter is that there is more to cell-based regenerative medicine than just the stem cells. Precursor cells and fully differentiated nullipotent cells are also capable of regrowth and regeneration. Although limited, there are several instances of regeneration and functional repairs happening naturally. Some regenerative options are also clinically available that do not rely predominantly on the stem cells. Enormous amount of research is being done on both stem and non-stem cells to develop clinical therapies for tissue regeneration and repairs. An effective clinical regenerative approach will likely consist of multiple components including cells and acellular elements.

References

Abbas AK, Aster JC (2015) Robbins and Cotran pathologic basis of disease. Saunders

Ahangar P, Cooke ME, Weber MH, Rosenzweig DH (2019) Current biomedical applications of 3D printing and additive manufacturing. Appl Sci 9:1713

Anderson KD, Guest JD, Dietrich WD, Bartlett Bunge M, Curiel R, Dididze M, Green BA, Khan A, Pearse DD, Saraf-Lavi E, Widerström-Noga E, Wood P, Levi AD (2017) Safety of autologous human schwann cell transplantation in subacute thoracic spinal cord injury. J Neurotrauma 34: 2950–2963

Anonymous (2021) Regenerative medicine. Nature Portfolio: Nature. https://www.nature.com/subjects/regenerative-medicine. Accessed Oct 2021

Asahara T, Murohara T, Sullivan A, Silver M, Van Der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM (1997) Isolation of putative progenitor endothelial cells for angiogenesis. Science 275:964–966

Atala A (2006) Recent applications of regenerative medicine to urologic structures and related tissues. Curr Opin Urol 16:305–309

Atala A, Irvine DJ, Moses M, Shaunak S (2010) Wound healing versus regeneration: role of the tissue environment in regenerative medicine. MRS Bull 35

Avraham O, Deng PY, Jones S, Kuruvilla R, Semenkovich CF, Klyachko VA, Cavalli V (2020) Satellite glial cells promote regenerative growth in sensory neurons. Nat Commun 11:4891

Awong G, Zuniga-Pflucker JC (2011) Thymus-bound: the many features of T cell progenitors. Front Biosci 3:961–969

Bachelin C, Lachapelle F, Girard C, Moissonnier P, Serguera-Lagache C, Mallet J, Fontaine D, Chojnowski A, Le Guern E, Nait-Oumesmar B (2005) Efficient myelin repair in the macaque spinal cord by autologous grafts of Schwann cells. Brain 128:540–549

Badhiwala JH, Ahuja CS, Fehlings MG (2018) Time is spine: a review of translational advances in spinal cord injury. J Neurosurg Spine 30:1–18

Barber CL, Iruela-Arispe ML (2006) The ever-elusive endothelial progenitor cell: identities, functions and clinical implications. Pediatr Res 59:26–32

Barber PC, Lindsay RM (1982) Schwann cells of the olfactory nerves contain glial fibrillary acidic protein and resemble astrocytes. Neuroscience 7:3077–3090

Barnett SC, Riddell JS (2004) Olfactory ensheathing cells (OECs) and the treatment of CNS injury: advantages and possible caveats. J Anat 204:57–67

Barrientos S, Brem H, Stojadinovic O, Tomic-Canic M (2014) Clinical application of growth factors and cytokines in wound healing. Wound Repair Regen 22:569–578

Bartolomei JC, Greer CA (2000) Olfactory ensheathing cells: bridging the gap in spinal cord injury. Neurosurgery 47:1057–1069

Barton MJ, John JS, Clarke M, Wright A, Ekberg J (2017) The glia response after peripheral nerve injury: a comparison between schwann cells and olfactory ensheathing cells and their uses for neural regenerative therapies. Int J Mol Sci 18:287

Beenken A, Mohammadi M (2009) The FGF family: biology, pathophysiology and therapy. Nat Rev Drug Discov 8:235–253

Bettinger CJ, Bruggeman JP, Misra A, Borenstein JT, Langer R (2009) Biocompatibility of biodegradable semiconducting melanin films for nerve tissue engineering. Biomaterials 30: 3050–3057

Bhatheja K, Field J (2006) Schwann cells: origins and role in axonal maintenance and regeneration. Int J Biochem Cell Biol 38:1995–1999

Bianconi V, Sahebkar A, Kovanen P, Bagaglia F, Ricciuti B, Calabrò P, Patti G, Pirro M (2018) Endothelial and cardiac progenitor cells for cardiovascular repair: a controversial paradigm in cell therapy. Pharmacol Ther 181:156–168

Biehl JK, Russell B (2009) Introduction to stem cell therapy. J Cardiovasc Nurs 24:98–103; quiz 104–5

Bischoff R (1994) The satellite cell and muscle regeneration. Myology

Blanes T (1898) Sobre algunos puntos dudosos de la estructura del bulbo olfatorio
Boerboom A, Dion V, Chariot A, Franzen R (2017) Molecular mechanisms involved in Schwann cell plasticity. Front Mol Neurosci 10:38
Büttner R, Schulz A, Reuter M, Akula AK, Mindos T, Carlstedt A, Riecken LB, Baader SL, Bauer R, Morrison H (2018) Inflammaging impairs peripheral nerve maintenance and regeneration. Aging Cell 17:e12833
Cao W, Helder MN, Bravenboer N, Wu G, Jin J, Ten Bruggenkate CM, Klein-Nulend J, Schulten E (2020) Is there a governing role of osteocytes in bone tissue regeneration? Curr Osteoporos Rep 18:541–550
Carotta S (2008) Losing B cell identity. BioEssays 30:203–207
Carr MJ, Johnston AP (2017) Schwann cells as drivers of tissue repair and regeneration. Curr Opin Neurobiol 47:52–57
Carroll L, Mridha AR, Tuch BE (2019) Encapsulation and transplantation of pancreatic progenitor cells. Methods Mol Biol 2029:93–102
Chen L, Huang H, Xi H, Zhang F, Liu Y, Chen D, Xiao J (2014) A prospective randomized double-blind clinical trial using a combination of olfactory ensheathing cells and Schwann cells for the treatment of chronic complete spinal cord injuries. Cell Transplant 23(Suppl 1):S35–S44
Cheng L, Wang C, Feng L, Yang K, Liu Z (2014) Functional nanomaterials for phototherapies of cancer. Chem Rev 114:10869–10939
Cloutier F, Kalincik T, Lauschke J, Tuxworth G, Cavanagh B, Meedeniya A, Mackay-Sim A, Carrive P, Waite P (2016) Olfactory ensheathing cells but not fibroblasts reduce the duration of autonomic dysreflexia in spinal cord injured rats. Auton Neurosci 201:17–23
Coleman MP, Freeman MR (2010) Wallerian degeneration, wlds, and nmnat. Annu Rev Neurosci 33:245–267
De Bari C, Dell'accio F, Vanlauwe J, Eyckmans J, Khan IM, Archer CW, Jones EA, Mcgonagle D, Mitsiadis TA, Pitzalis C (2006) Mesenchymal multipotency of adult human periosteal cells demonstrated by single-cell lineage analysis. Arthritis Rheum 54:1209–1221
De Ruijter M, Ribeiro A, Dokter I, Castilho M, Malda J (2019) Simultaneous micropatterning of fibrous meshes and bioinks for the fabrication of living tissue constructs. Adv Healthc Mater 8: 1800418
Deumens R, Van Gorp SF, Bozkurt A, Beckmann C, Fuhrmann T, Montzka K, Tolba R, Kobayashi E, Heschel I, Weis J, Brook GA (2013) Motor outcome and allodynia are largely unaffected by novel olfactory ensheathing cell grafts to repair low-thoracic lesion gaps in the adult rat spinal cord. Behav Brain Res 237:185–189
Donato MT, Lahoz A, Montero S, Bonora A, Pareja E, Mir J, Castell JV, Gomez-Lechon MJ (2008) Functional assessment of the quality of human hepatocyte preparations for cell transplantation. Cell Transplant 17:1211–1219
Dong C, Goldschmidt-Clermont PJ (2007) Endothelial progenitor cells: a promising therapeutic alternative for cardiovascular disease. J Interv Cardiol 20:93–99
Doucette R (1989) Development of the nerve fiber layer in the olfactory bulb of mouse embryos. J Comp Neurol 285:514–527
Doucette R (1990) Glial influences on axonal growth in the primary olfactory system. Glia 3: 433–449
Egea-Guerrero JJ, Carmona G, Correa E, Mata R, Arias-Santiago S, Alaminos M, Gacto P, Cuende N (2019) Transplant of tissue-engineered artificial autologous human skin in andalusia: an example of coordination and institutional collaboration. Transplant Proc 51:3047–3050
Ekberg JA, Amaya D, Mackay-Sim A, John JAS (2012) The migration of olfactory ensheathing cells during development and regeneration. Neurosignals 20:147–158
Ekberg JA, St John JA (2014) Crucial roles for olfactory ensheathing cells and olfactory mucosal cells in the repair of damaged neural tracts. Anat Rec 297:121–128
El-Ghalbzouri A, Gibbs S, Lamme E, Van Blitterswijk CA, Ponec M (2002) Effect of fibroblasts on epidermal regeneration. Br J Dermatol 147:230–243

Ezoe Y, Muto M, Uedo N, Doyama H, Yao K, Oda I, Kaneko K, Kawahara Y, Yokoi C, Sugiura Y, Ishikawa H, Takeuchi Y, Kaneko Y, Saito Y (2011) Magnifying narrowband imaging is more accurate than conventional white-light imaging in diagnosis of gastric mucosal cancer. Gastroenterology 141:2017–2025.e3

Féron F, Perry C, Cochrane J, Licina P, Nowitzke A, Urquhart S, Geraghty T, Mackay-Sim A (2005) Autologous olfactory ensheathing cell transplantation in human spinal cord injury. Brain 128:2951–2960

Fry EJ, Ho C, David S (2007) A role for Nogo receptor in macrophage clearance from injured peripheral nerve. Neuron 53:649–662

Fu S, Hu H, Chen J, Zhu Y, Zhao S (2020) Silicone resin derived larnite/C scaffolds via 3D printing for potential tumor therapy and bone regeneration. Chem Eng J 382:122928

Fujihara C, Kanai Y, Masumoto R, Kitagaki J, Matsumoto M, Yamada S, Kajikawa T, Murakami S (2019) Fibroblast growth factor-2 inhibits CD40-mediated periodontal inflammation. J Cell Physiol 234:7149–7160

Gant KL, Guest JD, Palermo AE, Vedantam A, Jimsheleishvili G, Bunge MB, Brooks AE, Anderson KD, Thomas CK, Santamaria AJ, Perez MA, Curiel R, Nash MS, Saraf-Lavi E, Pearse DD, Widerström-Noga E, Khan A, Dietrich WD, Levi AD (2021) Phase 1 safety trial of autologous human schwann cell transplantation in chronic spinal cord injury. J Neurotrauma

Golgi C (1875) Sulla fina anatomia del bulbi olfattorii. Ti Rivista Sperimentale di Freniatria 1: 405–425

Gomes ED, Mendes SS, Assuncao-Silva RC, Teixeira FG, Pires AO, Anjo SI, Manadas B, Leite-Almeida H, Gimble JM, Sousa N, Lepore AC, Silva NA, Salgado AJ (2018) Co-transplantation of adipose tissue-derived stromal cells and olfactory ensheathing cells for spinal cord injury repair. Stem Cells 36:696–708

Gomes ED, Mendes SS, Leite-Almeida H, Gimble JM, Tam RY, Shoichet MS, Sousa N, Silva NA, Salgado AJ (2016) Combination of a peptide-modified gellan gum hydrogel with cell therapy in a lumbar spinal cord injury animal model. Biomaterials 105:38–51

Gomez-Sanchez JA, Carty L, Iruarrizaga-Lejarreta M, Palomo-Irigoyen M, Varela-Rey M, Griffith M, Hantke J, Macias-Camara N, Azkargorta M, Aurrekoetxea I (2015) Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves. J Cell Biol 210:153–168

Goodarzi P, Falahzadeh K, Nematizadeh M, Farazandeh P, Payab M, Larijani B, Tayanloo Beik A, Arjmand B (2018) Tissue engineered skin substitutes. Adv Exp Med Biol 1107:143–188

Granger N, Blamires H, Franklin RJM, Jeffery ND (2012) Autologous olfactory mucosal cell transplants in clinical spinal cord injury: a randomized double-blinded trial in a canine translational model. Brain 135:3227–3237

Gratte FD, Pasic S, Olynyk JK, Yeoh GCT, Tosh D, Coombe DR, Tirnitz-Parker JEE (2018) Transdifferentiation of pancreatic progenitor cells to hepatocyte-like cells is not serum-dependent when facilitated by extracellular matrix proteins. Sci Rep 8:4385

Graziadei P, Graziadei GM (1980) Neurogenesis and neuron regeneration in the olfactory system of mammals. III. Deafferentation and reinnervation of the olfactory bulb following section of thefila olfactoria in rat. J Neurocytol 9:145–162

Graziadei P, Graziadei GM (1985) Neurogenesis and Plasticity of the Olfactory Sensory Neurons a. Ann N Y Acad Sci 457:127–142

Graziadei PP, Graziadei GA (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 8:1–18

Halban PA, German MS, Kahn SE, Weir GC (2010) Current status of islet cell replacement and regeneration therapy. J Clin Endocrinol Metab 95:1034–1043

Hamilton GA, Jolley SL, Gilbert D, Coon JD, Barros S, Lecluyse EL (2001) Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. Cell Tissue Res 306:85–99

Herberts CA, Kwa MSG, Hermsen HPH (2011) Risk factors in the development of stem cell therapy. J Transl Med 9:29

Hespel AM, Wilhite R, Hudson J (2014) Invited review—applications for 3D printers in veterinary medicine. Vet Radiol Ultrasound 55:347–358

Higginson JR, Barnett SC (2011) The culture of olfactory ensheathing cells (OECs)—a distinct glial cell type. Exp Neurol 229:2–9

Hirayama M, Oshima M, Tsuji T (2013) Development and prospects of organ replacement regenerative therapy. Cornea 32:S13–S21

Huang J, Wang K, Shiflett LA, Brotto L, Bonewald LF, Wacker MJ, Dallas SL, Brotto M (2019) Fibroblast growth factor 9 (FGF9) inhibits myogenic differentiation of C2C12 and human muscle cells. Cell Cycle 18:3562–3580

Isomoto H, Yamaguchi N, Minami H, Nakao K (2013) Management of complications associated with endoscopic submucosal dissection/endoscopic mucosal resection for esophageal cancer. Dig Endosc 25(Suppl 1):29–38

Jessen K, Mirsky R (2016) The repair Schwann cell and its function in regenerating nerves. J Physiol 594:3521–3531

Jessen KR, Mirsky R (2005) The origin and development of glial cells in peripheral nerves. Nat Rev Neurosci 6:671–682

Jessen KR, Mirsky R (2019) Schwann cell precursors; multipotent glial cells in embryonic nerves. Front Mol Neurosci 12:69

Kalka C, Masuda H, Takahashi T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T (2000) Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci 97:3422–3427

Kalra K, Tomar PC (2014) Stem cell: basics, classification and applications. Am J Phytomed Clin Therap 2:919–930

Kamada T, Koda M, Dezawa M, Yoshinaga K, Hashimoto M, Koshizuka S, Nishio Y, Moriya H, Yamazaki M (2005) Transplantation of bone marrow stromal cell-derived Schwann cells promotes axonal regeneration and functional recovery after complete transection of adult rat spinal cord. J Neuropathol Exp Neurol 64:37–45

Kang XW, Hu JL, Wang SK, Wang J (2015) Effectiveness of muscle basal lamina carrying neural stem cells and olfactory ensheathing cells in spinal cord repair. Genet Mol Res 14:13437–13455

Kaushik K, Das A (2019) Endothelial progenitor cell therapy for chronic wound tissue regeneration. Cytotherapy 21:1137–1150

Kawamoto A, Gwon H-C, Iwaguro H, Yamaguchi J-I, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM (2001) Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. Circulation 103:634–637

Kelly E (2017) FDA regulation of 3D-printed organs and associated ethical challenges. U Pa L Rev 166:515

Kocher A, Schuster M, Szabolcs M, Takuma S, Burkhoff D, Wang J, Homma S, Edwards N, Itescu S (2001) Neovascularization of ischemic myocardium by human bone-marrow–derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat Med 7:430–436

Kohama I, Lankford KL, Preiningerova J, White FA, Vollmer TL, Kocsis JD (2001) Transplantation of cryopreserved adult human Schwann cells enhances axonal conduction in demyelinated spinal cord. J Neurosci 21:944–950

Krafts KP (2010) Tissue repair: the hidden drama. Organogenesis 6:225–233

Ku HT (2008) Minireview: pancreatic progenitor cells—recent studies. Endocrinology 149: 4312–4316

Leung J, Chapman J, Harris J, Hale D, Chung R, West A, Chuah M (2008) Olfactory ensheathing cells are attracted to, and can endocytose, bacteria. Cell Mol Life Sci 65:2732–2739

Li BC, Xu C, Zhang JY, Li Y, Duan ZX (2012) Differing Schwann cells and olfactory ensheathing cells behaviors, from interacting with astrocyte, produce similar improvements in contused rat spinal cord's motor function. J Mol Neurosci 48:35–44

Li HC, Stoicov C, Rogers AB, Houghton J (2006) Stem cells and cancer: evidence for bone marrow stem cells in epithelial cancers. World J Gastroenterol 12:363–371
Li L, Xie T (2005) Stem cell niche: structure and function. Annu Rev Cell Dev Biol 21:605–631
Li PH (2014) 3D bioprinting technologies: patents, innovation and access. Law Innov Technol 6: 282–304
Li R, Li D, Wu C, Ye L, Wu Y, Yuan Y, Yang S, Xie L, Mao Y, Jiang T (2020) Nerve growth factor activates autophagy in Schwann cells to enhance myelin debris clearance and to expedite nerve regeneration. Theranostics 10:1649
Liu CY, Yin G, Sun YD, Lin YF, Xie Z, English AW, Li QF, Lin HD (2020) Effect of exosomes from adipose-derived stem cells on the apoptosis of Schwann cells in peripheral nerve injury. CNS Neurosci Ther 26:189–196
Liu X, Cao J, Li H, Li J, Jin Q, Ren K, Ji J (2013) Mussel-inspired polydopamine: a biocompatible and ultrastable coating for nanoparticles in vivo. ACS Nano 7:9384–9395
Llames SG, Del Rio M, Larcher F, García E, García M, José Escamez M, Jorcano JL, Holguín P, Meana A (2004) Human plasma as a dermal scaffold for the generation of a completely autologous bioengineered skin. Transplantation 77:350–355
Lo B, Parham L (2009) Ethical issues in stem cell research. Endocr Rev 30:204–213
Lutz AB, Chung W-S, Sloan SA, Carson GA, Zhou L, Lovelett E, Posada S, Zuchero JB, Barres BA (2017) Schwann cells use TAM receptor-mediated phagocytosis in addition to autophagy to clear myelin in a mouse model of nerve injury. Proc Natl Acad Sci 114:E8072–E8080
Ma H, Luo J, Sun Z, Xia L, Shi M, Liu M, Chang J, Wu C (2016a) 3D printing of biomaterials with mussel-inspired nanostructures for tumor therapy and tissue regeneration. Biomaterials 111: 138–148
Ma KH, Hung HA, Svaren J (2016b) Epigenomic regulation of Schwann cell reprogramming in peripheral nerve injury. J Neurosci 36:9135–9147
Maddaluno L, Urwyler C, Werner S (2017) Fibroblast growth factors: key players in regeneration and tissue repair. Development 144:4047–4060
Martini R, Schachner M, Faissner A (1990) Enhanced expression of the extracellular matrix molecule J1/tenascin in the regenerating adult mouse sciatic nerve. J Neurocytol 19:601–616
Mihara Y, Matsuura K, Sakamoto Y, Okano T, Kokudo N, Shimizu T (2017) Production of pancreatic progenitor cells from human induced pluripotent stem cells using a three-dimensional suspension bioreactor system. J Tissue Eng Regen Med 11:3193–3201
Min Q, Parkinson DB, Dun X-P (2021) Migrating Schwann cells direct axon regeneration within the peripheral nerve bridge. Glia 69:235–254
Modrak M, Talukder MAH, Gurgenashvili K, Noble M, Elfar JC (2020) Peripheral nerve injury and myelination: potential therapeutic strategies. J Neurosci Res 98:780–795
Morgan JE, Partridge TA (2003) Muscle satellite cells. Int J Biochem Cell Biol 35:1151–1156
Mukai N, Akahori T, Komaki M, Li Q, Kanayasu-Toyoda T, Ishii-Watabe A, Kobayashi A, Yamaguchi T, Abe M, Amagasa T, Morita I (2008) A comparison of the tube forming potentials of early and late endothelial progenitor cells. Exp Cell Res 314:430–440
Murphy SV, Atala A (2014) 3D bioprinting of tissues and organs. Nat Biotechnol 32:773–785
Murtaza M, Chacko A, Delbaz A, Reshamwala R, Rayfield A, Mcmonagle B, St John JA, Ekberg JAK (2019) Why are olfactory ensheathing cell tumors so rare? Cancer Cell Int 19:260
Nategh M, Firouzi M, Naji-Tehrani M, Zanjan LO, Hassannejad Z, Nabian MH, Zadega SA, Karimi M, Rahimi-Movaghar V (2016) Subarachnoid space transplantation of schwann and/or olfactory ensheathing cells following severe spinal cord injury fails to improve locomotor recovery in rats. Acta Med Iran 54:562–569
Nazareth L, Lineburg KE, Chuah MI, Tello Velasquez J, Chehrehasa F, St John JA, Ekberg JA (2015) Olfactory ensheathing cells are the main phagocytic cells that remove axon debris during early development of the olfactory system. J Comp Neurol 523:479–494
Nissen SE, Nicholls SJ, Sipahi I, Libby P, Raichlen JS, Ballantyne CM, Davignon J, Erbel R, Fruchart JC, Tardif JC, Schoenhagen P, Crowe T, Cain V, Wolski K, Goormastic M, Tuzcu EM (2006) Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. JAMA 295:1556–1565

Nocera G, Jacob C (2020) Mechanisms of Schwann cell plasticity involved in peripheral nerve repair after injury. Cell Mol Life Sci 77:3977–3989
Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE (2007) Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J 21:1345–1357
Ohki T, Yamamoto M (2020) Esophageal regenerative therapy using cell sheet technology. Regen Ther 13:8–17
Ohki T, Yamato M, Ota M, Takagi R, Murakami D, Kondo M, Sasaki R, Namiki H, Okano T, Yamamoto M (2012) Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. Gastroenterology 143:582–588.e2
Ono S, Fujishiro M, Niimi K, Goto O, Kodashima S, Yamamichi N, Omata M (2009) Long-term outcomes of endoscopic submucosal dissection for superficial esophageal squamous cell neoplasms. Gastrointest Endosc 70:860–866
Orlando G, Wood KJ, Stratta RJ, Yoo JJ, Atala A, Soker S (2011) Regenerative medicine and organ transplantation: past, present, and future. Transplantation 91:1310–1317
Ornitz DM, Itoh N (2015) The fibroblast growth factor signaling pathway. Wiley Interdiscip Rev Dev Biol 4:215–266
Park JY, Choi YJ, Shim JH, Park JH, Cho DW (2017) Development of a 3D cell printed structure as an alternative to autologs cartilage for auricular reconstruction. J Biomed Mater Res B Appl Biomater 105:1016–1028
Pellegrini G, De Luca M (2014) Eyes on the prize: limbal stem cells and corneal restoration. Cell Stem Cell 15:121–122
Penna V, Stark GB, Wewetzer K, Radtke C, Lang EM (2012) Comparison of Schwann cells and olfactory ensheathing cells for peripheral nerve gap bridging. Cells Tissues Organs 196: 534–542
Potten CS, Loeffler M (1990) Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. Development 110:1001–1020
Qiu Y, Zhang C, Zhang G, Tao J (2018) Endothelial progenitor cells in cardiovascular diseases. AGING Med 1:204–208
Quijano JC, Tremblay JR, Rawson J, Ku HT (2019) Isolation and characterization of colony-forming progenitor cells from adult pancreas. Methods Mol Biol 2029:63–80
Rafii S, Lyden D (2003) Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 9:702–712
Ramón-Cueto A, Avila J (1998) Olfactory ensheathing glia: properties and function. Brain Res Bull 46:175–187
Ramon-Cueto A, Cordero MI, Santos-Benito FF, Avila J (2000) Functional recovery of paraplegic rats and motor axon regeneration in their spinal cords by olfactory ensheathing glia. Neuron 25: 425–435
Ramon-Cueto A, Nieto-Sampedro M (1994) Regeneration into the spinal cord of transected dorsal root axons is promoted by ensheathing glia transplants. Exp Neurol 127:232–244
Reinke JM, Sorg H (2012) Wound repair and regeneration. Eur Surg Res 49:35–43
Reshamwala R (2020) Novel surgical approaches for transplanting three-dimensional constructs of olfactory ensheathing cells to repair the injured spinal cord. PhD, Griffith University
Reshamwala R, Shah M, Belt L, Ekberg JAK, John ST, J. A. (2020a) Reliable cell purification and determination of cell purity: crucial aspects of olfactory ensheathing cell transplantation for spinal cord repair. Neural Regen Res 15:2016–2026
Reshamwala R, Shah M, St John J, Ekberg J (2019) Survival and integration of transplanted olfactory ensheathing cells are crucial for spinal cord injury repair: insights from the last 10 years of animal model studies. Cell Transplant 28:132S–159S
Reshamwala R, Shah M, St John J, Ekberg J (2020b) The link between olfactory ensheathing cell survival and spinal cord injury repair: a commentary on common limitations of contemporary research. Neural Regen Res 15:1848–1849

Riethmacher D, Sonnenberg-Riethmacher E, Brinkmann V, Yamaai T, Lewin GR, Birchmeier C (1997) Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. Nature 389: 725–730

Ringe J, Leinhase I, Stich S, Loch A, Neumann K, Haisch A, Häup T, Manz R, Kaps C, Sittinger M (2008) Human mastoid periosteum-derived stem cells: promising candidates for skeletal tissue engineering. J Tissue Eng Regen Med 2:136–146

Robling AG, Bonewald LF (2020) The osteocyte: new insights. Annu Rev Physiol 82:485–506

Roet KC, Verhaagen J (2014) Understanding the neural repair-promoting properties of olfactory ensheathing cells. Exp Neurol 261:594–609

Rohban R, Pieber TR (2017) Mesenchymal stem and progenitor cells in regeneration: tissue specificity and regenerative potential. Stem Cells Int 2017:5173732

Romanazzo S, Vedicherla S, Moran C, Kelly D (2018) Meniscus ECM-functionalised hydrogels containing infrapatellar fat pad-derived stem cells for bioprinting of regionally defined meniscal tissue. J Tissue Eng Regen Med 12:e1826–e1835

Sakaguchi Y, Sekiya I, Yagishita K, Muneta T (2005) Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. Arthritis Rheum 52:2521–2529

Sandhu K, Mamas M, Butler R (2017) Endothelial progenitor cells: exploring the pleiotropic effects of statins. World J Cardiol 9:1–13

Sato H, Inoue H, Kobayashi Y, Maselli R, Santi EG, Hayee B, Igarashi K, Yoshida A, Ikeda H, Onimaru M, Aoyagi Y, Kudo SE (2013) Control of severe strictures after circumferential endoscopic submucosal dissection for esophageal carcinoma: oral steroid therapy with balloon dilation or balloon dilation alone. Gastrointest Endosc 78:250–257

Schofield R (1983) The stem cell system. Biomed Pharmacother 37:375–380

Seaberg RM, van der Kooy D (2003) Stem and progenitor cells: the premature desertion of rigorous definitions. Trends Neurosci 26:125–131

Sedó J, Saiz-Poseu J, Busqué F, Ruiz-Molina D (2013) Catechol-based biomimetic functional materials. Adv Mater 25:653–701

Simkin J, Seifert AW (2018) Concise review: translating regenerative biology into clinically relevant therapies: are we on the right path? Stem Cells Transl Med 7:220–231

Stich S, Loch A, Leinhase I, Neumann K, Kaps C, Sittinger M, Ringe J (2008) Human periosteum-derived progenitor cells express distinct chemokine receptors and migrate upon stimulation with CCL2, CCL25, CXCL8, CXCL12, and CXCL13. Eur J Cell Biol 87:365–376

Stratton JA, Holmes A, Rosin NL, Sinha S, Vohra M, Burma NE, Trang T, Midha R, Biernaskie J (2018) Macrophages regulate Schwann cell maturation after nerve injury. Cell Rep 24(2561–2572):e6

Su Z, Chen J, Qiu Y, Yuan Y, Zhu F, Zhu Y, Liu X, Pu Y, He C (2013) Olfactory ensheathing cells: the primary innate immunocytes in the olfactory pathway to engulf apoptotic olfactory nerve debris. Glia 61:490–503

Sullivan R, Dailey T, Duncan K, Abel N, Borlongan CV (2016) Peripheral nerve injury: stem cell therapy and peripheral nerve transfer. Int J Mol Sci 17

Sun T, Ye C, Zhang Z, Wu J, Huang H (2013) Cotransplantation of olfactory ensheathing cells and Schwann cells combined with treadmill training promotes functional recovery in rats with contused spinal cords. Cell Transplant 22(Suppl 1):S27–S38

Tabakow P, Raisman G, Fortuna W, Czyz M, Huber J, Li D, Szewczyk P, Okurowski S, Miedzybrodzki R, Czapiga B, Salomon B, Halon A, Li Y, Lipiec J, Kulczyk A, Jarmundowicz W (2014) Functional regeneration of supraspinal connections in a patient with transected spinal cord following transplantation of bulbar olfactory ensheathing cells with peripheral nerve bridging. Cell Transplant 23:1631–1655

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Tanner Y, Grose RP (2016) Dysregulated FGF signalling in neoplastic disorders. Seminars in cell & developmental biology. Elsevier, pp 126–135

Tasnim N, de la Vega L, Anil Kumar S, Abelseth L, Alonzo M, Amereh M, Joddar B, Willerth SM (2018) 3D bioprinting stem cell derived tissues. Cell Mol Bioeng 11:219–240

Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE (1998) HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev Biol 194:114–128

Tsourdi E, Jähn K, Rauner M, Busse B, Bonewald LF (2018) Physiological and pathological osteocytic osteolysis. J Musculoskelet Neuronal Interact 18:292–303

Vaquié A, Sauvain A, Duman M, Nocera G, Egger B, Meyenhofer F, Falquet L, Bartesaghi L, Chrast R, Lamy CM (2019) Injured axons instruct Schwann cells to build constricting actin spheres to accelerate axonal disintegration. Cell Rep 27(3152–3166):e7

Wang B, Zhao Y, Lin H, Chen B, Zhang J, Zhang J, Wang X, Zhao W, Dai J (2006) Phenotypical analysis of adult rat olfactory ensheathing cells on 3-D collagen scaffolds. Neurosci Lett 401: 65–70

Wang Z, Huang P, Jacobson O, Wang Z, Liu Y, Lin L, Lin J, Lu N, Zhang H, Tian R (2016) Biomineralization-inspired synthesis of copper sulfide–ferritin nanocages as cancer theranostics. ACS Nano 10:3453–3460

Weiss S, Reynolds BA, Vescovi AL, Morshead C, Craig CG, Kooy DER, D. V. (1996) Is there a neural stem cell in the mammalian forebrain? Trends Neurosci 19:387–393

Werbowetski-Ogilvie TE, Bossé M, Stewart M, Schnerch A, Ramos-Mejia V, Rouleau A, Wynder T, Smith MJ, Dingwall S, Carter T, Williams C, Harris C, Dolling J, Wynder C, Boreham D, Bhatia M (2009) Characterization of human embryonic stem cells with features of neoplastic progression. Nat Biotechnol 27:91–97

Wewetzer K, Kern N, Ebel C, Radtke C, Brandes G (2005) Phagocytosis of O4+ axonal fragments in vitro by p75− neonatal rat olfactory ensheathing cells. Glia 49:577–587

Xia D, Jin D, Wang Q, Gao M, Zhang J, Zhang H, Bai J, Feng B, Chen M, Huang Y (2019) Tissue-engineered trachea from a 3D-printed scaffold enhances whole-segment tracheal repair in a goat model. J Tissue Eng Regen Med 13:694–703

Xu JY, Lee YK, Wang Y, Tse HF (2014) Therapeutic application of endothelial progenitor cells for treatment of cardiovascular diseases. Curr Stem Cell Res Ther 9:401–414

Yang Y, Liu J, Liang C, Feng L, Fu T, Dong Z, Chao Y, Li Y, Lu G, Chen M (2016) Nanoscale metal–organic particles with rapid clearance for magnetic resonance imaging-guided photothermal therapy. ACS Nano 10:2774–2781

You H, Wei L, Liu Y, Oudega M, Jiao SS, Feng SN, Chen Y, Chen JM, Li BC (2011) Olfactory ensheathing cells enhance Schwann cell-mediated anatomical and functional repair after sciatic nerve injury in adult rats. Exp Neurol 229:158–167

Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z (2019) Stem cells: past, present, and future. Stem Cell Res Ther 10:68

Zhang J, Chen H, Duan Z, Chen K, Liu Z, Zhang L, Yao D, Li B (2017) The effects of co-transplantation of olfactory ensheathing cells and schwann cells on local inflammation environment in the contused spinal cord of rats. Mol Neurobiol 54:943–953

Zhang N, Fang M, Chen H, Gou F, Ding M (2014) Evaluation of spinal cord injury animal models. Neural Regen Res 9:2008

Zhao Y, Wang J, Yan X, Li D, Xu J (2008) Preliminary survival studies on autologous cultured skin fibroblasts transplantation by injection. Cell Transplant 17:775–783

Zigmond RE, Echevarria FD (2019) Macrophage biology in the peripheral nervous system after injury. Prog Neurobiol 173:102–121

Zou C, Lu Y, Teng X, Wang S, Sun X, Huang F, Shu G, Huang X, Guo H, Chen Z, Zhang J, Zhang YA (2017) MRI tracking of autologous pancreatic progenitor-derived insulin-producing cells in monkeys. Sci Rep 7:2505

Immunological Perspectives Involved in Tissue Engineering

3

Anita Hansda, Sayan Mukherjee, Krishna Dixit, Santanu Dhara, and Gayatri Mukherjee

3.1 Introduction

Tissue engineering is a multidisciplinary field that combines science and engineering principles to develop artificial biologic constructs aimed at restoring or regenerating damaged tissue or organs to help maintain their proper biological functions in the body (Langer and Vacanti 1999). It involves the integration of scaffolds derived from various biomaterials, on which cells and different bioactive molecules are seeded, to aid in the restoration and regeneration process (Sadtler et al. 2016). Furthermore, biomaterials provide physical support for endogenous host cells to adhere to, proliferate, and secrete extracellular matrix (ECM), and eventually build tissue, leading to tissue regeneration. Importantly, the host immune system recognizes the foreignness of the different components of the transplanted biomaterials or the cells within a cell-seeded construct and mounts a response toward it (Antonios G Mikos et al. 2006; Boehler et al. 2011). Besides, different effector molecules secreted by the host immune system in response to the engineered tissue further determine the biocompatibility of the material. Thus, all these factors

Anita Hansda and Sayan Mukherjee contributed equally to this work.

A. Hansda · G. Mukherjee (✉)
Immunology and Inflammation Research Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India
e-mail: gayatri.mukherjee@smst.iitkgp.ac.in

S. Mukherjee · K. Dixit · S. Dhara (✉)
Biomaterials and Tissue Engineering Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India
e-mail: sdhara@smst.iitkgp.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_3

together contribute to the overall success or failure of an engineered tissue to be integrated within the host.

In modern medicine, organ or tissue grafting is one of the most advanced practices to overcome severe injury or trauma challenges. Grafting in humans can be classified into following groups: (1) autograft or autologous graft is where organs are obtained from the patient's own body; (2) in the case of allograft or heterologous graft, organs are obtained from another donor; and (3) in xenograft or heterograft, the grafting organs are obtained from other species, such as pigs or primates. Allogeneic organ or tissue transplantation is potentially lifesaving, but some serious complications might happen to the immunogenic reaction of the recipient body. Even after prolonged immunosuppression, immune-mediated rejection occurs and limits the life span of transplanted allografts, and immune cells obtained from donated tissue can trigger graft-versus-host disease (GVHD), threatening the recipient's life. To overcome these grafting-related challenges, the concept of engineered tissue has been introduced. To minimize the chance of rejection of the grafts containing biomaterials, these can be fabricated with different bioactive molecules (i.e., amino acids, growth factors, enzymes, hydroxyapatite).

The process of implanting bioactive scaffolds can stimulate the immune reaction, leading to inflammation. This initial inflammatory response to the implantation-mediated injury can lead to tissue damage, hindering regeneration (Fig. 3.1) (Julier et al. 2017). Therefore, the development of immunomodulatory strategies that harness the beneficial aspects of the immune response while limiting the potentially deleterious side effects is necessary for the successful integration of engineered tissue and enhanced regeneration of endogenous tissue.

In this chapter, we detailed the different kinds of biomaterials used in tissue engineering, the role of the immune system in response to transplanted biomaterials, and the potential for biomaterial scaffolds to modulate immune signaling to create a pro-regenerative environment.

3.2 Biomaterials and Tissue Engineering

A biomaterial is a biologically inert or active component that can be introduced into a living organism with minimal chance of immune rejection. It is an integral component of tissue engineering derived from synthetic or natural precursors to regenerate biological function in damaged tissue. Based on physicochemical characteristics, biomaterials can be broadly classified into metallics, ceramics, and polymers (Fig. 3.2).

3.2.1 Metallic Biomaterials

Metallic biomaterials are generally used to develop implants providing load-bearing capabilities, especially in orthopedics and dentistry. Challenges with knee and hip joint replacement, or maxillofacial and cranial bone reconstruction can be resolved

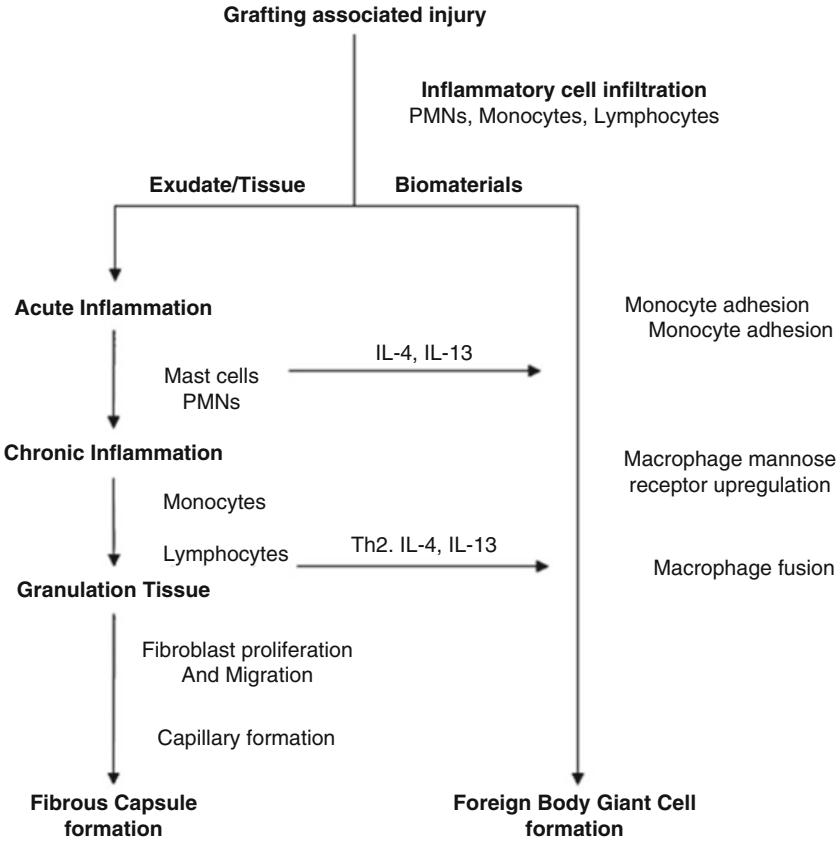


Fig. 3.1 Schematic diagram of immune reaction in graft associated injury

using metallic implants. Alloys like stainless steel (304, 316, 316 L), CoCr, and Ti_6Al_4V are the most frequently explored load-bearing implants. But many complications arise from metallic implants due to compromised biocompatibility and a relatively long time for tissue response (Vroman and Tighzert 2009). Moreover, during implantation, these materials may cause localized wounds at the implant site, stress shielding, stress-induced corrosion, release of metal ions, and debris particle generation that would trigger an allergic response, osteolysis, and eventually fibrotic encapsulation and immune rejection. Therefore, the metal-based implant surface must be modulated to minimize the chance of rejection and impart adequate host response through augmenting bioactivity.

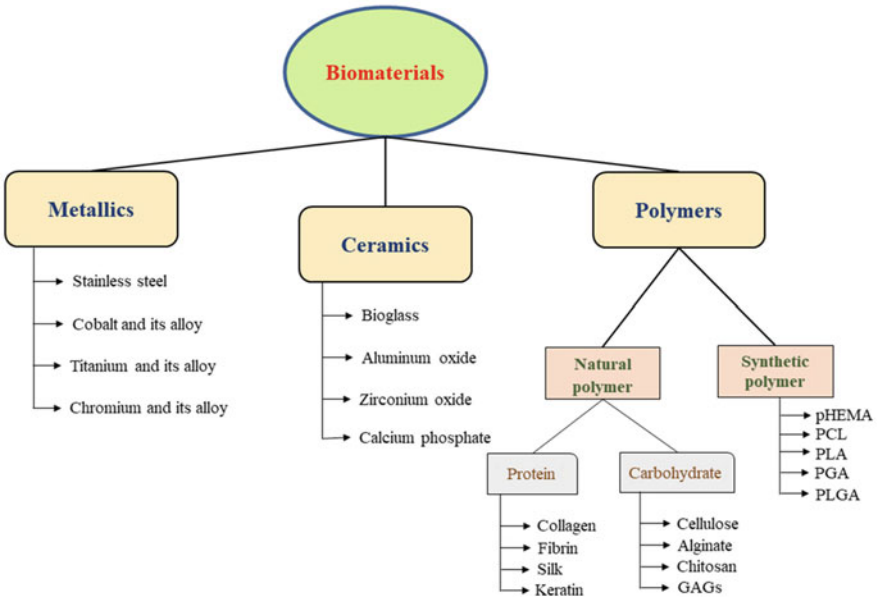


Fig. 3.2 Schematic diagrams showing the general classification of biomaterials

3.2.2 Ceramic Biomaterials

Similarly, ceramic biomaterials are also mostly used in orthopedic and dental reconstructions. They are also widely used as bone cavity fillers. These materials are characterized by high hardness, high temperature resistance, and high fragility. Surface modifications using bioactive cues (e.g., hydroxyapatite, peptides) can make these more suitable host responses thereby minimizing immune rejection. Bioglass, aluminum oxide, zirconium oxide, and calcium phosphate are widely explored as osteoconductive ceramics (Gao et al. 2014). Debris generation during relative motion or catastrophic failure may trigger an immune response as well.

3.2.3 Polymer Biomaterials

Irrespective of the above-discussed advantages of metallics and ceramic materials, they may cause localized inflammatory responses by the generation of particles, stress-induced osteolysis, and high ion flux on the surrounding tissues. These shortcomings can be addressed by polymer-based biomaterials, which have well-characterized biomechanical properties. Polymer biomaterials can be categorized into synthetic and natural based on their source materials.

Synthetic polymers are chemically synthesized organics having monomeric units functionalized for biological responses. Depending on their chemical bonding nature, degradation of these materials would differ in terms of erosion, enzymatic

degradation, and hydrolysis, whereas some are non-biodegradable as well mainly due to high hydrophobicity with the absence of any reactive molecular epitopes under in vivo conditions. Though biodegradable polymers have advantages like degradation over time through enzymatic reactions, biodegraded elements should not trigger any enzymatic or toxic responses. It may be favorable if the degraded products clean away through Krebs cycle. Poly(caprolactone) (PCL), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(lactic-co-glycolic acid) (PLGA) are the most common biodegradable synthetic polymers used in scaffold construction and drug delivery applications (Gao et al. 2014; Stratton et al. 2016). Whereas non-biodegradable polymers like polyhydroxyethylmethacrylate (pHEMA) and high-density polyethylene (HDPE) are commonly used for ocular lens and non-load-bearing implants, respectively.

However, tissue regeneration is hampered due to the unavailability of bioactive cues. These polymers are modified too through grafting bioactive peptides to impart adequate host response. Besides these, upon degradation of synthetic polymer, they release chemical residues that can further induce low pH at tissue microenvironment, tissue necrosis, and even immune response (Nair and Tang 2017). To overcome such limitations, synthetic polymers were further blended with natural polymers and or bioactive glass.

Natural polymers are broadly divided into polysaccharides (such as agarose, alginate, chitosan, hyaluronic acid, and glycosaminoglycan [GAG]) and proteins (such as collagen, fibrin, silk, and keratin). One of the polysaccharide-based natural polymers, alginate, has turned up as a hemostatic agent, betters wound dressing, and is known for controlled drug release. Similarly, glycosaminoglycans (GAGs) promote tissue growth and help in maintaining homeostasis and in extracellular matrix (ECM) construction (Croisier and Jérôme 2013). Another ECM component, collagen, has been explored in multidomain applications like a drug, gene, and protein delivery matrix and burn/wound sponges (Gasperini et al. 2014). Thus, natural biopolymers are more immunocompatible due to their anti-inflammatory and antioxidant properties.

3.3 Immunological Response to Biomaterials Used in Tissue Engineering

The immune system is a complex network of biological processes that protects and maintains homeostasis in the host, and plays a predominant role in successful integration of biomaterial. It is made up of two parts: the innate, and the adaptive immune systems (Lewis et al. 2014). Biomaterials once implanted come in contact with cells, blood, and surrounding tissues of the host evoking an immune response due to the foreign nature of the implanted material. Following the implantation, the initial reaction involves the engagement of innate immune cells at the site of implantation, and also around the implanted biomaterial itself. Concomitantly, activation of the initial steps of the adaptive immune system in conjunction with

innate immune cells can either positively or negatively affect the tissue regeneration and integration of the implanted biomaterial.

Following the surgical implantation of a biomaterial, the tissue injury leads to a set of biological processes: homeostasis, inflammation, and fibrotic tissue formation (Rodrigues et al. 2019).

- **Homeostasis:** This occurs immediately after tissue injury. Tissue injury leads to exposure of endothelial tissues along with the underlying vascular basement membrane leading to platelet factors adhesion, activation, and finally the coagulation cascade. This slows down the further hemorrhage.
- **Inflammation:** Simultaneously, tissue injury causes the secretion of different cytokines and chemokines by damaged cells, initiating acute inflammation. During this phase, an influx of neutrophils and macrophages occurs and becomes activated. These activated cells with phagocytic properties secrete proteolytic enzymes and degrade the cell debris and extracellular matrices (ECMs). Moreover, they engulf, process, and present any foreign peptide, and present the antigenic peptide to thymocytes (T-cells) involved in adaptive immunity.
- **Fibrotic tissue formation:** At later stages of the innate immune response, fibroblast and endothelial cell proliferation conduct the way to granulation tissue formation. Fibroblast proliferation conducts the synthesis of collagen and proteoglycans that remodel the ECM. Meanwhile, endothelial cells lead the way for new blood vessel formation for nutrient supply (Corradetti et al. 2017).

3.3.1 Innate Immune Response to Biomaterials

The immune system through some physicochemical reactions has advanced itself for identifying harmful microbes or any non-self or foreign agents for protecting the body from invaders. These foreign agents consist of bioactive materials, nanoparticles, nanocomposites, or biomimetic materials in the field of tissue engineering (Matzinger 2002). Pattern-recognition receptors (PRRs) are one of the vital machinery of innate immunity that provides swift, but not as many, specific responses. Pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) are detected through the PRRs present on the antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs) (Cooper and Hausman 2016). Macrophages provide the initial line of defense against any foreign material, and, unlike T-cells, possess remarkable plasticity upon activation, and they are the first population among mononuclear cells to be recruited in response to the biomaterial. They display heterogeneous phenotypes ranging from M1 (pro-inflammatory) to M2 (anti-inflammatory) macrophages (Mantovani et al. 2004; Gordon and Taylor 2005). Prominently, M1 macrophages are activated by well-known pro-inflammatory signals such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS) and produce characteristic pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12, IL-23, and tumor necrosis factor alpha (TNF-α), having low production of anti-inflammatory cytokines such as IL-10. They

also produce high levels of reactive oxygen species (ROS). In contrast, M2 cells are activated by molecular cues such as IL-4, IL-13, and IL-10. They induce high IL-10 secretion, display increased scavenger molecule expression, produce ornithine and polyamines in place of ROS, and engage in polarized anti-inflammatory reactions (Gordon 2003; Mosser and Edwards 2008). The composition of biomaterial determines the induction of specific phenotypes of macrophages and decides the successful integration or rejection of the material.

Another antigen-presenting entity, the dendritic cell (DC), also responds to external signals such as foreign pathogens, and mounts an adaptive immune response. Thus, these DCs recognize PRRs on implanted biomaterial in an innate response, get activated, and present to T-cells, resulting in a T-cell stimulation that is involved in the adaptive response. Moreover, DCs may recognize carbohydrate motifs and other toll-like receptor (TLR) ligands on biomaterial surfaces through TLRs, and induce an immune response (Szeto and Lavik 2016). Thus, the biomaterial component influences the involvement of different types of immune cells during an immune response.

3.3.2 Adaptive Immune Response to Biomaterials

The bioactive particles must reach the lymph nodes and tissues that are responsible for adaptive immune response through B- and T-cell interaction with APCs. The bioavailability of biomaterials in the lymph nodes and the drainage through lymphatics entirely rely on the size of the particles (Reddy et al. 2006). Macrophage and dendritic cells get drawn to the site of transplantation due to injury and the presence of foreign material. Just like invading pathogens, particles, ions, or cellular debris of the implanted material are also recognized, internalized, processed, and presented by macrophages and DCs to T-cells through the major histocompatibility complex (MHC) molecules (Anderson et al. 2008). This in turn causes the activation of T-cells.

A subset of activated CD4+ T-cells, termed helper T-cells, releases cytokines that regulate inflammation. These helper T-cells can be triggered to display pro-inflammatory (Th1) or anti-inflammatory (Th2) secretory contours like macrophages (Shanley et al. 2021). A Th1-mediated immune response is commonly associated with a pro-inflammatory response to xenogeneic materials, materials with cytotoxic deprivation products, and/or non-degradable synthetic materials, while Th2 responses naturally support better acceptance of the implant (Badylak 2007). Th2 cells are also involved in interaction with macrophages and are allied with an anti-inflammatory M2 macrophage phenotype (Mills and Ley 2014). Thus, the combined contribution of these immune cells involving both innate and adaptive immune responses influences the integration of the implanted materials into tissues.

3.4 Need for Graft and Their Interaction with the Recipient's Immune System

For the last few decades, grafting has become common in organs like skin, bone, teeth, heart valves, and blood vessels. Besides allografting, artificial organs or implant grafting has become a more advanced and immunologically safe practice in current days. Unfortunately, the need for organ donors is much greater than the number of people who donate. The scarcity of donors is an important cause of moving toward artificial implants from allografting.

After successful engraftment, the recipient may experience some post-grafting consequences like infection or inflammation. Interaction of blood with the grafted material initiates the provisional matrix formation. This matrix formation during the early stage of grafting occurs by adsorption of protein to the graft surface through blood–material interactions, commencing thrombus at the tissue–graft interface. Also, this matrix formation is characterized by fibrin predominance and protein adsorption, which are closely correlated in the perspective of their mechanistic responses (Luttikhuizen et al. 2006). In brief, the injury to blood vessels and connective tissues during or post engraftment activates the innate immunity, consequently activating the extrinsic and intrinsic coagulation systems to initiate thrombus formation. These protein cascades of the complement system, the fibrinolytic system, and the kinin-generating system are intricate in the circumstances of protein adsorption and desorption, also known as the Vroman Effect (Tsai et al. 2004) (Fig. 3.3a). Moreover, this temporary matrix helps to induce foreign body reaction (FBR) by providing biochemical, cellular, and structural ingredients and provides a bioactive-agent-rich microenvironment full of cytokines, chemo-attractants, mitogens, or growth factors, activating and inhibiting reactions harmonizing macrophage activity for inflammatory response (Zdolsek et al. 2007). Post matrix formation, both acute and chronic inflammatory responses occurred due to the graft's interactions with blood. The polymorphonucleocytes (PMNs) or neutrophils, localized in the tissue or organ into which the implant is grafted, decide the degree of the inflammatory response. The acute inflammatory response with grafted biomaterials is usually determined quickly, usually in less than 1 week, dependent on the extent of the injury at the implant site. At the injured region, monocytes naturally activate "M1" macrophages, consequently introducing phagocytic response through the discharge of proteolytic enzymes and reactive oxygen species (ROS) (Fig. 3.3b). During this time, activated PMNs also secrete IL-1β, IL-6, IL-8, TNF-α, monocyte chemotactic protein (MCP-1), and macrophage inflammatory protein (MIP-1β) like chemokines, which act as chemo-attractants and eventually activate macrophages, monocytes, lymphocytes, and immature DCs (Christo et al. 2015; Vasconcelos et al. 2019). In due course, macrophages adhered to grafts ultimately evolve into an alternatively activated or "M2" phenotype, same as in wound-healing events. These types of macrophages are characterized by releasing anti-inflammatory cytokines like IL-10 and they have enhanced tissue-remodeling capacity. The overlying actions of the M1 to M2 phenotype switching conduct the fusion of M2 macrophages forming a foreign body giant cell (FBGC) on the

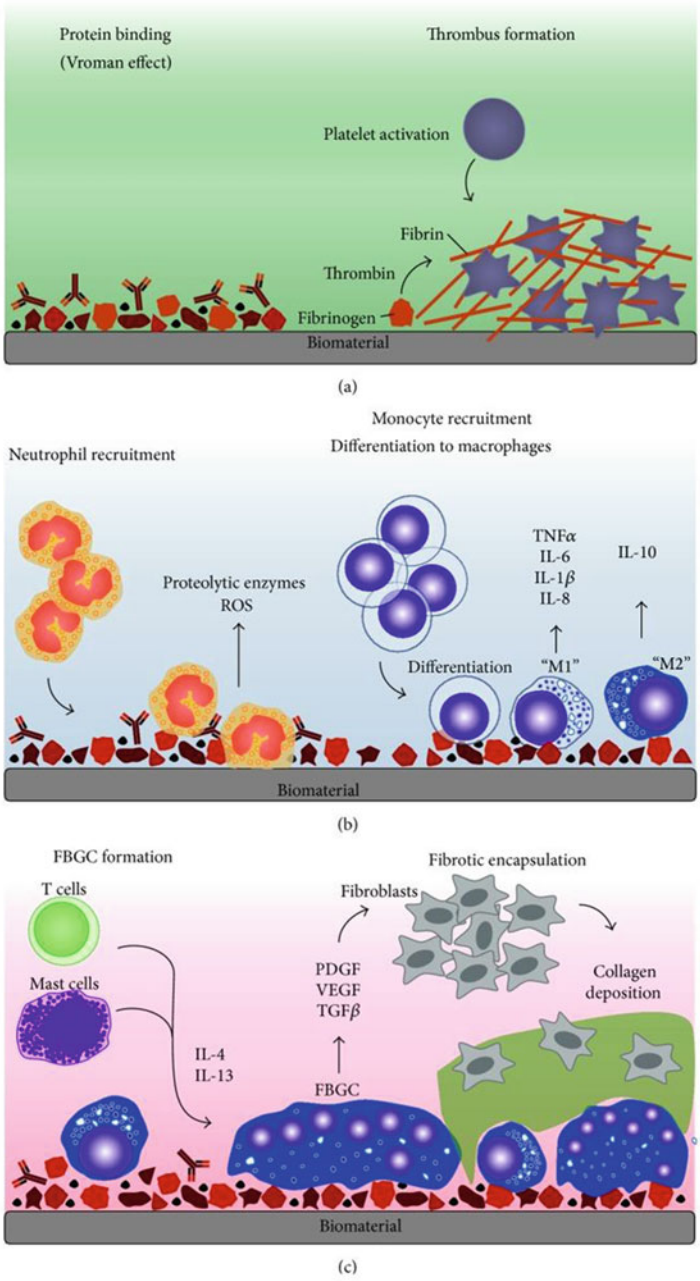


Fig. 3.3 Foreign body response after introducing biomaterials into the body. This immune response pursues an acute inflammatory reaction by means of tissue remodeling and vascularization and eventually fibrotic encapsulation of implant. (**a**) Plasma proteins are instantly adsorbed and induce the thrombus formation, initiating a provisional matrix formation rich in leukocyte-recruiting factors. (**b**) Neutrophils are employed to the niche of implant and introduced to biomaterial degradation. Monocytes are activated into macrophages, and M1 and M2 phenotypical

engrafted surface, enhancing their phagocytic capacity. Chronic inflammation is characterized by the presence of monocytes and lymphocytes in the grafted region. This chronic inflammatory response to grafts is typical to a brief extent and is limited to the implant site. The FBGC formation happens during this chronic inflammatory response at the biomaterial interface (Christo et al. 2015).

Furthermore, mast cell activation induces the degranulation near engrafted region consistently through the secretion of both anti- and pro-inflammatory cytokines. On top of this, different profibrotic and angiogenic factors, including vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β), are secreted for enhancing the degranulation process (Nakayama et al. 2004; Ribatti and Crivellato 2012). This process in turn results in the isolation of engrafted biomaterial from the recipient tissue by a phenomenon called fibrotic encapsulation through releasing profibrogenic factors such as platelet-derived growth factor (PDGF), VEGF, and TGF-β that further recruit fibroblasts to the engrafted region (Grainger 2013) (Fig. 3.3c). Those recruited and activated fibroblasts deposit collagen to form encapsulation around the implanted biomaterials in an attempt to repair the engraftment associated with tissue damage (Diegelmann and Evans 2004).

3.5 Modulation of the Host Immune Response by Biomaterials

The triumph of biomaterial grafting through the tissue-engineering approach is highly determined by the implementation of host defense mechanisms. Failure of the interactions between implantable scaffolds and cellular bioactive molecules can cause rejection of implant and medical consequences. Biomaterials can be used in various arenas of tissue engineering significantly with appropriate surface modification approaches as discussed below.

3.5.1 Immune Pathways Modulated by Foreign Molecules and Biomaterials

The inflammasome is an important component of immune activation. It is an intricate part of cytoplasmic proteins that trigger the caspase-induced IL-1 release, eventually entangled in introducing inflammatory responses (Martinon et al. 2009). Fascinatingly, immune activation associated with inflammasomes is often allied with adjuvants, chemical entities that usually surge the immunogenicity or effectiveness by adding them to vaccine formulations. Recently, in the advancement of

Fig. 3.3 (continued) differentiation occurred that finally drained their phagocytic activity. (**c**) T-cells and mast cells like adaptive leukocytes were employed and induce cytokine secretion that ultimately formed foreign body giant cell (FBGC). FBGCs secrete fibroblast-recruiting factors and activate them to deposit collagen, creating a capsule surrounding the biomaterial to avoid the host tissue interaction. (Copyright © 2015 Susan N. Christo et al.)

immunotherapies and vaccines, several microparticles- and nanoparticles-based technologies are evolving (Eisenbarth et al. 2008). Synthetic polymers having different shapes, sizes, and topographic characteristics have been used in tissue engineering. These different structural parameters can promote or inhibit inflammasome activations by generating a tunable bioactive construct.

In specific cases, the repeating patterns of polymer chains and hydrophobic entities activate immune reactions, generally resembling microbial polysaccharides (Seong and Matzinger 2004). Some researchers have discovered that immune pathways can be activated by any bioactive materials even when other immunostimulatory signals are not present, and the materials' characteristic features might modulate the degree of the immune response. For instance, DCs cultured on alginate, chitosan, or hyaluronic acid like natural polymers, and PLGA like synthetic polymer-derived thin films enhance the expression of some surface markers. These surface markers include CD40 (maturation marker), major histocompatibility class II (MHC II) complexes, and CD80 and CD86 co-stimulatory markers (Babensee and Paranjpe 2005). TLR-agonist-like inflammatory signal conjugated silica, PLGA, or polystyrene-based particle systems and the response of DC were evaluated in some other studies (Demento et al. 2009). The result has shown synergistic enhancement of DC activation in treated particles than the polymer or the TLR agonist alone. These exposures have impelled us to understand the impact of physicochemical characteristics on intrinsic immune response and how these interactions occur.

In recent years, researchers are mining the knowledge about the properties of bioactive materials used in vaccines and immuno-therapeutics, triggering innate immune reactions. Many researchers recently established some ideas (Vishwakarma et al. 2016) to dodge the body's defense system by modulating the biomaterials-based systems, providing a valuable insight that should enrich the arena of tissue engineering, and regenerative medicine. Chemical modification of biomaterial surface through employing surface charge and hydrophilic functional groups is broadly used to tune its property. Hydrophilic chemical entities of biomaterials are essential for interacting with biological molecules (Morent et al. 2011). Hydrophilicity can also be mediated by possessing the surface charge of biomaterials, influencing significantly osteogenesis (Boyan et al. 1996). Surface roughness and definite chemical modification can influence the degree of immunogenicity and immune interactions (Chen et al. 2017). The hydrophobic part of biomaterials has modulated the immune system by recognizing them as foreign particles. This property can lead to elimination through triggering PRRs. Bioactive hydrophobic particles have shown enhanced expression of TNF- or IFN-like pro-inflammatory cytokines in the tissue-engineering application. Combating the immunomodulatory responses of hydrophobic surfaces, polyethylene oxide (PEO) and polyethylene glycol (PEG)-like hydrophilic molecules are frequently used as transport systems and tissue-engineering scaffolds through reducing protein absorption (Peppas et al. 2006).

3.5.2 Macrophage Polarization-Mediated Immune Modulation

Macrophagic phenotypic modification epitomizes a fascinating scope in tissue engineering. Inducing stem cell migration, T-cell activation, enhancing angiogenesis, and remodeling extracellular matrix, macrophages help in the inflammatory response in recovering from injury progressions. This functionality of macrophages is initially modulated through phenotype plasticity. Based on the nearby environmental signals, the phenotypic malleability switches the same macrophage between pro-inflammatory (M1) and pro-healing (M2) states. The macrophages arriving first at the site of injury reveal an inflammatory phenotype recognized as M1. By producing inducible nitric oxide synthase (iNOS), IL-12, and TNF-like pro-inflammatory factors, these M1 macrophages engulf foreign bodies and injured cells. These secreted pro-inflammatory factors endorse inflammation and employ lymphocytes tangled in activating adaptive immunity. After persisting for 2–3 days at the implant or injury site, functions of M1 macrophages polarize toward the M2 phenotype, which helps in repairing tissue damage and angiogenesis (Spiller et al. 2015). Functionally, the M1 phenotypes are entirely dissimilar from the M2 phenotypes. The expression of key genetic factors involved in wound healing, cytokine secretion, and growth factors is induced by the M2 phenotype, stimulating cellular propagation and depositing extracellular matrix for providing tissue growth (Vishwakarma et al. 2016). Macrophages also impact on producing specific foreign body reactions in response to implantation. Isolating those implants from the surrounding niche of the body, macrophages surround the material by a fibrotic capsule. M1 and M2 macrophages have an immunomodulatory prospect, responding to tissue-engineering strategies (Mosser and Edwards 2008) (Fig. 3.4).

Immune modulation of the macrophage population has been studied in different disease models to aid recovery through a tissue-engineering approach. Myocarditis is an inflammation of the myocardium, commonly triggered by a viral infection. In this disease, macrophages help in the progression of eliminating infected myocardium, by TGF-β1 (Gong et al. 2012), endorsing the expression of extracellular matrix (ECM) and inhibiting the expression of matrix metalloproteinases (MMPs) that initiate post-infarction fibrosis (Nakamura et al. 2016). In the case of viral myocarditis infection, microRNA-155 silencing modulates macrophagic polarization by inducing M2-like macrophage levels and declining levels of M1-like macrophages, signifying a conceivable treatment option (Zhang et al. 2016). Bone tissue damage is repaired through a multifaceted process including inflammation, soft and hard callus construction, and finally remodeling (Schindeler et al. 2008). Immediately after bone injury, through cytokine signals macrophages infiltrate into the wound site and initiate tissue restoration by prompting an inflammatory cascade (Schindeler et al. 2008). Modulating the physical, mechanical, and chemical properties of smart orthopedic scaffolds can be manipulated in such a way that can polarize the macrophage toward the M2 phenotype (Anderson et al. 2008). A study has shown polarization of J774 macrophages toward M2 phenotype through a combination of surface alteration using both divalent cations and nanostructured

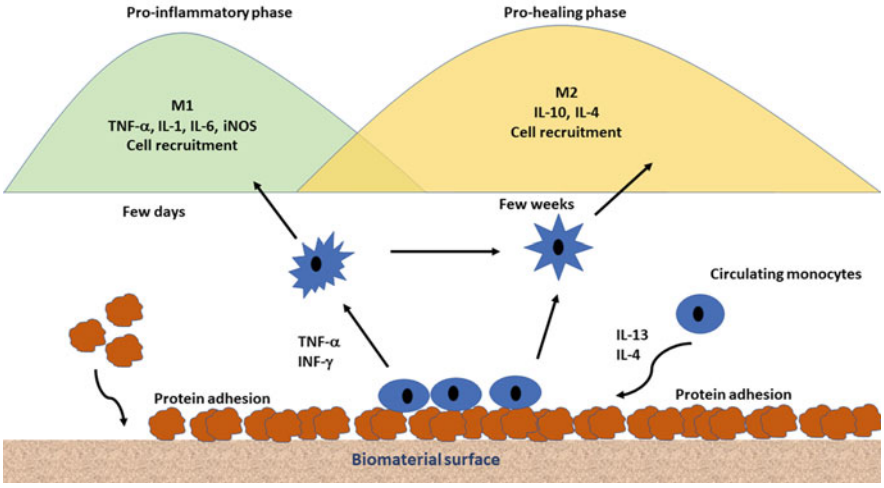


Fig. 3.4 Schematic diagram presentation factors intricated in macrophage activation as well as polarization into M1 and M2 phenotypes that secrete specific cytokines and chemokines to regulate the type of succeeding inflammatory response

titanium (Ti). That study concluded the reduction of the inflammatory response by ion-mediated surface modification (Lee et al. 2016).

3.5.3 Immune Responses to Nanomaterials in Tissue Engineering

Advanced techniques in the field of nanotechnology and fabrication have evolved the synthesis of nanomaterials, enhancing tissue-engineering capability for its good biocompatibility.

Nanoparticles, biomimetic nanopatterned surface, nanoscale porous scaffolds, carbon nanodots, nanowires, nanofibers, and nanotubes are (Biswas et al. 2012) having properties like higher surface area compared to size, enhanced cell proliferation, porosity-dependent diffusion, increased protein adsorption, etc. These properties make them a game-changer in the scope of tissue engineering (Drury and Mooney 2003; Ramiro-Gutiérrez et al. 2014). Dendritic cells can also facilitate the immune reaction, which afterward leads to acceptance or rejection of implants fabricated with the specific nanomaterials (Chan and Mooney 2008). The extent of nanoparticle-mediated immune response is dependent on particle size. The relation between hydrodynamic size and the inflammatory response with polyacrylic acid-coated gold nanoparticles evidenced the particle size-dependent immune response (Deng et al. 2011). The fibrinogen-induced stimulation of monocyte Mac-1 receptor has been upregulated by the particles less than 20 nm in size. Mac-1 activation eventually upregulates the nuclear factor (NF) mediated downstream expression of inflammatory cytokines, but when nanoparticles have a size greater than 20 nm, this

phenomenon becomes absent. Some in vivo experiments also have been validating the nanoparticle size dependency of inflammatory response. For instance, PLGA nanoparticles have shown size-dependent conscription of polymorphonucleocytes (PMNs) (Dailey et al. 2006).

In a recent study, researchers have shown shape-dependent DC activation, cytokine expression, and ROS production in 70–150 nm long titanium oxide nanotubes of 10–15 nm diameter (Gultepe et al. 2010). Specifically, among all nanostructures the most efficient immune modulation has been shown in the case of nanotubes, representing the shape dependency on immunogenicity. Nanoporous biomaterial scaffolds also could escape inflammatory responses by mimicking natural matrix architecture. In the bone tissue-engineering field, one study explained the reduced immune cell activation by nanoporous silica and hydroxyapatite in comparison to cancellous bone implants (Abshagen et al. 2009). Promoting biomaterial incorporation and controlling inflammatory responses, nanopatterned surfaces could be used through regulating fibrotic cell adhesion. Nanoridge-patterned polyacrylamide hydrogels revealed abridged adhesion of macrophages after immune interaction (Takahashi et al. 2012). Nanofibers and nanowires are also other categories of nanostructures having nanoscale surface structure and interrelated nanopores making suitable scaffolds for immune modulations. Tuning the material surface chemistry, functionalization, and mechanical strength of biopolymers, the immunological efficiency and compatibility of electrospunned scaffolds with the extracellular matrix can be modulated. Among these versatile nanomaterials, the nanofiber is one of the important components that help to obtain substantial anti-inflammatory features. Researchers have proven the immune-modulatory response of nanofibers depends on the morphology, diameter, pore size, and alignment. Macrophagic pro-inflammatory cytokine secretion can be downregulated by the reduction in fiber diameter, as has been proven in vitro (Saino et al. 2011). Attenuating the immune activation, inflammatory response, and implant-mediated fibrosis, biomaterial's surface nanoarchitecture can temper the mechanical stiffness of scaffolds for corresponding to the tissue-engineered microenvironment.

3.5.4 Immunomodulation by Stem Cells Used in Engineered Tissue

Stem cells have the ability for self-renewal, differentiation, and regeneration. They also create an immunoregulatory environment in response to tissue injury (Mahla 2016). Mesenchymal stem cells (MSCs), a type of adult stem cells, exert their pro-inflammatory and anti-inflammatory properties via secretion of different chemokines and cytokines by immune cells' involvement in innate and adaptive immunity (Kyurkchiev 2014; Mahla 2016). This cell population exhibits an inhibitory effect through the expression of immunosuppressive molecules like Fas Ligand (Fas L) and programmed death-ligand (PD-L1). Additionally, TGF-β expression promotes regulatory T (Tregs) cell induction (Engela et al. 2013). MSCs suppress the cell's proliferation by increasing the IL-10 expression and downregulating

TNF-α expression (Klyushnenkova et al. 2005). In addition, MSCs also reportedly reduce the M1 phenotype and polarize M1 toward M2 (Zheng et al. 2018).

MSCs' regeneration potential was exploited for tissue regenerative strategies. MSCs have been used for treating cartilage, bone, skin, and nerve defects (Li et al. 2019). As mentioned above, biomaterial imbibes intrinsic anti-inflammatory properties too. Valles et al. reported that biomaterial topographical cues have very well modulated the MSC response; according to the report, the 3D-cultured MSCs have shown decrement in macrophage response via IL-6 expression compared to 2D monolayer cultures (Vallés et al. 2015). Likewise, other biomaterial surface properties have made a great impact on MSC immunomodulation and accelerating tissue repair. To mimic the complex and well-coordinated interaction of the biomaterial with the host cells Caires et al. designed an ex-vivo model. On this model, chitosan scaffolds were co-cultured with MSC and fibroblast and cultured with macrophages; interestingly macrophages promoted the fibroblast recruitment and no effect was marked for MSCs (Caires et al. 2018). So, from this ex vivo model, researchers inferred that immune cells' crosstalk with resident cells is highly regulated by the different cell types involved, and also the timings of each cell encounter play a crucial role.

3.6 Future Perspectives and Conclusion

The interfaces between grafted biomaterials and the host tissue were illuminated through diversified viewpoints in this last decade. The ideal outcome depends on the immunomodulatory response exerted by different immune cells on implanted material. There have been continuous efforts made to understand the involvement of host immune cells in response to biomaterial, and this affects the way biomaterials are designed and manufactured. Not only exploring the construction of different biomaterials, but scientists also have been designing novel materials considering the impact of their physicochemical properties on bioinspired and biomimetic factors to reduce the chance of immune rejection. In the clinical field, allogeneic transplantation of bone marrow or umbilical cord-derived MSCs has been evaluated in cartilage defect, spinal cord injury, corneal injuries, cardiomyopathy, intervertebral disc (IVD) degeneration, and bone defect with a minimal chance of immune rejection. Worldwide researchers have also studied different biomaterials for regenerative medicine tissue reconstruction applications. Further, allogeneic stem cell transplantation will enlighten the future of tissue engineering by reducing the occurrence of inflammatory reactions. Moreover, a human tissue-derived decellularized extracellular matrix enriched with collagen has become a game-changer in the field of regenerative medicine. It is not so far when in situ tissue engineering using gene therapy and immunotherapy will promote the healing and regeneration of tissues within the body in near future (Han et al. 2020). The field of tissue engineering may resolve serious problems like autoimmune diseases or cancer using biomaterial-based gene therapy and immunotherapy. Though tissue engineering and biomaterial research have grown in number this decade, in the future we still

have an extended journey to blend tissue engineering with clinics, with an in-depth understanding of immunobiology to increase the successful integration of engineered construct.

References

Abshagen K, Schrodi I, Gerber T, Vollmar B (2009) In vivo analysis of biocompatibility and vascularization of the synthetic bone grafting substitute NanoBone®. J Biomed Mater Res A 91: 557–566. https://doi.org/10.1002/jbm.a.32237

Anderson JM, Rodriguez A, Chang DT (2008) Foreign body reaction to biomaterials. Semin Immunol 20:86–100. https://doi.org/10.1016/j.smim.2007.11.004

Mikos AG, Herring SW, Ochareon P, Elisseeff J, Lu HH, Kandel R, Schoen FJ, Toner M, Mooney D, Atala A, Van Dyke ME, David Kaplan GV-N (2006) Engineering complex tissues. Tissue Eng 12:3307–3339. https://doi.org/10.1089/ten.2006.12.3307

Babensee JE, Paranjpe A (2005) Differential levels of dendritic cell maturation on different biomaterials used in combination products. J Biomed Mater Res A 74:503–510. https://doi.org/10.1002/jbm.a.30429

Badylak SF (2007) The extracellular matrix as a biologic scaffold material. Biomaterials 28:3587–3593. https://doi.org/10.1016/j.biomaterials.2007.04.043

Biswas A, Bayer IS, Biris AS et al (2012) Advances in top-down and bottom-up surface nanofabrication: techniques, applications & future prospects. Adv Colloid Interf Sci 170:2–27. https://doi.org/10.1016/j.cis.2011.11.001

Boehler RM, Graham JG, Shea LD (2011) Tissue engineering tools for modulation of the immune response. BioTechniques 51:239–254. https://doi.org/10.2144/000113754

Boyan BD, Hummert TW, Dean DD, Schwartz Z (1996) Role of material surfaces in regulating bone and cartilage cell response. Biomaterials 17:137–146. https://doi.org/10.1016/0142-9612(96)85758-9

Caires HR, Barros da Silva P, Barbosa MA, Almeida CR (2018) A co-culture system with three different primary human cell populations reveals that biomaterials and MSC modulate macrophage-driven fibroblast recruitment. J Tissue Eng Regen Med 12:e1433–e1440. https://doi.org/10.1002/term.2560

Chan G, Mooney DJ (2008) New materials for tissue engineering: towards greater control over the biological response. Trends Biotechnol 26:382–392. https://doi.org/10.1016/j.tibtech.2008.03.011

Chen Z, Bachhuka A, Han S et al (2017) Tuning chemistry and topography of nanoengineered surfaces to manipulate immune response for bone regeneration applications. ACS Nano 11: 4494–4506. https://doi.org/10.1021/acsnano.6b07808

Christo SN, Diener KR, Bachhuka A et al (2015) Innate immunity and biomaterials at the nexus: friends or foes. Biomed Res Int 2015:342304. https://doi.org/10.1155/2015/342304

Corradetti B, Scarritt ME, Londono R, Badylak SF (2017) The immune response to implanted materials and devices. Springer US

Croisier F, Jérôme C (2013) Chitosan-based biomaterials for tissue engineering. Eur Polym J 49: 780–792. https://doi.org/10.1016/j.eurpolymj.2012.12.009

Dailey LA, Jekel N, Fink L et al (2006) Investigation of the proinflammatory potential of biodegradable nanoparticle drug delivery systems in the lung. Toxicol Appl Pharmacol 215: 100–108. https://doi.org/10.1016/j.taap.2006.01.016

Demento SL, Eisenbarth SC, Foellmer HG et al (2009) Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy. Vaccine 27:3013–3021. https://doi.org/10.1016/j.vaccine.2009.03.034

Deng ZJ, Liang M, Monteiro M et al (2011) Nanoparticle-induced unfolding of fibrinogen promotes Mac-1 receptor activation and inflammation. Nat Nanotechnol 6:39–44. https://doi.org/10.1038/nnano.2010.250

Diegelmann RF, Evans MC (2004) Departments of Biochemistry, Anatomy, Emergency Medicine and Virginia Commonwealth University, Richmond Virginia. Front Biosci:283–289

Drury JL, Mooney DJ (2003) Hydrogels for tissue engineering: scaffold design variables and applications. Biomaterials 24:4337–4351. https://doi.org/10.1016/S0142-9612(03)00340-5

Eisenbarth SC, Colegio OR, O'Connor W et al (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 453:1122–1126. https://doi.org/10.1038/nature06939

Engela AU, Hoogduijn MJ, Boer K et al (2013) Human adipose-tissue derived mesenchymal stem cells induce functional de-novo regulatory T cells with methylated FOXP3 gene DNA. Clin Exp Immunol 173:343–354. https://doi.org/10.1111/cei.12120

Gao C, Deng Y, Feng P et al (2014) Current progress in bioactive ceramic scaffolds for bone repair and regeneration. Int J Mol Sci 15:4714–4732. https://doi.org/10.3390/ijms15034714

Gasperini L, Mano JF, Reis RL (2014) Natural polymers for the microencapsulation of cells. J R Soc Interface 11. https://doi.org/10.1098/rsif.2014.0817

Cooper GM, Hausman RE (2016) Focus: microbiome: the cell: a molecular approach, seventh edition. Yale J Biol Med 89:424

Gong D, Shi W, Yi S et al (2012) TGFβ signaling plays a critical role in promoting alternative macrophage activation. BMC Immunol 13:31. https://doi.org/10.1186/1471-2172-13-31

Gordon S (2003) Alternative activation of macrophages. Nat Rev Immunol 3:23–35. https://doi.org/10.1038/nri978

Gordon S, Taylor PR (2005) Monocyte and macrophage heterogeneity. Nat Rev Immunol 5:953–964. https://doi.org/10.1038/nri1733

Grainger DW (2013) All charged up about implanted biomaterials. Nat Biotechnol 31:507–509. https://doi.org/10.1038/nbt.2600

Gultepe E, Nagesha D, Sridhar S, Amiji M (2010) Nanoporous inorganic membranes or coatings for sustained drug delivery in implantable devices. Adv Drug Deliv Rev 62:305–315. https://doi.org/10.1016/j.addr.2009.11.003

Han F, Wang J, Ding L et al (2020) Tissue engineering and regenerative medicine: achievements, future, and sustainability in Asia. Front Bioeng Biotechnol 8:1–35. https://doi.org/10.3389/fbioe.2020.00083

Julier Z, Park AJ, Briquez PS, Martino MM (2017) Promoting tissue regeneration by modulating the immune system. Acta Biomater 53:13–28. https://doi.org/10.1016/j.actbio.2017.01.056

Klyushnenkova E, Mosca JD, Zernetkina V et al (2005) T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci 12:47–57. https://doi.org/10.1007/s11373-004-8183-7

Kyurkchiev D (2014) Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells 6:552. https://doi.org/10.4252/wjsc.v6.i5.552

Langer RS, Vacanti JP (1999) Tissue engineering: the challenges ahead. Sci Am 280:86–89

Lee CH, Kim YJ, Jang JH, Park JW (2016) Modulating macrophage polarization with divalent cations in nanostructured titanium implant surfaces. Nanotechnology 27:85101. https://doi.org/10.1088/0957-4484/27/8/085101

Lewis JS, Roy K, Keselowsky BG (2014) Materials that harness and modulate the immune system. MRS Bull 39:25–34. https://doi.org/10.1557/mrs.2013.310

Li H, Shen S, Fu H et al (2019) Immunomodulatory functions of mesenchymal stem cells in tissue engineering. Stem Cells Int 2019:9671206. https://doi.org/10.1155/2019/9671206

Luttikhuizen DT, Harmsen MC, Van Luyn MJA (2006) Cellular and molecular dynamics in the foreign body reaction. Tissue Eng 12:1955–1970

Mahla R (2016) Adipose stem cells applications in regenerative medicine. Int J Cell Biol 3:1–24

Mantovani A, Sica A, Sozzani S et al (2004) The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol 25:677–686. https://doi.org/10.1016/j.it.2004.09.015

Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: guardians of the body. Annu Rev Immunol 27:229–265. https://doi.org/10.1146/annurev.immunol.021908.132715

Matzinger P (2002) The danger model: a renewed sense of self. Science (1979) 296:301–305. https://doi.org/10.1126/science.1071059

Mills CD, Ley K (2014) M1 and M2 macrophages: the chicken and the egg of immunity. J Innate Immun 6:716–726. https://doi.org/10.1159/000364945

Morent R, De Geyter N, Desmet T et al (2011) Plasma surface modification of biodegradable polymers: a review. Plasma Process Polym 8:171–190. https://doi.org/10.1002/ppap.201000153

Mosser DM, Edwards JP (2008) Exploring the full spectrum of macrophage activation. Nat Rev Immunol 8:958–969. https://doi.org/10.1038/nri2448

Nair A, Tang L (2017) Influence of scaffold design on host immune and stem cell responses. Semin Immunol 29:62–71. https://doi.org/10.1016/j.smim.2017.03.001

Nakamura K, Sano S, Fuster JJ et al (2016) Secreted frizzled-related protein 5 diminishes cardiac inflammation and protects the heart from ischemia/reperfusion injury. J Biol Chem 291:2566–2575. https://doi.org/10.1074/jbc.M115.693937

Nakayama T, Yao L, Tosato G (2004) Mast cell-derived angiopoietin-1 plays a critical role in the growth of plasma cell tumors. J Clin Investig 114:1317–1325. https://doi.org/10.1172/JCI22089

Peppas NA, Hilt JZ, Khademhosseini A, Langer R (2006) Hydrogels in biology and medicine: from molecular principles to bionanotechnology. Adv Mater 18:1345–1360. https://doi.org/10.1002/adma.200501612

Ramiro-Gutiérrez ML, Will J, Boccaccini AR, Díaz-Cuenca A (2014) Reticulated bioactive scaffolds with improved textural properties for bone tissue engineering: nanostructured surfaces and porosity. J Biomed Mater Res A 102:2982–2992. https://doi.org/10.1002/jbm.a.34968

Reddy ST, Rehor A, Schmoekel HG et al (2006) In vivo targeting of dendritic cells in lymph nodes with poly(propylene sulfide) nanoparticles. J Control Release 112:26–34. https://doi.org/10.1016/j.jconrel.2006.01.006

Ribatti D, Crivellato E (2012) Mast cells, angiogenesis, and tumour growth. Biochim Biophys Acta Mol basis Dis 1822:2–8. https://doi.org/10.1016/j.bbadis.2010.11.010

Rodrigues M, Kosaric N, Bonham CA, Gurtner GC (2019) Wound healing: a cellular perspective. Physiol Rev 99:665–706. https://doi.org/10.1152/physrev.00067.2017

Sadtler K, Singh A, Wolf MT et al (2016) Design, clinical translation and immunological response of biomaterials in regenerative medicine. Nat Rev Mater 1:16040. https://doi.org/10.1038/natrevmats.2016.40

Saino E, Focarete ML, Gualandi C et al (2011) Effect of electrospun fiber diameter and alignment on macrophage activation and secretion of proinflammatory cytokines and chemokines. Biomacromolecules 12:1900–1911. https://doi.org/10.1021/bm200248h

Schindeler A, McDonald MM, Bokko P, Little DG (2008) Bone remodeling during fracture repair: the cellular picture. Semin Cell Dev Biol 19:459–466. https://doi.org/10.1016/j.semcdb.2008.07.004

Seong SY, Matzinger P (2004) Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol 4:469–478. https://doi.org/10.1038/nri1372

Shanley LC, Mahon OR, Kelly DJ, Dunne A (2021) Harnessing the innate and adaptive immune system for tissue repair and regeneration: considering more than macrophages. Acta Biomater 133:208–221. https://doi.org/10.1016/j.actbio.2021.02.023

Spiller KL, Nassiri S, Witherel CE et al (2015) Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. Biomaterials 37:194–207. https://doi.org/10.1016/j.biomaterials.2014.10.017

Stratton S, Shelke NB, Hoshino K et al (2016) Bioactive polymeric scaffolds for tissue engineering. Bioactive Mater 1:93–108. https://doi.org/10.1016/j.bioactmat.2016.11.001

Szeto GL, Lavik EB (2016) Materials design at the interface of nanoparticles and innate immunity. J Mater Chem B 4:1610–1618. https://doi.org/10.1039/c5tb01825k

Takahashi M, Heo YJ, Shibata H, et al (2012) Nano-patterned hydrogel reduced inflammatory effects in subcutaneous tissue. In: Proceedings of the IEEE international conference on micro electro mechanical systems (MEMS), pp 973–976. https://doi.org/10.1109/MEMSYS.2012.6170195

Tsai WB, Shi Q, Grunkemeier JM et al (2004) Platelet adhesion to radiofrequency glow-discharge-deposited fluorocarbon polymers preadsorbed with selectively depleted plasmas show the primary role of fibrinogen. J Biomater Sci Polym Ed 15:817–840. https://doi.org/10.1163/1568562041271093

Vallés G, Bensiamar F, Crespo L et al (2015) Topographical cues regulate the crosstalk between MSCs and macrophages. Biomaterials 37:124–133. https://doi.org/10.1016/j.biomaterials.2014.10.028

Vasconcelos DP, Águas AP, Barbosa MA et al (2019) The inflammasome in host response to biomaterials: bridging inflammation and tissue regeneration. Acta Biomater 83:1–12. https://doi.org/10.1016/j.actbio.2018.09.056

Vishwakarma A, Bhise NS, Evangelista MB et al (2016) Engineering immunomodulatory biomaterials to tune the inflammatory response. Trends Biotechnol 34:470–482. https://doi.org/10.1016/j.tibtech.2016.03.009

Vroman I, Tighzert L (2009) Biodegradable polymers. Materials 2:307–344. https://doi.org/10.3390/ma2020307

Zdolsek J, Eaton JW, Tang L (2007) Histamine release and fibrinogen adsorption mediate acute inflammatory responses to biomaterial implants in humans. J Transl Med 5:1–6. https://doi.org/10.1186/1479-5876-5-31

Zhang Y, Zhang M, Li X et al (2016) Silencing microRNA-155 attenuates cardiac injury and dysfunction in viral myocarditis via promotion of M2 phenotype polarization of macrophages. Sci Rep 6:1–13. https://doi.org/10.1038/srep22613

Zheng YH, Deng YY, Lai W et al (2018) Effect of bone marrow mesenchymal stem cells on the polarization of macrophages. Mol Med Rep 17:4449–4459. https://doi.org/10.3892/mmr.2018.8457

Advances in Medical Imaging for Wound Repair and Regenerative Medicine

4

Biswajoy Ghosh and Jyotirmoy Chatterjee

4.1 Need for Imaging

A wound is a breach of the physiological continuity in the body that the body's biological defense attempts to bridge. To do so, the body has extremely intricate systems in place to categorically stop excessive bleeding, remove unwanted pathogens and dead cells, encourage the multiplication of new cells, and restore the lost tissue integrity. Unfortunately, in mammals, the restoration achieved does not usually reconstitute all original functions and this sub-optimally restored patch of healed tissue is called a scar (Clark 2021; Gurtner et al. 2008; Shah et al. 1992; Jiang et al. 2020). Regenerative medicine aims to achieve quality healing by assisting one or all the four phases of wound healing that could enable wound to heal faster with minimum scarring. The more challenging goal of regenerative medicine however is to fully restore all original functions of the tissue.

The socio-economic burden of wound management especially for chronic wounds today is immense. Chronic wounds are the wounds associated with diabetes, chronic kidney disease, hypertension and vascular inadequacies, and infections. A 2018 study on Medicare beneficiaries reported that total medical expenses for all wound types range between 28.1 and 96.8 billion USD including the cost of infection management (Nussbaum et al. 2018). Among all wound types, surgical wounds and diabetic foot ulcers (DFU) incurred the highest expenses. In 2020, Coronavirus Disease (COVID) dramatically disrupted health care systems

B. Ghosh (✉)
Indian Institute of Technology Kharagpur, Kharagpur, India

UiT—The Arctic University of Norway, Tromsø, Norway
e-mail: biswajoy.ghosh@uit.no

J. Chatterjee
Indian Institute of Technology Kharagpur, Kharagpur, India
e-mail: jchatterjee@smst.iitkgp.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_4

worldwide, including wound care systems (Sen 2021). Needless to say, wound care and management also put a significant social and psychological burden on the patients.

4.2 The Process of Skin Wound Healing

In response to cuts, punctures, bruises, and burns on the skin, our body immediately attempts to mend the damages. This process of repairing the wounds progresses over several days, depending on the degree of the injury (Gurtner et al. 2008). The body first stops the bleeding by constricting the blood vessels (vasoconstriction) and forming clots to plug the rupture—a process called hemostasis. As soon as the clot forms, the blood vessels dilate to enable fast disposal of white blood cells (WBCs) like neutrophils and macrophages to engulf foreign particles and germs that might have entered the body during wounding. The WBCs further recall many more healing molecules and substances that result in local swelling, redness, pain, and a temperature elevation—a process called inflammation. The arsenal of inflammatory molecules motivates the rebuilding of new cells of the skin in the next stage of proliferation. During proliferation, we observe mitotic multiplication of the cells that slowly uproots the clots from underneath (scab). As the outermost epidermal layer of cells fully forms, we achieve re-epithelialization. Besides the epidermal cells, the cells of the connective tissue (dermis)—fibroblasts—multiply and deposit the extracellular matrix (ECM) that fills up space between cells (Sidgwick et al. 2015). Once sufficient ECM forms, the connective tissue of the skin enters a stage of continuous matrix deposition and degradation equilibrium in the stage called remodeling, which results in a scar (Gurtner et al. 2008). The scar tissue, in many cases, is permanent and is a result of the fibrotic repair. The reasons scarring is not desirable are that (1) specialized functions like thermoregulation and moisturization by oil/sweat glands are permanently lost, (2) structures like the fat and hair follicles never form back, and (3) the scar may progress to have pathological consequences like in case of hypertrophic scars and keloids (Ogawa 2017).

4.3 Scar Fibrosis

A skin scar is the inevitable outcome of the full-thickness adult mammalian excision wound healing and has functional and psychological repercussions (Brown et al. 2008; Sen et al. 2009; Lo et al. 2020). The high clinical burden of scars has ushered in a huge market for scarless wound healing (Sen et al. 2009). Medical approaches are targeting biochemical factors associated with scarless healing observed in the fetus and adult oral mucosa to achieve scarless healing in adult mammalian tissues (Moore et al. 2018). Despite this, achieving regenerative or fully scarless wound healing has a long way to go. In addition to biochemical factors, mechanical forces regulate cellular functions and gene expressions in all phases of wound healing making it a powerful tool for the scarless healing (Aarabi et al. 2007). Current

biomedical and tissue engineering research is employing a range of materials and fabrication modules to design scaffolds and hydrogels with tissue-mimicking features to alleviate the scarring (Yang et al. 2014; Khetan et al. 2013).

The healthy skin of vertebrates consists of the epidermis, dermis, and hypodermis. Hypodermis or the subcutaneous tissue comprises a fat-rich adipose layer (panniculus adiposus) followed by a layer of striated muscles (panniculus carnosus) to primarily provide structural support and act as shock buffers from external impacts. In humans, however, the *panniculus carnosus* is retained only at a few anatomical locations with high variability between individuals. The scar tissue is devoid of the hypodermal layers and consists only of the scar matrix covered by the epidermal layer. The scar differs from the healthy skin due to variations in the ratio of collagen-I and collagen-III (Gurtner et al. 2008), collagen post-translational modifications and protein cross-linking (Lau et al. 2006), fiber arrangement and orientation, hydration (hyaluronic acid) (Freitas et al. 1996), ground substance composition (proteoglycans) (Blundell et al. 2006), and fibroblast differentiation (Huang et al. 2012). These multifaceted differences in the scar matrix cause them to seldom regain the mechanical strength and integrity of the healthy skin (Corr and Hart 2013). Despite the difference in the layered structural and compositional diversity between the scar and the skin, how the normal cutaneous scars are well integrated with all the layers of the adjoining skin with prolonged duration is unknown.

Scars are either normal or pathological. Pathological scars like the hypertrophic scars and keloids are different from the normal or non-pathological scars as they have bulged morphologies and tend to spread out from their boundaries over time. Usually, pathological scars are associated with injuries occurring in areas with continuous movements like the abdomen, chest, and thighs. Therefore, mechanical stresses impact the structure and morphology of the scars. Mechanical forces like tension, compression, shear, and osmotic forces are known to play a key role in wound healing and scar formation (Agha et al. 2011). Further, skin movements associated with different body parts determine the proclivity of scar tissue to be "normal" or hyperproliferative/pathological. Hence mechanical offloading approaches have seen the potential to reduce scars in both animals and humans (Aarabi et al. 2007; Agha et al. 2011; Huang et al. 2013). Although mechanical isolation of wounds created by offloading mechanisms can buffer the site-specific forces on the wound bed, a full-thickness skin wound during its maturation is continuously interacting with adjoining multilayered tissue with each layer having its respective mechanical property. Studies have shown that the final shape of the scar is related to the tension/stress lines impending in the wound during healing. In some shallow wounds or partial-thickness wounds (only epidermis) like minor bruises, scars disappear entirely (Moore et al. 2018). However, deeper cuts like full-thickness wounds (epidermis, dermis, hypodermis) develop long-term scars.

4.4 Imaging in Wound Healing

Wounds usually heal by either primary or secondary intention. Primary intention wounds are incisional wounds in which no tissue is lost. In secondary intention wounds, a chunk of the tissue is lost, and healing requires the formation of one or more new tissues. The scar is the new tissue formed that replaces the lost tissue and typically lacks specialized structures like hair follicles and functions like secretion. In many cases, the wounds heal "normally," that is, do not grow beyond the margins of the lost tissue. However, in several cases scars are hyperproliferative and they continue to grow and spread, that is, hypertrophic scars and keloids, and can even lead to severe consequences. Thus, it is important to reduce the number of scar formations. There are several therapeutic approaches to reducing scar tissue formation today. These include novel drug candidates as well as biomaterial scaffolds. On the other hand, there are wounds that do not heal easily or take indefinitely long time to heal; such wounds are called chronic wounds. Chronic wounds can be caused due to metabolic conditions such as diabetes or due to infection. In both cases, careful and timely monitoring of the wounds is essential. Thus, visualization of wound healing is important to assess the quality of healing, rate of healing, and efficacy of therapy and provide a more informed prognosis for intermediate therapeutic interventions.

Specifically for wounds, it is important that imaging needs to be non-invasive to evaluate the quality of healing. Several imaging techniques have been developed over the years that can image wounds non-invasively. The most widely used clinical imaging modality includes surface photography and is used to assess wound contraction. However, surface imaging methods cannot image deeper wounds and related features such as underlying inflammation, formation of new blood vessels, and organization of the healed tissue. But the optical imaging method uses light to penetrate deeper into the tissues non-invasively; the optical coherence tomography (OCT) is one such powerful imaging tool to image subsurface microstructures with a high lateral resolution of 5–15 μm. Although OCT can provide structural information label-free, it cannot accurately quantify the functional state of wound or wound healing. Advanced OCT-based imaging methods like OCT angiography (OCTA) can measure functional blood flow behavior in wounds that can be important to the healing quality. Polarization-sensitive OCT (PS-OCT) utilizes the polarizing ability of the tissues to quantify the functional state of healing. Here, we discuss a range of different imaging modalities that are used today for the assessment of wounds with their potential advantages and limitations and give an idea to the reader about the choice of imaging modality based on the requirement for the type and characteristics of a given wound.

Among optical imaging techniques for wound imaging, surface photography with digital cameras, multispectral imaging (MSI) (Basiri et al. 2010), hyperspectral imaging (HSI) (Calin et al. 2015a, b), near-infrared spectroscopy (NIRS) (Weingarten et al. 2010), diffuse reflectance spectroscopy (DRS) (Anand et al. 2014), laser Doppler imaging (LDI) (Hoeksema et al. 2009), laser speckle imaging (LSI) (Stewart et al. 2005; Ponticorvo et al. 2014), spatial frequency domain imaging

(SFDI) (Burmeister et al. 2015), fluorescence imaging (Wu et al. 2016), and optical coherence tomography (OCT) (Jayachandran et al. 2016; Cobb et al. 2006) have demonstrated promise in identifying different structural and functional aspects of healing. Spectral imaging methods (MSI, HSI, NIRS, DRS) have been found to closely measure blood oxygen in wounds (Jayachandran et al. 2016). Further, some of these methods like the NIRS provide images from tissue deep down. The compromise is the requirement of direct contact with the wound surface, which may be painful. Thus, non-contact and patient-comfort-centric methods are preferable. A quantitative approach to evaluate wound-healing efficiency is therefore needed to determine the functional aspects of healing like the healing rate and time for wound closure (Sidgwick et al. 2015).

4.4.1 Surface Imaging (Digital Photography)

Surface digital photography is the most used imaging method to assess wound healing. This is accompanied by measuring different parameters of the wound like size, contraction of margins, and their changes with time. However, surface imaging is limited as the wound closes by re-epithelialization quite early, that is, 5–6 days post injury (dpi), but it takes more time to heal in the deeper regions of the tissue. These deep regions are inaccessible to digital photography also because the clot/scab restricts the visible light to go deeper and image beneath the clot. This is a problem, especially during the most active stages of healing (4–10 dpi), when new cells are formed, and a new matrix is deposited. This also limits the study of the true temporal development of the healing in the wound bed. Therefore, although the surface measurement of wound features from digital photographs is an inexpensive and simple method, any assessment of wound-healing characteristics and efficacy of new treatment candidates is inadequate (Kislevitz et al. 2020). Therefore, imaging methods that can resolve and quantify changes in deeper tissues are essential for the proper wound-healing assessment. Another limitation of the tool is its inability to estimate the functional parameters of healing such as blood parameters such as oxygenation of the healing tissues and the formation of new blood vessels.

Thus, additional imaging methods are required to provide (a) information on deeper tissues during healing, (b) assessment of healing quality based on functional parameters, and (c) temporal development of the healing wounds.

4.4.2 Optical Coherence Tomography

OCT is a standard clinical imaging modality widely used in ophthalmology and has recently seen application in a variety of medical applications like dermatology, gastroenterology, and cardiology. OCT uses near-infrared (NIR) laser to image subsurface structures and image tomograms up to a depth of 2–4 mm from the surface. Although the clinical application of OCT in dermatology was explored in the mid-1990s, since then OCT has increasingly seen application in wound imaging

with variations that can image both the structural and functional features of wound healing. This was particularly because it is a non-contact non-invasive imaging mode. OCT is however limited as it cannot show cellular resolution otherwise easily visible in histological images taken in brightfield microscopes after staining. However, several early studies have matched tissue architectural features between OCT and histology for all the four stages of wound healing—(a) hemostasis, (b) inflammation, (c) proliferation, and (d) remodeling.

The earliest OCT was the time-domain OCT (TD-OCT) that demonstrated sufficient potential in imaging subsurface with micron-scale resolution. However, the images were prone to artifacts and the system was both speed and sensitivity limited due to the need for a mechanically moving reference mirror (Fig. 4.1). The Fourier-domain optical coherence tomography (FD-OCT) was an improvement over

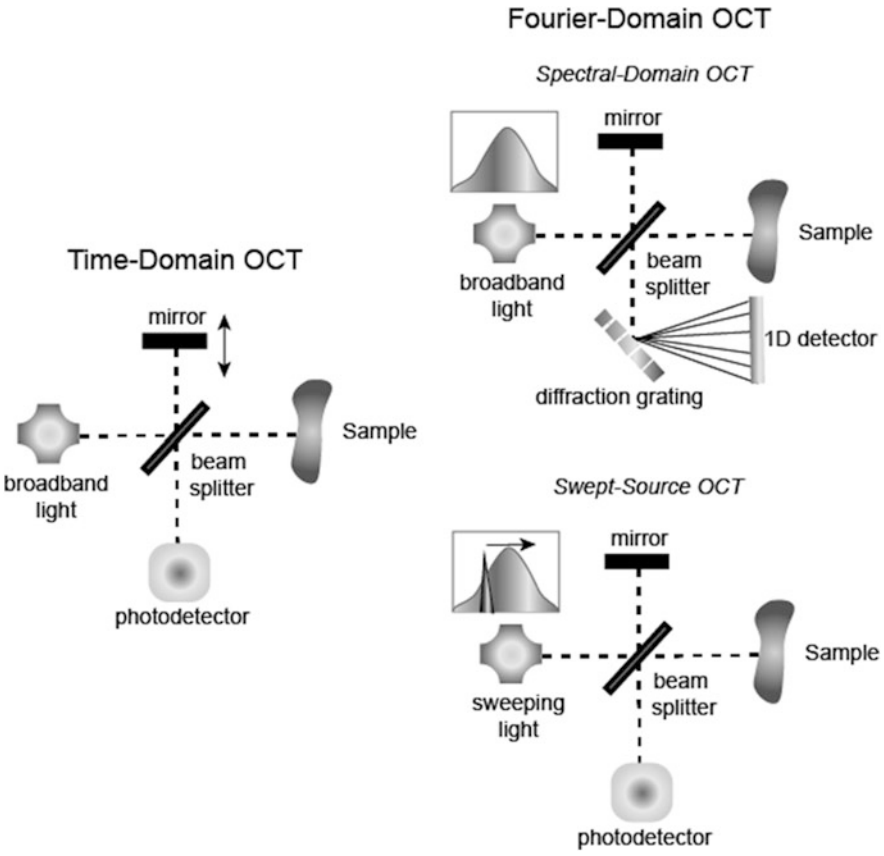


Fig. 4.1 Schematic diagram of different types of OCT systems. The time-domain OCT was the earliest form of OCT but was prone to imaging artifacts as it required the reference mirror to be mechanically moved to capture depth information of tissues. On the other hand, the Fourier-Domain OCT does not require the reference mirror to be mechanically moved and hence is much faster, highly sensitive, and produces much fewer artifacts

the TD-OCT with respect to both speed and sensitivity (hence better images), providing underlying tissue structures with a depth of up to 3 mm and a resolution of 3–15 μm (de Boer et al. 2017a) (Fig. 4.1). The OCT imaging mainly helps visualize tissue structures in tomogram (cross-section), but other formats for en face and 3D are also achieved. Although OCT is widely used in ophthalmology, cardiology, and gastroenterology, it is emerging as a valuable tool in dermatology also (Gambichler et al. 2015; Schwartz et al. 2017) due to its easy accessibility. In addition to cutaneous wound healing, skin conditions such as cancer, scleroderma, psoriasis, and dermatitis can be observed with high resolution with OCT (Gambichler et al. 2015; Sattler et al. 2013a). OCT modifications, to enable functional imaging, provide structural details of tissues and have potential in detecting the functional aspects like blood flow (OCT-angiography [OCTA], Dynamic-OCT (Olsen et al. 2018), Doppler-OCT (Leitgeb et al. 2014)), stiffness changes (OCT elastography (Liu et al. 2019)), and light polarizability (polarization-sensitive OCT [PS-OCT] (De Boer et al. 2017b)). For wound-healing applications, OCT also augments surface imaging by providing complementary information such as tissue conditions in deep structures (Fig. 4.2).

PS-OCT measures tissue birefringence and phase changes introduced by tissue matrix components such as collagen (Sakai et al. 2011). PS-OCT therefore can reveal differences in tissues due to healing or various skin conditions, as an outcome of changes in collagen density and orientation (Pierce et al. 2004a, b, c). Earlier demonstrations of PS-OCT recorded in skin burns have shown to quantify burn depth (Park et al. 2001; Srinivas et al. 2004) and amount of denaturation of collagen (Pierce et al. 2004a). The very first execution of wound healing after cut in the skin done by Oh et al. in 2006 showed that phase retardation in wounds considerably changes when suitable drugs are administered (Oh et al. 2006). Alternatively, Golberg et al. used PS-OCT of burn images to measure degree of polarization (DOP) and tissue birefringence upon a non-invasive electroporation (Golberg et al.

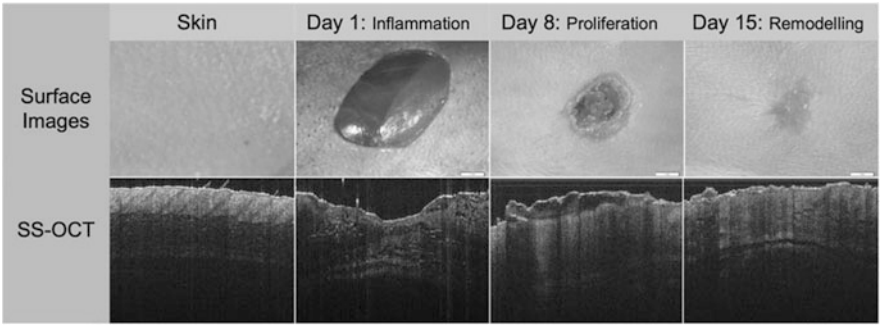


Fig. 4.2 A comparison of surface digital photographs and SS-OCT images at different stages of skin wound healing in mice. The normal skin OCT shows the layers of skin and the hair follicles; the inflammatory stage shows the clot deposition; in the proliferation stage, the OCT image records new tissue formation and the scab tissue coming off gradually from the center of the wound bed; and the remodeling stage shows the healed skin with a normotrophic scar

2015, 2016). Recently, Park et al. included using multifunctional PS-OCT to determine phase retardation and relative axis orientation of the collagen fibers in the tissues (Park et al. 2018). They also were able to correlate the PS-OCT findings with structural features on swept-source OCT (SS-OCT) and blood vessel structures from OCTA. Kim et al., in 2012, studied both shallow and deep burns and compared SS-OCT and PS-OCT images. They established the relation between burn depth and phase retardation in these wounds (Kim et al. 2012). Recent advances in PS-OCT include monitoring of the optic axis (Li et al. 2020) to measure collagen content and orientation in scarred tissues. It is a common practice to use SS-OCT for providing anatomical references for PS-OCT images. SS-OCT mainly provides information on tissue reflectivity. However, the true reflectivity information is mixed with noises such as the attenuation artifact with tissue depth, and this restricts the quantitative measurement of reflectivity in OCT images. It is, therefore, crucial to eliminate the attenuation for achieving SS-OCT reflectivity that is accurate and quantifies true reflectivity. PS-OCT also is not artifact-free. Gong et al. have used a novel technique referred to as vascular-masking on PS-OCT burn scars to decrease scattering artifacts from blood vessels and presented the birefringence improvement on scars (Gong et al. 2013, 2014).

The main benefit of using FD-OCT (spectral-domain OCT and swept-source OCT) in wound healing is that it can be correlated with histological images—a ground truth in the clinical assessment of the healing (Cobb et al. 2006; Greaves et al. 2015; Singer et al. 2007) (Fig. 4.3). Since the early application, the clinical evaluation of OCT images uses the mean gray value (MGV) to quantify the degree of scar tissue accumulation (Greaves et al. 2014) and rate of healing (Sattler et al. 2013b). The OCT uses the near-infrared photons scattered back from intact tissues and reconstructs a tomographic image with an up to 4 mm penetration achievable due to the use of specific NIR windows, that is, 650–950 and 1000–1350 nm (Smith

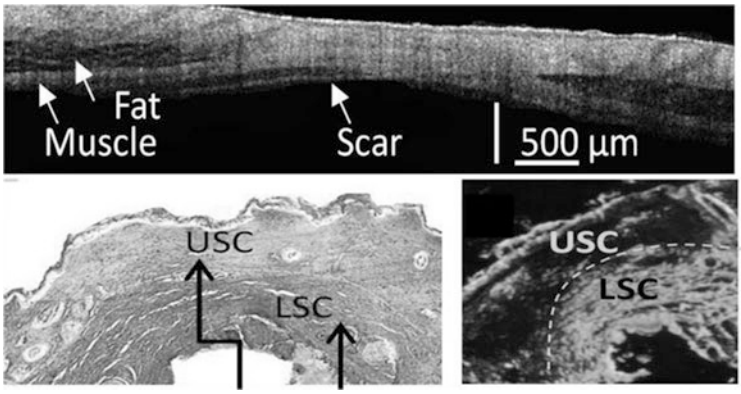


Fig. 4.3 Comparison of the scar as seen in SS-OCT (top), histology (bottom left), and dark-field microscopy (bottom right). As the OCT image is taken in the intact stage, we can observe a distinct bridge-like structure of the scar that has mechanobiological benefits for adapting to external stresses. (Image adapted with permission from Ghosh et al. (Ghosh et al. 2021b))

et al. 2009). However, in deeper tissue, the OCT signals experience heavy exponential attenuation following the Lambert–Beer law. This intensity loss renders deep-tissue visualization and reflectivity quantification with MGV unsuitable. Thus, advanced methods of attenuation correction have enabled the use of OCT to derive quantitative information related to wound-healing characteristics like healing rate and scar deposition (Ghosh et al. 2021a).

4.4.3 Laser Doppler Imaging (LDI)

LDI is a sensitive velocimetric technique that uses the frequency shift due to the Doppler effect when the laser is reflected from moving objects like blood cells to image the blood flow rate in the vessels. LDI functions by exciting the tissue with monochromatic laser light that is scattered back by static and dynamic/moving tissue structures. The scattered light from moving structures (like red blood cells [RBCs]) experiences a small change in frequency that is then collected by the detector like a complementary metal oxide semiconductor (CMOS) camera that is then processed to detect the blood perfusion parameters. The number of blood cells and the speed of blood flow both are directly proportional to the amount of frequency shift in the laser light, and this is used to calculate the blood flow rate of the microcirculation in the blood capillaries.

LDI has been widely used to assess burn wounds (Hoeksema et al. 2009; Pape et al. 2001; Holland et al. 2002; Droog et al. 2001; Kloppenberg et al. 2001). The popularity of LDI in burn depth assessment is due to the changes in the microvascular blood flow related to the depth of the burn injury (Yeong et al. 1996). In shallow burns, the onset of the inflammatory phase causes the blood flow to increase; however, in the case of deeper burns the microvasculature of the dermis is destroyed, and hence the flow of blood is relatively less. With the advancement of the LDI systems over the years, it is now established as a non-invasive, non-contact, real-time method to register perfusion changes over a broad field of view ($>1000\ cm^2$: by scanning), which is patient comfort centric (Leutenegger et al. 2011) and with reproducible diagnostic accuracy (Wang et al. 2020). Besides this, LDI can also be used to determine the need for excision and grafting (Jeng et al. 2003), clinical assessment of endothelial function (Kubli et al. 2000), diabetes (Kingwell et al. 2003), and rheumatic diseases (Murray et al. 2004).

4.4.4 Laser Speckle Imaging (LSI)

Like LDI, LSI uses a similar laser excitation on the tissues. However, unlike LDI, LSI detects the speckles generated by the backscattered light instead of frequency shifts (Fig. 4.4). Speckles are the random patterns generated due to the interference of the backscattered light. The speckles generated from the dynamic structures vary temporally and are different from the speckles generated by the static tissue structures. The speckles are captured by a charge-coupled device (CCD) detector

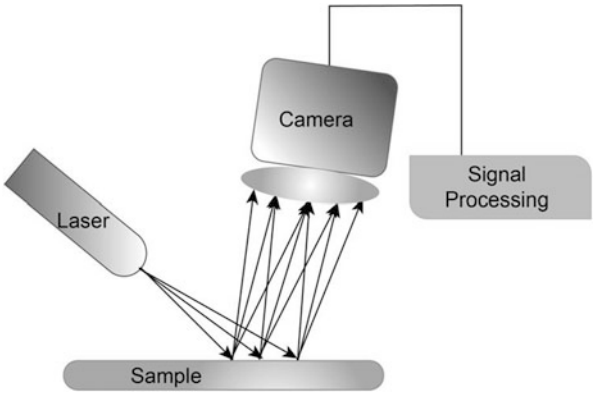


Fig. 4.4 Schematic diagram of laser speckle imaging. The light scattered from the sample is collected by the camera detector and is processed to identify moving structures such as flowing blood based on image contrast over a period of small-time

and are then processed. A speckle pattern generated from both static and dynamic tissue components produces a high contrast image. However, as the speckle patterns are constantly changing for the dynamically scattered components, when integrated over a small-time interval, it results in "blurring" of the image. The amount of the "blurring" depends on the speed and volume of the moving structures (like blood) and thus can be used to estimate flow measurements. The LSI has also been used for perfusion studies to non-invasively monitor the burn wounds (Stewart et al. 2005; Ponticorvo et al. 2014). LSI is alternatively also called laser speckle contrast imaging (LSCI), laser speckle perfusion imaging, and laser speckle contrast analysis (LASCA).

4.4.5 Fluorescence Imaging

Fluorescence imaging is a non-contact and real-time imaging tool that images tissues of interest by virtue of autofluorescence in the tissue components. The region of interest (wound) is typically illuminated by white light. Several biological molecules like RBCs, collagen, and keratin exhibit autofluorescence. The tissues give out fluorescent light that is collected and is separated by the diffused reflected source light by virtue of the difference in contrast. A few studies have been performed with fluorescence imaging to monitor diabetic foot ulcers (DFUs) (Wu et al. 2016; DaCosta et al. 2015). Some useful clinical insights provided by fluorescence imaging include microbiological contamination of windshield (DaCosta et al. 2015; Rennie et al. 2017) and neutrophil infiltration dynamics (Kim et al. 2008).

4.4.6 Spectral Imaging

This is a type of photographic imaging but instead of using visible white light to capture an image, spectral imaging collects light from a variety of wavelengths in the

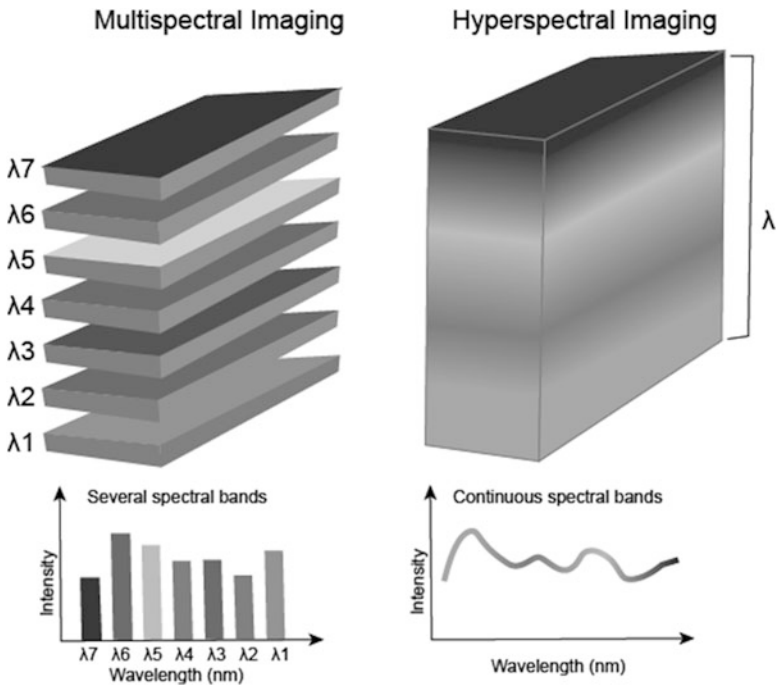


Fig. 4.5 Illustration of the difference between multispectral and hyperspectral imaging. While MSI collects images at a discrete set of wavelengths, HSI collects images over a continuous spread of wavelengths

visible and the infrared region of the electromagnetic spectra. Depending on whether the individual frames are captured at continuous or discrete spectral bands, we have two types of spectral imaging, that is, hyperspectral imaging (HSI) and multispectral imaging (MSI) (Fig. 4.5).

(a) *Hyperspectral imaging* captures continuous images over a broad spectral range. The broadband light source illuminates the wound or skin surface ranging across the visible and near-infrared (NIR) region of the electromagnetic spectra. A tunable filter captures frames over narrow bands across the spectral range of the light source. As different wavelengths have different depths of penetration, complementary information about a 3D volume of the skin can be obtained from the 3D data stack. Further, optics-based models are used to derive functional cutaneous parameters from the 3D data stack on a 2D map. These functional parameters quantify the amount of oxyhemoglobin (HbO) and deoxyhemoglobin (HbR) in the blood (Yudovsky et al. 2010; Khaodhiar et al. 2007). HSI application has been shown on several kinds of skin injuries including burns (Calin et al. 2015b), open wounds (Calin et al. 2015a), diabetic foot ulcers (Yudovsky et al. 2010; Khaodhiar et al. 2007; López-Moral et al. 2021), and venous ulcers (Denstedt et al. 2013). Since HSI uses longer

wavelengths than digital photography, the resultant image contains information from deeper regions and is a projection of the deep tissues (2–3 mm) where the longer wavelengths can reach. Depending on the wavelength range the resolution of the HSI images ranges between 0.4 and 1 μm (Albert 2012). Portability, low cost, and non-contact approach to imaging are some of the practical benefits of HSI imaging.

(b) *Multispectral imaging* works on the same principle of getting different information using specific wavelength filters but unlike HSI uses only discrete set of filters instead of a range. Skin optical models entailing the tissue–photon interaction of the skin layers are used to map the functional parameters like blood oxygenation (Basiri et al. 2010). Recent studies have also shown that MSI when integrated with computational machine learning can be used to predict with high accuracy the non-healing amputation sites in chronic wounds (Squiers et al. 2021).

(c) *Near-Infrared Spectroscopy (NIRS)* is a vibrational spectroscopy tool to map tissue oxygenation states based on the different absorptions of light differently by oxygenated and the deoxygenated blood (Afara et al. 2021). The wavelength of NIR ranges between 780 and 2500 nm.

NIRS is a label-free, non-destructive, and rapid tool for biochemical assessment of the wounds like Raman spectroscopy and Fourier-transform infrared spectroscopy (FTIR). For the measurement of the tissue oxygenation, the knowledge that oxyhemoglobin (HbO) and deoxyhemoglobin (HbR) have absorption maxima at 900 and 760 nm, respectively, is used to quantify the specific tissue oxygenation state of the new tissue that is forming on the wound bed (Weingarten et al. 2006, 2010, 2012; Papazoglou et al. 2006a, b, 2007, 2008, 2009). The most important feature of NIRS in addition to the determination of the tissue oxygenation however is the ability to create a biochemical map of the tissue components like proteoglycans, water, and collagen (Afara et al. 2021). These components have specific relevance to wound healing and regenerative medicine as they are the most important components of the tissue matrix and hence can be used to quantify the degree and quality of healing in the new tissue.

(d) *Spatial frequency domain imaging (SFDI)* is an extension of NIRS wherein the chromophores present in the tissue absorb or scatter light differently at different wavelengths. However, the key difference in SFDI that makes it unique is the modulation of the incident light falling on the tissues. Different patterns of light are illuminated on the sample (typically in the NIR range of 650–950 nm) and the remitted light from the sample is collected over a period (Fig. 4.6). The video is then processed to determine the type and concentration of the chromophores such as HbO and HbR. SFDI is also able to determine the coefficients of absorption and scattering, which can be important signatures for tissue composition, wound severity, and healing stage. SFDI has been widely used to study scarring and burn wounds (Burmeister et al. 2015; Ramella-Roman et al. 2015; Mazhar et al. 2014; Lin et al. 2013; Nguyen et al. 2013a, b). In scar tissues, SFDI can be used to determine oxygen saturation, water content, and blood volume fraction. In burn wounds, SFDI has been exhaustively used for measuring burn depth and oxygen saturation.

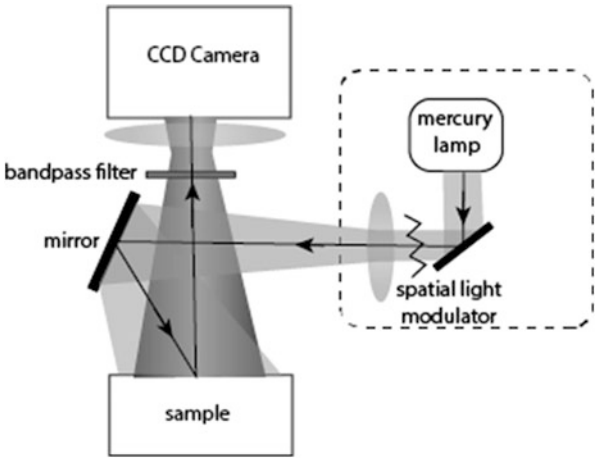


Fig. 4.6 Schematic diagram of SFDI showing how a modulator is used to illuminate the sample with different patterns, and the scattered light from the sample is then used to measure the tissue properties based on differential absorption and scattering of light

4.4.7 Ultrasonography

It is a less widely used modality to monitor and assess the healing of wounds but due to several advantages can be a powerful tool to measure several useful attributes of wound healing. Ultrasound uses a transducer probe that emits ultrasound into the tissues and collects the echoes with the same probe as a receiver. The echoes are more prominent at the different layers/interfaces (epidermis, dermis, subcutaneous fat, muscles) and thereby provide images of affected tissues and layers in the wound as a cross-section image (B-mode).

Usually, ultrasound for cutaneous wound-healing monitoring uses a high-frequency transducer in the range of 20–60 MHz to visualize high-resolution structures (60–100 μm) and microcirculation (Foster et al. 2002). Further, with the help of Doppler mode, it is also possible to quantify the flow rate of the blood in the capillaries in and around the wound bed. Recent studies on high-resolution ultrasound have shown its use in determining wound tissue morphology, biomechanics, and hemodynamics (Gnyawali et al. 2015, 2020). Despite these advantages, the major limitation still includes its inability to image in non-contact mode and the need for more rigorous clinical studies.

4.4.8 Photoacoustic Imaging (PAI)

The principle of PAI is essentially exciting the target tissue with short pulses of light that are absorbed by chromophores in tissues (like blood cells). This results in the increase in adiabatic temperature of the chromophores, which causes the emission of ultrasound waves through the thermoelastic process (Fig. 4.7). The acoustic waves generated from the tissues are captured using an ultrasound receiver and processed to visualize the chromophores. As blood can function as excellent chromophores, PAI can be very useful to determine the microvascular changes in the tissues in the

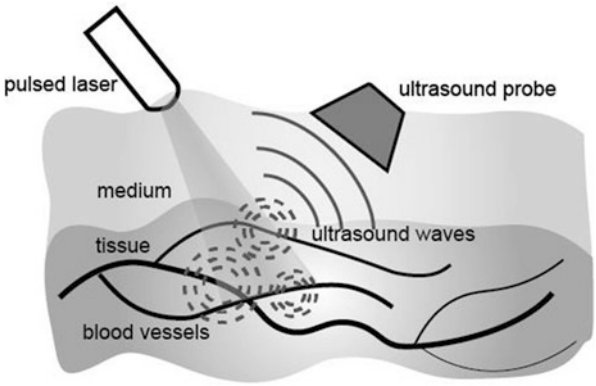


Fig. 4.7 Schematic diagram of photoacoustic imaging. A short, pulsed laser is allowed to excite the chromophores such as RBCs in the blood vessels. The chromophores upon absorbing the energy emit acoustic waves that are captured by an ultrasound probe to image the localization and perfusion characteristics of the chromophores

wounds. PAI has been performed on burn wounds (Sato et al. 2005; Yamazaki et al. 2005) and new blood vessel formation on grafted skin (Yamazaki et al. 2006), chronic foot ulcers (Wang et al. 2019), pressure ulcers (Hariri et al. 2019), and monitoring angiogenesis (Mantri et al. 2021). Thus, a wide application, non-invasive and non-contact imaging mode, and real-time high-resolution imaging make PAI a very desirable tool to monitor wounds.

4.4.9 Thermal Imaging

Thermal imagers are simple devices that aid in visualizing the temperature distribution of the skin by detecting infrared emissions from the tissue. Thermal imaging has seen an enormous application in burn wounds (Liddington and Shakespeare 1996; Burke-Smith et al. 2015; Medina-Preciado et al. 2012; Wearn et al. 2018), venous leg ulcers (Monshipouri et al. 2021), and diabetic foot ulcers (Aliahmad et al. 2019). The studies have shown that thermal imaging can be used to measure wound-healing effect.

4.5 Prospects for Advancements in Current Systems for Assessment of Healing

4.5.1 Imaging for Biochemical Analysis of Wounds by Micro-spectroscopy

Most imaging methods discussed in the chapter provide structural or blood vascular information and therefore have a direct relation with the clinical monitoring of wounds. However, at a biomolecular level it can also be characterized and imaged. Raman and FTIR spectroscopy are vibrational spectroscopy methods used to biochemically characterize biological tissues in a non-destructive and label-free way (Crane and Elster 2012; Krafft et al. 2007, 2008). Vibrational spectroscopy can be used to predict the occurrence of molecular markers in the wound healing (Crane and

Elster 2012; Chan et al. 2008). In the spectroscopy methods, laser light is allowed to interact with the samples, and, based on the interaction (absorption, transmission), a one-dimensional spectra plot is obtained that shows the absorption/transmission peaks for a range of wavelengths (typically 2.5–25 μm). The peak characteristics like intensity, breadth, and wavelength location determine the biochemical properties of the tissues. However, to get 2D biochemical maps, micro-spectroscopy is used. In micro-spectroscopy, an objective lens scans the sample and provides a 2D plot of the scanned region for every wavelength of the spectrometer. Typically, spectroscopy can be performed on samples collected from the patient-like tissues, with limited scope for it to be used as a handheld scanner. However, with appropriate signal processing, there is a lot of scope for the discovery of new spectral biomarkers of healing and wound severity thereby making micro-spectroscopy a powerful adjunct tool for wound assessment.

4.5.2 Monitoring Drug Delivery for Identifying Therapeutic Efficacy

Regenerative medicine aims to restore organ function, and with advancement in the field of tissue engineering and regenerative medicine we have a host of options to deal with wounds including severe chronic ones while minimizing the scarring. Besides the use of conventional drugs, different novel drug delivery mechanisms are now being implemented ranging from drug-laden biomaterial implants and scaffolds (Biswas et al. 2019) to nanoparticles as efficient and targeted drug delivery machinery (Parveen et al. 2012). Along with the development of such technologies, it is important to image the healing potential of these candidates continuously or at definite intervals during wound healing.

Nanomaterials are readily taken up by the cells and need to be imaged in a way that is both fast and sensitive. The most suitable imaging modality in such a case is fluorescence microscopes. However, nanostructures require a far superior resolution than even the conventional optical microscopes, that is, 200 nm. Super-resolution microscopy also called optical nanoscopy breaks this optical diffraction limit and with the help of such technologies as stochastic optical reconstruction microscopy (STORM), stimulated emission depletion (STED) microsocpy, and photo-activated localization microscopy (PALM), it is now possible to resolve structures that are 20–30 nm apart. Fluorescent probes are used to label specific target biomolecule of interest and are imaged for gaining meaningful insights. The labels include: (a) molecular bodies with defined structures such as organic dyes, metal–ligand complexes, bio-fluorophores; (b) quantum nanocrystals with size-dependent optical properties such as quantum dots, carbon nanoparticles, and silicon nanoparticles (Ben Moshe et al. 2011); and (c) entities with size-independent optical properties (Sadhanala and Nanda 2016). Targeted and controlled release of drugs is often crucial for therapy and hence the imaging systems should be able to track the fidelity of the drug targets and the release kinetics for proper assessment of the therapeutic candidate. Furthermore, the imaging systems should be also able to determine the retention, degradation, and removal of the delivery systems from the cells and tissues.

References

Aarabi S et al (2007) Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis. FASEB J 21(12):3250–3261

Afara IO et al (2021) Characterization of connective tissues using near-infrared spectroscopy and imaging. Nat Protoc 16(2):1297–1329

Agha R et al (2011) A review of the role of mechanical forces in cutaneous wound healing. J Surg Res 171(2):700–708

Albert H (2012) A survey of optical imaging techniques for assessing wound healing. Int J Intell Control Syst 17:79–85

Aliahmad B et al (2019) Is thermal imaging a useful predictor of the healing status of diabetes-related foot ulcers? A pilot study. J Diabetes Sci Technol 13(3):561–567

Anand S et al (2014) Diffuse reflectance spectroscopy for monitoring diabetic foot ulcer—a pilot study. Opt Lasers Eng 53:1–5

Basiri A et al (2010) Use of a multi-spectral camera in the characterization of skin wounds. Opt Express 18(4):3244–3257

Ben Moshe A, Szwarcman D, Markovich G (2011) Size dependence of chiroptical activity in colloidal quantum dots. ACS Nano 5(11):9034–9043

Biswas A, Shukla A, Maiti P (2019) Biomaterials for interfacing cell imaging and drug delivery: an overview. Langmuir 35(38):12285–12305

Blundell CD, Deangelis PL, Almond A (2006) Hyaluronan: the absence of amide–carboxylate hydrogen bonds and the chain conformation in aqueous solution are incompatible with stable secondary and tertiary structure models. Biochem J 396(3):487–498

Brown B et al (2008) The hidden cost of skin scars: quality of life after skin scarring. J Plast Reconstr Aesthet Surg 61(9):1049–1058

Burke-Smith A, Collier J, Jones I (2015) A comparison of non-invasive imaging modalities: infrared thermography, spectrophotometric intracutaneous analysis and laser Doppler imaging for the assessment of adult burns. Burns 41(8):1695–1707

Burmeister DM et al (2015) Utility of spatial frequency domain imaging (SFDI) and laser speckle imaging (LSI) to non-invasively diagnose burn depth in a porcine model. Burns 41(6): 1242–1252

Calin M-A et al (2015a) Hyperspectral imaging-based wound analysis using mixture-tuned matched filtering classification method. J Biomed Opt 20(4):046004

Calin MA et al (2015b) Characterization of burns using hyperspectral imaging technique—a preliminary study. Burns 41(1):118–124

Chan KA et al (2008) A coordinated approach to cutaneous wound healing: vibrational microscopy and molecular biology. J Cell Mol Med 12(5b):2145–2154

Clark RA (2021) To scar or not to scar. N Engl J Med 385(5):469–471

Cobb MJ et al (2006) Noninvasive assessment of cutaneous wound healing using ultrahigh-resolution optical coherence tomography. J Biomed Opt 11(6)

Corr DT, Hart DA (2013) Biomechanics of scar tissue and uninjured skin. Adv Wound Care 2(2): 37–43

Crane NJ, Elster EA (2012) Vibrational spectroscopy: a tool being developed for the noninvasive monitoring of wound healing. J Biomed Opt 17(1):010902

DaCosta RS et al (2015) Point-of-care autofluorescence imaging for real-time sampling and treatment guidance of bioburden in chronic wounds: first-in-human results. PLoS One 10(3): e0116623

de Boer JF, Leitgeb R, Wojtkowski M (2017a) Twenty-five years of optical coherence tomography: the paradigm shift in sensitivity and speed provided by Fourier domain OCT [Invited]. Biomed Opt Express 8(7):3248–3280

De Boer JF, Hitzenberger CK, Yasuno Y (2017b) Polarization sensitive optical coherence tomography—a review. Biomed Opt Express 8(3):1838–1873

Denstedt M, et al (2013) Hyperspectral imaging as a diagnostic tool for chronic skin ulcers. in Photonic Therapeutics and Diagnostics IX. International Society for Optics and Photonics
Droog E, Steenbergen W, Sjöberg F (2001) Measurement of depth of burns by laser Doppler perfusion imaging. Burns 27(6):561–568
Foster F et al (2002) A new ultrasound instrument for in vivo microimaging of mice. Ultrasound Med Biol 28(9):1165–1172
Freitas J et al (1996) Hyaluronic acid in progressive systemic sclerosis. Dermatology 192(1):46–49
Gambichler T, Pljakic A, Schmitz L (2015) Recent advances in clinical application of optical coherence tomography of human skin. Clin Cosmet Investig Dermatol 8:345–353
Ghosh B et al (2021a) Attenuation corrected-optical coherence tomography for quantitative assessment of skin wound healing and scar morphology. J Biophotonics 14(4):e202000357
Ghosh B et al (2021b) Structural mechanics modeling reveals stress-adaptive features of cutaneous scars. Biomech Model Mechanobiol 20(1):371–377
Gnyawali SC et al (2015) High-resolution harmonics ultrasound imaging for non-invasive characterization of wound healing in a pre-clinical swine model. PLoS One 10(3):e0122327
Gnyawali SC et al (2020) High resolution ultrasound imaging for repeated measure of wound tissue morphometry, biomechanics and hemodynamics under fetal, adult and diabetic conditions. PLoS One 15(11):e0241831
Golberg A et al (2015) Skin rejuvenation with non-invasive pulsed electric fields. Sci Rep 5(1):1–18
Golberg A et al (2016) Preventing scars after injury with partial irreversible electroporation. J Investig Dermatol 136(11):2297–2304
Gong P et al (2013) Assessment of human burn scars with optical coherence tomography by imaging the attenuation coefficient of tissue after vascular masking. J Biomed Opt 19(2):021111
Gong PJ et al (2014) Imaging of skin birefringence for human scar assessment using polarization-sensitive optical coherence tomography aided by vascular masking. J Biomed Opt 19(12)
Greaves NS et al (2014) Optical coherence tomography: a reliable alternative to invasive histological assessment of acute wound healing in human skin? Br J Dermatol 170(4):840–850
Greaves NS et al (2015) Skin substitute-assisted repair shows reduced dermal fibrosis in acute human wounds validated simultaneously by histology and optical coherence tomography. Wound Repair Regen 23(4):483–494
Gurtner GC et al (2008) Wound repair and regeneration. Nature 453(7193):314–321
Hariri A et al (2019) Noninvasive staging of pressure ulcers using photoacoustic imaging. Wound Repair Regen 27(5):488–496
Hoeksema H et al (2009) Accuracy of early burn depth assessment by laser Doppler imaging on different days post burn. Burns 35(1):36–45
Holland A, Martin H, Cass D (2002) Laser Doppler imaging prediction of burn wound outcome in children. Burns 28(1):11–17
Huang X et al (2012) Matrix stiffness–induced myofibroblast differentiation is mediated by intrinsic mechanotransduction. Am J Respir Cell Mol Biol 47(3):340–348
Huang C et al (2013) Mechanotherapy: revisiting physical therapy and recruiting mechanobiology for a new era in medicine. Trends Mol Med 19(9):555–564
Jayachandran M et al (2016) Critical review of noninvasive optical technologies for wound imaging. Adv Wound Care 5(8):349–359
Jeng J et al (2003) Laser Doppler imaging determines need for excision and grafting in advance of clinical judgment: a prospective blinded trial. Burns 29(7):665–670
Jiang D et al (2020) Injury triggers fascia fibroblast collective cell migration to drive scar formation through N-cadherin. Nat Commun 11(1):1–13
Khaodhiar L et al (2007) The use of medical hyperspectral technology to evaluate microcirculatory changes in diabetic foot ulcers and to predict clinical outcomes. Diabetes Care 30(4):903–910
Khetan S et al (2013) Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. Nat Mater 12(5):458–465
Kim M-H et al (2008) Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. J Investig Dermatol 128(7):1812–1820

Kim KH et al (2012) In vivo imaging of human burn injuries with polarization-sensitive optical coherence tomography. J Biomed Opt 17(6)
Kingwell BA et al (2003) Type 2 diabetic individuals have impaired leg blood flow responses to exercise: role of endothelium-dependent vasodilation. Diabetes Care 26(3):899–904
Kislevitz M et al (2020) Use of optical coherence tomography (OCT) in aesthetic skin assessment—a short review. Lasers Surg Med 52(8):699–704
Kloppenberg F, Beerthuizen G, Ten Duis H (2001) Perfusion of burn wounds assessed by laser Doppler imaging is related to burn depth and healing time. Burns 27(4):359–363
Krafft C et al (2007) Methodology for fiber-optic Raman mapping and FTIR imaging of metastases in mouse brains. Anal Bioanal Chem 389(4):1133–1142
Krafft C et al (2008) Raman and FTIR microscopic imaging of colon tissue: a comparative study. J Biophotonics 1(2):154–169
Kubli S et al (2000) Reproducibility of laser Doppler imaging of skin blood flow as a tool to assess endothelial function. J Cardiovasc Pharmacol 36(5):640–648
Lau Y-KI, Gobin AM, West JL (2006) Overexpression of lysyl oxidase to increase matrix crosslinking and improve tissue strength in dermal wound healing. Ann Biomed Eng 34(8):1239–1246
Leitgeb RA et al (2014) Doppler optical coherence tomography. Prog Retin Eye Res 41:26–43
Leutenegger M et al (2011) Real-time full field laser Doppler imaging. Biomed Opt Express 2(6):1470–1477
Li Q, Sampson DD, Villiger M (2020) In vivo imaging of the depth-resolved optic axis of birefringence in human skin. Opt Lett 45(17):4919–4922
Liddington M, Shakespeare P (1996) Timing of the thermographic assessment of burns. Burns 22(1):26–28
Lin AJ et al (2013) Visible spatial frequency domain imaging with a digital light microprojector. J Biomed Opt 18(9):096007
Liu CH et al (2019) Translational optical coherence elastography for assessment of systemic sclerosis. J Biophotonics 12(12):e201900236
Lo ZJ et al (2020) Clinical and economic burden of wound care in the tropics: a 5-year institutional population health review. Int Wound J 17(3):790–803
López-Moral M et al (2021) A comparison of hyperspectral imaging with routine vascular noninvasive techniques to assess the healing prognosis in patients with diabetic foot ulcers. J Vasc Surg
Mantri Y et al (2021) Photoacoustic monitoring of angiogenesis predicts response to therapy in healing wounds. Wound Repair Regen
Mazhar A et al (2014) Noncontact imaging of burn depth and extent in a porcine model using spatial frequency domain imaging. J Biomed Opt 19(8):086019
Medina-Preciado JD et al (2012) Noninvasive determination of burn depth in children by digital infrared thermal imaging. J Biomed Opt 18(6):061204
Monshipouri M et al (2021) Thermal imaging potential and limitations to predict healing of venous leg ulcers. Sci Rep 11(1):1–11
Moore AL et al (2018) Scarless wound healing: transitioning from fetal research to regenerative healing. Wiley Interdiscip Rev Dev Biol 7(2):e309
Murray AK, Herrick A, King T (2004) Laser Doppler imaging: a developing technique for application in the rheumatic diseases. Rheumatology 43(10):1210–1218
Nguyen JQM et al (2013a) Spatial frequency domain imaging of burn wounds in a preclinical model of graded burn severity. J Biomed Opt 18(6):066010
Nguyen TT et al (2013b) Novel application of a spatial frequency domain imaging system to determine signature spectral differences between infected and noninfected burn wounds. J Burn Care Res 34(1):44–50
Nussbaum SR et al (2018) An economic evaluation of the impact, cost, and medicare policy implications of chronic nonhealing wounds. Value Health 21(1):27–32

Ogawa R (2017) Keloid and hypertrophic scars are the result of chronic inflammation in the reticular dermis. Int J Mol Sci 18(3)
Oh J-T et al (2006) Quantification of the wound healing using polarization-sensitive optical coherence tomography. J Biomed Opt 11(4):041124
Olsen J, Holmes J, Jemec GB (2018) Advances in optical coherence tomography in dermatology—a review. J Biomed Opt 23(4):040901
Papazoglou ES et al (2006a) Optical properties of wounds: diabetic versus healthy tissue. IEEE Trans Biomed Eng 53(6):1047–1055
Papazoglou ES, et al (2006b) Monitoring diabetic wound healing by NIR spectroscopy. In: 2005 IEEE Engineering in Medicine and Biology 27th Annual Conference. IEEE
Papazoglou ES et al (2007) Near infrared diffuse optical tomography: improving the quality of care in chronic wounds of patients with diabetes. Biomed Instrum Technol 41(1):83–87
Papazoglou ES, et al (2008) Assessment of diabetic foot ulcers with diffuse near infrared methodology. In: 2008 8th IEEE International Conference on BioInformatics and BioEngineering. IEEE
Papazoglou ES et al (2009) Noninvasive assessment of diabetic foot ulcers with diffuse photon density wave methodology: pilot human study. J Biomed Opt 14(6):064032
Pape SA, Skouras CA, Byrne PO (2001) An audit of the use of laser Doppler imaging (LDI) in the assessment of burns of intermediate depth. Burns 27(3):233–239
Park BH et al (2001) In vivo burn depth determination by high-speed fiber-based polarization sensitive optical coherence tomography. J Biomed Opt 6(4):474–479
Park KS et al (2018) Multifunctional in vivo imaging for monitoring wound healing using swept-source polarization-sensitive optical coherence tomography. Lasers Surg Med 50(3):213–221
Parveen S, Misra R, Sahoo SK (2012) Nanoparticles: a boon to drug delivery, therapeutics, diagnostics and imaging. Nanomedicine 8(2):147–166
Pierce MC et al (2004a) Collagen denaturation can be quantified in burned human skin using polarization-sensitive optical coherence tomography. Burns 30(6):511–517
Pierce MC et al (2004b) Advances in optical coherence tomography imaging for dermatology. J Investig Dermatol 123(3):458–463
Pierce MC et al (2004c) Birefringence measurements in human skin using polarization-sensitive optical coherence tomography. J Biomed Opt 9(2):287–291
Ponticorvo A et al (2014) Quantitative assessment of graded burn wounds in a porcine model using spatial frequency domain imaging (SFDI) and laser speckle imaging (LSI). Biomed Opt Express 5(10):3467–3481
Ramella-Roman J et al (2015) Monitoring hypertrophic burn scars with a spectroscopic polarimetric optical system. SPIE Newsroom:1–3
Rennie M et al (2017) Point-of-care fluorescence imaging predicts the presence of pathogenic bacteria in wounds: a clinical study. J Wound Care 26(8):452–460
Sadhanala HK, Nanda KK (2016) Boron-doped carbon nanoparticles: size-independent color tunability from red to blue and bioimaging applications. Carbon 96:166–173
Sakai S et al (2011) In vivo evaluation of human skin anisotropy by polarization-sensitive optical coherence tomography. Biomed Opt Express 2(9):2623–2631
Shah M, Foreman DM, Ferguson MW (1992) Control of scarring in adult wounds by neutralising antibody to transforming growth factor β. Lancet 339(8787):213–214
Sen CK (2021) Human wound and its burden: updated 2020 compendium of estimates. Mary Ann Liebert, Inc., New York, pp 281–292
Sidgwick G, McGeorge D, Bayat A (2015) A comprehensive evidence-based review on the role of topicals and dressings in the management of skin scarring. Arch Dermatol Res 307(6):461–477
Sen CK et al (2009) Human skin wounds: a major and snowballing threat to public health and the economy. Wound Repair Regen 17(6):763–771
Yang C et al (2014) Mechanical memory and dosing influence stem cell fate. Nat Mater 13(6): 645–652

Weingarten MS et al (2010) Prediction of wound healing in human diabetic foot ulcers by diffuse near-infrared spectroscopy: a pilot study. Wound Repair Regen 18(2):180–185
Stewart C et al (2005) A comparison of two laser-based methods for determination of burn scar perfusion: laser Doppler versus laser speckle imaging. Burns 31(6):744–752
Wu YC et al (2016) Handheld fluorescence imaging device detects subclinical wound infection in an asymptomatic patient with chronic diabetic foot ulcer: a case report. Int Wound J 13(4): 449–453
Schwartz M, Levine A, Markowitz O (2017) Optical coherence tomography in dermatology. Cutis 100(3):163–166
Sattler E, Kastle R, Welzel J (2013a) Optical coherence tomography in dermatology. J Biomed Opt 18(6)
Srinivas SM et al (2004) Determination of burn depth by polarization-sensitive optical coherence tomography. J Biomed Opt 9(1):207–212
Singer AJ et al (2007) Optical coherence tomography: a noninvasive method to assess wound reepithelialization. Acad Emerg Med 14(5):387–391
Sattler ECE et al (2013b) Confocal laser scanning microscopy and optical coherence tomography for the evaluation of the kinetics and quantification of wound healing after fractional laser therapy. J Am Acad Dermatol 69(4):E165–E173
Smith AM, Mancini MC, Nie S (2009) Second window for in vivo imaging. Nat Nanotechnol 4(11):710–711
Yeong EK et al (1996) Improved accuracy of burn wound assessment using laser Doppler. J Trauma Acute Care Surg 40(6):956–962
Wang R et al (2020) Diagnostic accuracy of laser Doppler imaging for the assessment of burn depth: a meta-analysis and systematic review. J Burn Care Res 41(3):619–625
Yudovsky D, Nouvong A, Pilon L (2010) Hyperspectral imaging in diabetic foot wound care. J Diabetes Sci Technol 4(5):1099–1113
Squiers JJ et al (2021) Machine learning analysis of multispectral imaging and clinical risk factors to predict amputation wound healing. J Vasc Surg
Weingarten MS et al (2006) Measurement of optical properties to quantify healing of chronic diabetic wounds. Wound Repair Regen 14(3):364–370
Weingarten MS et al (2012) Diffuse near-infrared spectroscopy prediction of healing in diabetic foot ulcers: a human study and cost analysis. Wound Repair Regen 20(6):911–917
Sato S et al (2005) Photoacoustic diagnosis of burns in rats. J Trauma Acute Care Surg 59(6): 1450–1456
Yamazaki M et al (2005) Measurement of burn depths in rats using multiwavelength photoacoustic depth profiling. J Biomed Opt 10(6):064011
Yamazaki M et al (2006) Photoacoustic monitoring of neovascularities in grafted skin. Lasers Surg Med 38(3):235–239
Wang Y et al (2019) A portable three-dimensional photoacoustic tomography system for imaging of chronic foot ulcers. Quant Imaging Med Surg 9(5):799
Wearn C et al (2018) Prospective comparative evaluation study of Laser Doppler Imaging and thermal imaging in the assessment of burn depth. Burns 44(1):124–133

Role of Biosensors in Regenerative Therapeutics: Past, Present, and Future Prospects

5

Mukti Mandal, Jai Shukla, Brateen Datta, and Gorachand Dutta

5.1 Introduction

Regenerative medicine, an interdisciplinary field of research, is a significant improvement in medical therapy that uses stem cell therapy, and tissue engineering to repair, replace or regenerate human tissues and organs and restore their functioning. William Haseltine is widely credited with coining the term "regenerative medicine," where, during a 1999 conference on Lake Como, in an attempt to describe an emerging field that combined the knowledge from various disciplines, such as tissue engineering (TE), cell transplantation, stem cell biology, biomechanics, prosthetics, nanotechnology, and biochemistry. This word first appeared in a 1992 study by Leland Kaiser, who enumerated technologies that would have an impact on the future of hospitals (Sampogna et al. 2015).

Body part regeneration is a typical occurrence in nature; a salamander may regenerate an amputated limb in a matter of days. Humans have this ability as well, but it fades with age: a severed fingertip can regenerate until age 11. The ability of humans to regenerate was known even in ancient times, as evidenced by the Prometheus myth, in which his liver was eaten by an eagle during the day and then totally regenerated overnight (Sampogna et al. 2015).

Stem cells have the ability to self-renew as well as divide asymmetrically, resulting in one identical daughter stem cell and a second unique daughter cell. The cells have the ability to differentiate into lineage-specific programs. Determination of powerful stem cell is still far from resolved, but it is evident that long-recognized characterization hurdles persist without a universal stem cell type or method of distribution. Despite this, there is a growing consensus that alterations in

M. Mandal · J. Shukla · B. Datta · G. Dutta (✉)
NanoBiosensors and Biodevices Lab, School of Medical Sciences and Technology, Indian Institute of Technology, Kharagpur, West Bengal, India
e-mail: g.dutta@smst.iitkgp.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_5

DNA-binding core histones influence cell lineage commitment and may aid in the identification of self-renewing stem cells. Pretreatment in vitro alteration of therapeutic cells can decrease their later biological function, which is a genuine issue. Biomarkers that indicate cell stress responses can help guide optimal cell manipulation for their therapeutic context and improve clinical outcomes.

Tissue engineering is the study of how cells, organs, and engineered tissues grow and develop in order to restore damaged or sick tissues and organs. In the realm of tissue engineering, biosensors have shown significant promise. Biomolecule concentrations including glucose, adenosines, and hydrogen peroxide play important roles in the maintenance and growth of three-dimensional cell cultures and organs. In this case, biosensors aid in the detection of biological analytes in real time, providing additional information about the cellular activity (Kaur and Sharma 2015).

Vascularization and angiogenesis are a crucial step in building of tissues-engineered grafts. One of the most notable phases in regenerative medicine is blood vessel formation. In this phase, blood flow to ischemic tissues and rapid vascularization in a clinical-sized tissue-engineered graft are observed. New blood vessels are formed in therapeutic angiogenesis when blood supply to ischemic tissues is restored. Expediting vascularization in tissue-engineered grafts by transplanting in vitro-generated tissue for damaged or surgically treated tissues due to a lack of endogenous tissue perfusion is useful for treating ischemic illness (Jahani et al. 2020).

5.2 Envisaging Direction in Regenerative Medicine

Regenerative medicine might be characterized as the method involved with replacing or "recovering" human cells, tissues, or organs to re-establish or lay out normal physicochemical properties of tissues. Regenerative medicine allows researchers to generate tissues or organs in vitro and implant them in the damaged location of the human body, and aid in the healing. From a biomedical perspective, regenerative medicine covers a wide range of applications like immunomodulation, tissue engineering, and stem cell therapy. The idea of regenerative medicine is not just the implantation or regeneration of tissues, but the physicochemical and morphological parameters of original tissues must also be stabilized. For that complex biological systems must be identified to replicate, stimulate, and applied for better therapeutic efficacy. It involves a wide range of fields from cell biology and biomaterial science to biomedical engineering. The idea is to promote vascularization, the regeneration of new tissues that can secrete hormones and other growth factors and maintain the normal physiological characteristics of the human body. The two most important concepts of regenerative medicine are cells and biomaterials. In tissue engineering, biomaterials mimic the function of extracellular matrix (ECM) by providing three dimensional space for the cells to adhere, proliferate, differentiate, and form new tissues, which are appropriate for the particular function. Biomaterials can be natural as well as synthetic. Natural biomaterials have the advantage of escaping the immune response whereas synthetic biomaterials can

be mass generated. Typical control in the synthetic biomaterials allows for regulation of strength, mechanical and biological properties. In acellular matrices, the biomaterials get degraded slowly and newly developed cell-secreted ECM proteins replace the position.

5.3 Recent Perspective into the Role of Stromal Cell in Regenerative Medicine

In this decade, cellular treatment has progressed at rapid pace in preclinical research in vitro and in vivo, as well as clinical trials. Herein, there has been an increase interest in mesenchymal stem cells (MSCs), a type of adult stem cell, in the field of regenerative medicine because of their unique molecular properties (Wang et al. 2012). MSCs are a subset of mesenchymal cells that meet stringent criteria for stem cell activity. Differentiating connective tissue cells that sustain the parenchymal function of a certain organ are known as stromal cells. Stromal cells have been discovered to offer a tremendous promise in regenerative medicine due to their exceptional ability to self-renew and differentiate. Standardized methods for the creation and characterization of MSCs, as well as specified functional assays for assessing their biological potential, are critical factors in determining their therapeutic utility. Stem cells have the ability to self-renew and differentiate into a variety of cell lineages. They show an important standard prototype that can be used to treat a variety of illnesses. Embryonic and nonembryonic stem cells exist. Embryonic stem cells (ESCs) develop from the blastocyst's inner cell mass and differentiate into distinct germ layers. Nonembryonic stem cells, often known as adult stem cells, have a restricted ability to differentiate. However, they can be extracted from a range of tissues. Hence, they are extensively applied in regenerative medicine.

There are two basic types of stems cell one being embryonic and the other being nonembryonic stem cells. Embryonic stem cells (ESCs) that originate in the blastocysts' inner cell mass are capable enough to advance into cells that form all three germ layers. MSCs are multipotent stromal cells that can differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes (fat cells that give rise to marrow adipose tissue). MSCs have been studied in tissue culture (in vitro) as well as in living creatures (in vivo) (Lindner et al. 2010). Mesenchymal cells are colony-forming unit-fibroblasts (CFU-Fs) that were first identified in 1970, by Friedenstein and colleagues (Friedenstein et al. 1970). Pittenger and colleagues (Pittenger et al. 1999) were the first to describe MSCs' trilineage potential. The first MSC clinical trials were carried out in 1995, with a group of 15 patients receiving cultured MSCs to assess the treatment's safety (Wang et al. 2012).

Due to the lack of a distinguishing role, MSCs are referred to as "mesenchymal stem cells," "mesenchymal stromal cells," "BM (bone marrow) stromal cells," and "marrow stromal cells." MSCs are typically identified by their morphological appearance, such as the fibroblastoid phenotype, as well as their plastic adhesion. This technique produces a heterogeneous population that includes single stem cell-like cells as well as progenitor cells with varying lineage commitments, which is not

the case for hematopoietic stem cells (HSCs) and embryonic stem cells (ESCs). HSCs can repopulate bone marrow and produce all blood types, whereas ESCs can participate in embryonic development where tissues are developed only after re-injection into early embryos. MSCs have no established in vivo tests (Pittenger et al. 1999). MSCs are now frequently employed to treat immune-based illnesses such as Crohn's disease, rheumatoid arthritis, diabetes, and multiple sclerosis due to their ability to modulate immune responses, support hematopoiesis, and repair tissues. Nonembryonic stem cells, or adult stem cells, on the other hand, are highly specialized and have limited differentiation ability. They are the most commonly employed in regenerative medicine because they may be isolated from a variety of tissues.

5.4 General Biosensing Technologies

A biosensor is commonly characterized as a transducer that incorporates a biological entity and is coated with a chemically selective covering. This biological entity may consist of biomolecules found in both living and nonliving parts of various systems. These systems can be made up of living creatures with complex biological cycles that cause changes in the concentration of several biomarkers. Enzymes, proteins, DNA, RNA, and others are some common biomarkers of interest for detection, whose quantification may assist in the identification of various changes in a physiological system as well as the detection of anomalies. These biosensors can be printed on a circuit board and used for electrochemical detection of biomarkers (Dutta et al. 2018, 2019, 2020). Biosensors can thus aid in the improvement and optimization of several stages of regenerative medicine application and development. Often the sensor is used one time, this feat can be simplified by incorporating wash free biosensor (Dutta and Lillehoj 2018). The detection range, sensitivity, and specificity of a sensor are all determined by different sensor design strategies (Dutta et al. 2014; Dutta and Lillehoj 2017; Jiaul Haque et al. 2015).

Different techniques based on mechanical, electrical, chemical, and optical measurements can be used to create sensors (Dutta 2020). One example of a mechanical sensor is a microcantilever or a micropillar. The target analyte is attached to the surface of a microcantilever, which is commonly made of silicon. The deflection of the balance or the change in the cantilever's resonant frequency is used to make a mechanical observation. Photoacoustic (PA) imaging is based on the thermoelastic effect, which uses a laser to generate ultrasonic waves. It is capable of producing a noninvasive image of tissues, including blood vessels, down to several centimeters in-depth and with resolutions of a few micrometers. Piezoelectric sensors have a natural frequency of vibration, however, when an analyte is added to the surface of the piezoelectric sensor, the mass changes, resulting in a change in frequency. Quartz crystal microbalance is another technology used for sensing purposes.

Impedance-based measurement techniques are crucial in electrical biosensors. These systems offer advantage of real-time response and can be utilized to obtain

spatial resolution using an array of electrodes. Additionally, these techniques can be combined with microfluidics to achieve high-throughput flow cytometry without the requirement for labeling. Dielectrophoresis is a process that moves particles based on their polarizability using an inhomogeneous electric field. This is a useful strategy for influencing distinct cell populations using polarizability differences. This technique could be used to identify cells without causing harm to the one being studied. When combined with other techniques, this technology could be used to create sensors that improve the effectiveness of cell separation based on several features. Field-effect transistors (FETs) have a wide range of uses outside of the electronics sector, including high-sensitivity biosensors. Apart from the conventionally sensing methods of incorporating immunoactive biospecies, using aptamer-based sensors could assist in the simpler design of sensors with self-designed features and properties (Mandal et al. 2021). They work by changing the impedance of a semiconductor between a source and drain terminal via the field effect of an applied electric field. When a chemical attaches to a surface receptor, the surface potential changes, and the resulting change in channel width changes the current between the source and drain. FETs have been miniaturized (mostly through technologies developed for the electronics sector) and their detection limit has been considerably reduced (Chalklen et al. 2020).

5.5 Biomarkers in Regenerative Medicine

Cells grown on different materials respond differently; for example, cells grown on hard or stiff substrates like glass, or plastic substrate including various synthetic matrices, get attached to and pull them with an elastic moduli in the gigapascal range. These cells on stiff substrate form actin stress fibers and have a more distributed phenotype (Georges and Janmey 2005; Pelham and Wang 1997). Well-defined mechanical qualities often characterized by the elastic moduli can be used to determine the normal functioning organs in a healthy animal. This relies on the fact that elastic moduli depend on the age and type of the tissue. Hence this indicates the health of tissue as the value for elastic moduli lies within a range that is defined by these parameters. Several extrinsic and intrinsic factors are necessary for maintaining the microenvironment of the stromal cells (Table 5.1).

Cells were grown on glass or plastic surfaces, or in a variety of synthetic matrices, attach to and pull on materials with elastic moduli in the gigapascal range. Cells grown on different materials respond differently; for example, cells grown on stiff substrates form actin stress fibers and have a more distributed phenotype (Georges and Janmey 2005; Pelham and Wang 1997). Normally functioning organs in healthy animals often have well-defined mechanical qualities characterized by elastic moduli that fall within a small range that relies on tissue type and the age and health of the organisms. Several extrinsic and intrinsic factors are necessary for maintaining the microenvironment of the stromal cells (Table 5.1).

Table 5.1 Stem cell diagnosing biomarkers

Sl no			Stem cell markers	References
1	Embryonic stem cell	Transcription factors	Oct 3/4, c-Myc, NANOG, LEF-1/TCF1	Li (2010)
		Cell surface markers	SSEA-1,3,4, CD 133, TRA 1-60, TRA 1-81	Zhao et al. (2012)
2	Adult stem cell	Hematopoietic stem cell	CD34, ABCG2	Hawley et al. (2006)
		Mesenchymal stem cell	CD90, CD105, Stro-1	Maleki et al. (2014)
		Neural stem cell	Nestin VI, Msi 1	R&D Systems Tools for Cell Biology ResearchTM (n.d.)

Conventionally cells are studied on materials that are many orders of magnitude stiffer than the biochemical and genetic requirements for cells to live and work properly. Earlier, the role of compatibility of cell and its substrate in the survivability of cells had been overlooked. But recent advances in compatible materials and understanding of how cells respond to different changes in the environment have allowed demonstrations showing cells are extremely sensitive to the mechanical compatibility of cells with their substrates on which they are grown, in the same chemical environment.

There are two key aspects of mechanosensing that are commonly examined or regarded separately. Cells frequently respond to external influences in a predictable way. Hearing is likely the most prominent example, in which acoustic waves cause stereocilia on the hair cell to move. As a result, proteins that govern ion flux through the membrane are subjected to pressures and deformation, triggering biochemical reactions that rise to the sense of sound. The sense of touch is thought to be explained by similar but less well-defined systems. The other part of mechanosensing relies on forces created within the cell rather than external forces (Georges and Janmey 2005; Janmey et al. 2009). The pharmaceutical industry relies on biological systems' chemical sensitivity, but as tissue engineering and regenerative medicine become more common, it is critical that all aspects of biological signaling, chemical, mechanical, and electrical, are understood and managed.

Smith M. et al. showed that a high aspect ratio of the nanostructure can be employed to build "soft" piezoelectric surfaces that can directly interface with developing cells in research. Poly-L-lactic acid (PLLA) piezoelectric characteristics were combined with a nanofabrication approach in this study. The melt-press template wetting process was used to create the PLLA nanotubes. Polymer pellets were heated to 190 °C and pushed into a nanoporous membrane made of anodized aluminum oxide (AAO). The molten polymer flows into the template's pores, covering the walls and forming a nanotube. The diameter of the resultant nanotube was 305 ± 24 nm, while the wall thickness was 56 ± 10 nm (Smith et al. 2019).

5.6 Conventional Strategies for Sensing of Biomolecules

Different techniques such as high-performance liquid chromatography, ELISA, and mass spectrometry (MS) are used in traditional ways for quantifying biological chemical output. In the current development of stem cell-based products, ELISA and PCR-based measurement appear to be the methods of choice (Sue et al. 2014).

Because of their excellent detection limits, ELISA and PCR-based quantification appear to be the methods of choice in the current development of stem cell-based products. However, progress in biosensor design is increasingly meeting the challenge of ensuring high-quality measurements (Sue et al. 2014).

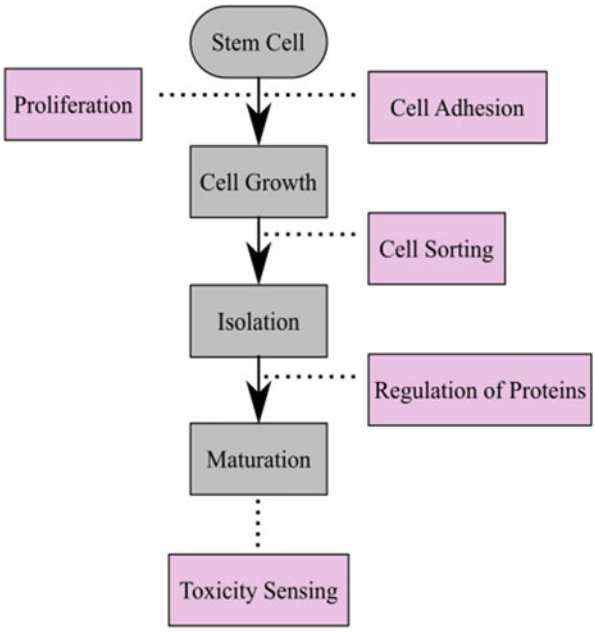
5.7 Biosensors in Regenerative Medicine

Regenerative medicine can be concentrated into tissue engineering, cell therapy, and transplantation of artificial organs. In the case of tissue engineering, biocompatible scaffolds are installed near the wound area where scaffolds mimic as if of extracellular matrix by attracting cells to proliferate, and differentiate and form tissues of proper geometric shape. On the other hand, the self-renewal property of stem cells is utilized by injecting adult stem cells into the wounded area so that the damaged area is reconstructed or repaired over time.

Regenerative medicine aims to solve the issues of untreatable diseases by regenerating the part that was diseased and now can be regenerated. This can act as a cure for ailments caused by disease, trauma, or congenital issues. Regenerative medicine is used in various areas like the treatment of cancer, abdominal adhesions (Carmichael et al. 2022) kidney regeneration (Ko et al. 2021). Regenerative medicine can be improved by improving electrical signaling, cell adhesion, alteration in mechanical properties, adhesion and integration with organs, differentiation, proliferation, vascularization, and maturation of cells (Yadid et al. 2019). Regenerative medicine is used to treat cancer, abdominal adhesions (Carmichael et al. 2022), and kidney regeneration, among other things (Ko et al. 2021). Improved electrical signaling, cell adhesion, mechanical characteristics, adhesion and integration with organs, and cell differentiation, proliferation, vascularization, and maturation can all help in regenerative medicine (Scheme 5.1) (Yadid et al. 2019).

The developing regenerative medicine therapy can be applied in various aspects mentioned below. Various noninvasive biosensing techniques especially electrochemical biosensing platforms are mentioned in this paper.

From this discussion, we can derive the workflow of sensors that can be developed for improving the results for regenerative medicines. First type of sensor can tell us about the proliferation of cells. CMOS-based sensors can detect the proliferation of the cell by measuring the capacitive behavior of cells in a counter ionic environment. Cell holds charge on the surface, this surface charge gets accumulated and can be mapped with the growth or death of cells making it a good indicator of cell proliferation (Prakash and Abshire 2008). Second type of sensors is the ones that can tell how cell is being attached to the surface. Cell adhesion is one of the most



Scheme 5.1 Requirement of sensors at different stages of regenerative medicine

important factors in the whole process, the detection of adhesion of cells is crucial, and it can be detected by measurement of the electrochemical impedance of surface of an electrode. This can also be achieved by measurement of change in the piezoelectric resonant frequency of the surface. Both these properties depend on the adhesion of cell that alters the electrical and mechanical properties of the surface. Third is required in the sorting of cells, like separating dead and alive cells or cells with different types. The cells are dielectric and can experience a force when made to flow through an electric gradient. This phenomenon is called cell dielectrophoresis and can be used to separate the cells of different types in a microfluidic channel. Fourth type of biosensors is the ones required for the quantization of regulation proteins in the culture (Ling et al. 2012). In the differentiation of cells, different regulation factors are responsible. Few of these factors are Oct4, Sox2, Klf4, and c-Myc, these factors can be detected and quantified by using electrochemical biosensors (Takahashi and Yamanaka 2006). Having this information will help in understanding the role of differentiation factors at different stages. Further, this will also be important once the differentiation process starts to proceed so that it can ensure that the process of development of regenerative medicine is going as planned. Finally, the toxicity of cells in the culture needs to be monitored. Cells with highly toxic contents cannot be used for regenerative medicine applications and will be leading to the failure of the whole process. Detectors for such cases can be made using cell-based sensors where cells are grown on electrode that can detect changes in the resistance of the surface during the presence of toxic material. When toxic material is present it may lead to the formation of lipid vacuoles that expose the

electrode surface resulting into low resistance and when the toxic material is removed the vacuoles are cured or are not formed leading to increase in the resistance of the sensor, hence detecting the presence of toxicity in the culture (Cho et al. 2009).

5.7.1 Biosensors to Detect Pluripotent Stromal Cells

The multipotent nature of stromal cells can give rise to various differentiated functional cells or tissues. To induce functionally organized tissue, the phenotypical environment of stromal cells is very crucial. Therefore, stimulus-responsive for differentiation has to be controlled. Any kind of genetic, epigenetic, and proteomic changes can alter the biological activity of stromal cells and hence the differentiation pattern. The vigorous self-renewal property of stem cells plays an important role in the process of development. The dynamic interaction between the extrinsic and intrinsic factors with the stromal cell microenvironment defines the self-renewal efficiency of the injured tissue in regenerative medicine (Fig. 5.1) (R&D Systems Tools for Cell Biology ResearchTM n.d.).

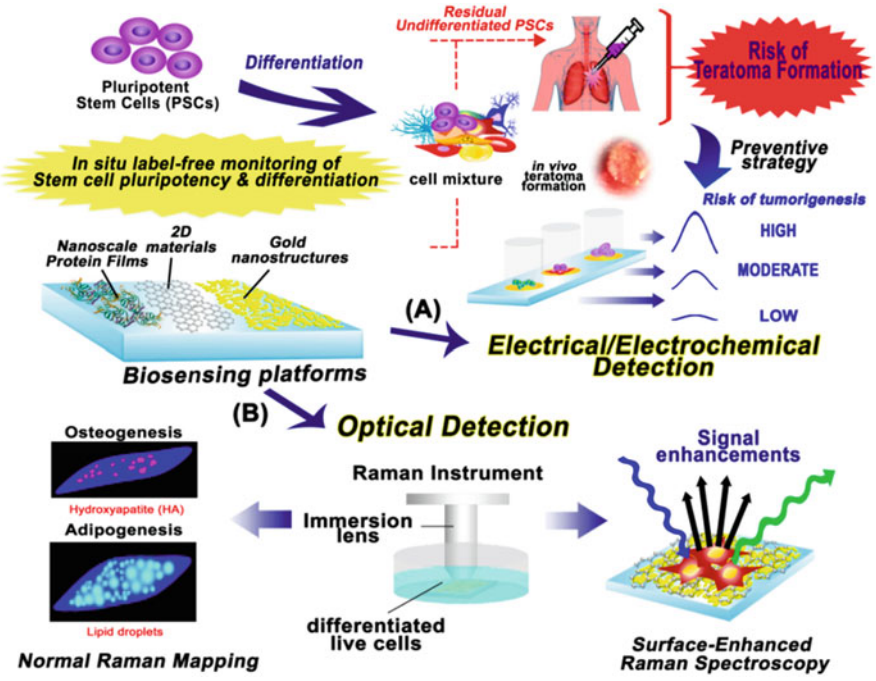


Fig. 5.1 Schematic representation of label-free biosensing platform for the detection of differentiation potential of stem cells. (**a**) Detection through electrochemical measurement. (**b**) Optical monitoring using Raman spectroscopy (Suhito et al. 2018)

Table 5.2 Electrochemical biosensing platform to detect the stem cells

Sl no	Differentiation type	Biosensing platform	Detection parameters	References
1	Neurogenesis	PEDOT-PSS/ITO microelectrode array	Impedance spectroscopy	Furukawa et al. (2013)
2	Neurogenesis	Au-nanodot/ITO	Cyclic voltammetry	An et al. (2015)
3	Vasculogenesis	L-cystine/Au electrode array	Electrochemical impedance spectroscopy	Szulcek et al. (2014)
		PDMS/platinum electrode	Cyclic voltammetry	Yea et al. (2016)
4	Neurogenesis	Gold electrode array	Electrical impedance analysis	Lee et al. (2014)
5	Neurogenesis	3D carbon scaffold	Amperometry	Amato et al. (2014)

Further, in the case of therapeutic application, in the field of regenerative medicine, continuous monitoring of differentiation potential is much needed to check the minimal loss of the differentiated tissues. Several biochemical and biophysical tools are already in use such as western blot, PCR, and flow cytometry to serve the purpose. However, such techniques are very time-consuming, invasive, and laborsome. Biosensors can be an excellent alternative to tracking the differentiation activity of stromal cells (Table 5.2).

The electrophysiological properties of nerve cells depend largely on the differentiation activity of neural cells. Any prognostic efficacy of traumatic brain injuries is also monitored by tracking the differentiation pattern of neural stem cells (NSC). Furukawa et al. fabricated poly(3,4-ethylenedioxythiophene)–poly (styrenesulfonate) (PEDOT–PSS) on an ITO microelectrode array to understand the differentiation activity of the NSC. NSC generally migrate in a wider area, which makes the detection of electrical activity a little difficult to analyze. However, examining the burst pattern in electrical cell activity analysis, the differentiated and undifferentiated NSCs are separated. Undifferentiated NSCs did not show any proper burst channel pattern whereas differentiated NSCs exhibited proper burst pattern. In another study, Lee et al. used a capacitive sensor to separate out differentiated and undifferentiated NSCs. Interdigitated gold electrodes were fabricated with poly-L-Lysine so that cells can easily adhere over the working surface. They observed that differentiated NSCs exhibit a steady increase of capacitance with peak formation whereas undifferentiated NSCs did not express any such peak. Thus, by observing the capacitance change they differentiated the NSCs. Bagnaninchi et al. have reported the differentiation pattern of osteoblast and adipocyte lineages using electrochemical impedance spectroscopy. Real-time electric cell–substrate impedance sensing (ECIS) is an established technique to track cell motility and differentiation efficiency. ECIS is a label-free, real-time impedance detection technique to examine the morphological properties of adherent cells. A small alternating current is applied across the ECIS array. Upon the adherence of

cells to the ECIS array, it forms an insulating layer and increases the impedance (Szulcek et al. 2014). They used adipose-derived stem cells (ADSC) to induce differentiation and stained them with alizarin red dye. Eight gold microelectrode arrays were used and with the implementation of ECIS Zθ software frequency-dependent resistance is measured (Bagnaninchi and Drummond 2011). A controlled environment in a miniaturized microfluidic chip helps in label-free, low sample analysis that induces a dramatic decrease in analysis time, and high efficiency. An et al. used an on-chip electrochemical platform to detect the differentiation activity of neural stem cells. They fabricated gold nanodots over an ITO chip to immobilize the cells on the working surface (Jung et al. 2011). The cells were induced through immunostaining and the differentiation pattern was checked by the electrochemical voltammetric response of the gold nanodot decorated ITO chip. They noticed that the E_{PC} peak in cyclic voltammogram was decreased in differentiated cells in comparison to undifferentiated cells whereas the E_{pa} peak of differentiated cells was much higher than in undifferentiated cells. In the field of regenerative medicine, such on-site monitoring platform is useful in the diagnosis of a particular disease, analysis of drug dosage response, and cell differentiation. In the field of regenerative medicine, Raman spectroscopy is a well-established method that offers high throughput sensing to measure the molecular characteristics of the differentiated cells. Nevertheless, this high throughput technique requires complex sample pretreatment and a benchtop instrument. In order to address the limitations, Yea et al. developed an electrochemical biosensor to monitor the differentiation efficiency of mouse embryonic stem cells. A gold microelectrode was used to immobilize 1-naphthyl phosphate (NP). In presence of embryonic stem cell surface biomarker alkaline phosphatase, NP gets dephosphorylated into 1-naphthol. Based on measuring the voltammetric electrochemical response of NP and 1-naphthol, the differentiation efficiency of mouse embryonic stem cells was measured. In another study, Yea et al. distinguished undifferentiated human induced pluripotent stem cells (hiPSC) from differentiated stem cells using the electrochemical cyclic voltammogram technique. Matrigel-modified gold working electrode was used to immobilize the stem cells. Depending upon the variance in the E_{pc} value, the differentiated and undifferentiated stem cells were distinguished. Further, they observed that cathodic peak current was increased with increasing undifferentiated cell number. Such a simple yet reliable technique supports the conventional FACS and real-time PCR analysis data (Jeong et al. 2017). Although an electrochemical on-chip detection system is a growing field, the output signal in electrochemistry depends very much upon the composition of the electrolytes. Alongside, reproducibility and stability are also a huge concern in electrochemical biosensor. In spite of the growing advantages of biosensors in recent times, the conventional optical detection system is much established in the detection of differentiation activity of stem cells.

Implanting supramolecular substances containing cell growth factor binding patterns into a diseased or traumatic tissue defect is one of the common practices in regenerative medicine. Supramolecular substances react to stimulate tissue healing, also sometimes accompanied by the recruitment of biological system

(Cheng et al. 2019). Cheng et al. employed the use of prolyl hydroxylase enzyme that can be used as both a regeneration-inducing treatment and a structure-directing agent in a supramolecular polymer system that makes shear-thinning nanofiber hydrogels.

Ear tissue regeneration, similar to epimorphic regeneration due to transitory activation of hypoxia-inducible factor-1 can be observed when mice with a critical-sized ear defect were injected with supramolecular hydrogel under their skins.

This drug-induced regeneration technique makes use of a simple and translatable supramolecular architecture that eliminates the necessity for biological delivery (e.g., growth factors, cells) and avoids the implantation of a foreign material directly into a tissue defect. Reprogramming somatic cells to pluripotent cells, which can subsequently develop into specialized cells to grow the injured tissue or organ in the body, is another generative medicine technique. This can be accomplished by reactivating dormant genes. Kazutoshi Takahashi and Shinya Yamanaka created murine ES-like cell lines from mouse embryonic fibroblasts (MEFs) and skin fibroblasts for the first time in 2006 by simply expressing four transcription factor genes encoding Oct4, Sox2, Klf4, and c-Myc (Takahashi and Yamanaka 2006). Induced pluripotent stem (iPS) cells were named after these somatic cell-derived cell lines. These iPS cell lines have shape and development characteristics comparable to ES cells and express ES cell-specific genes. The creation of germ-cell-tumor (teratoma)-containing tissues from all three germ layers was observed after iPS cells were transplanted into immunodeficient mice, indicating the pluripotent capacity of iPS cells. However, there were two issues: low iPS cell line efficiency and some differences in gene expression profile between iPS cells and ES cells. The latter sparked concerns that cell reprogramming alone might not be enough to restore full pluripotency in somatic cells, as seen in ES cells (Pluripotent Stem Cells, iPs cells | Learn Science at Scitable n.d.). Later, examination of the genes contained in the biosensors can be used to detect somatic cell transition, such as the detection of Oct4 utilizing biosensors (Ma et al. 2019). The cells can also develop cancer throughout development, therefore a sensor based on Sox2 detection can be used to monitor them (Tarimeri and Sezgintürk 2020).

5.7.2 Biosensor Prospect in Cartilage Regeneration

As a result of a deficient metabolic rate and very less blood supply, cartilage regeneration is a very slow process. Injuries such as osteochondritis, rheumatoid arthritis, polytonicities associated with cartilage damage may lead to exposure to bone tissues and upon repeated friction cause bone damage. Regenerative technologies can be an attractive area for cartilage regeneration. In that context, real-time monitoring of regenerative events and wound healing is much required. For that, the molecular markers associated with cartilage degeneration must be accessed and sensed. Over the last few decades, miniaturized biosensing platforms have gathered a lot of attention in the early diagnosis of cartilage regeneration.

Matrix metalloproteinase (MMP) is a type of collagenase 3 protein that is associated with the degradation of osteoarthritic cartilage in osteoarthritic patients. For that reason, MMP is studied to be a potential target in osteoarthritis. Deng et al. utilized phage-display technique to screen a highly specific phage library against MMP (Deng et al. 2000). Such a phage library can be used in regenerative medicine to screen inflammatory markers like MMP using a biosensor platform Furthermore, the recent advancement of luminescence biosensors has evolved as an emerging noninvasive technique to monitor cartilage regeneration (Je et al. 2017). Continuous monitoring of the biochemical and molecular properties in cartilage regeneration is very crucial for therapeutic interventions.

5.7.3 Biosensor for Prolotherapy

Prolotherapy is generally done to reduce muscle and joint pain by injecting an irritant solution. The irritant sugar solution induces the development of connective tissue in the joints. Depending upon the extent of injury, repeated exposure to an irritant, stabilize the ligaments.

The recent advancement of nanotechnology has developed microfluidic devices that can trigger immediately in response to the local environment. The smart sensor can be advanced, which can sense the local microenvironment of the joints such as pH, oxygen content, the extent of blood supply, and release irritant by a feedback loop mechanism to improve tissue regeneration.

5.7.4 Regenerative Medicine On Chip

On-chip technology is a microfabricated platform that can mimic normal human physiological systems. The physiological and biomechanical conditions in the human body are generally controlled in small microfluidic channels. To control the microenvironment and associated challenges, organoid models on a microfluidic chip can be an excellent approach. Furthermore, the real-time monitoring of drug usage and associated stimulus from organoids can also be measured using microfluidic biosensors. Shin et al. fabricated a drug dose doxorubicin-dependent microfluidic biosensor that can detect creatine kinase myocardial band from damaged cardiac tissue. With the use of the label-free electrochemical microfluidic device, they were able to detect CK-MB from cardiac spheroids at the picomolar level. Microfluidic control of extracellular parameters such as pH, temperature, and oxygen content is very crucial in organ-on-chip. Zhang et al. reported a microfluidic integrated platform that was completely automated to detect drug-dependent toxicity in liver and heart organoids on a chip device. They measured the immunogenic level of albumin, GST-α, and CK-MB using an electrochemical immunosensor secreted from the heart-organoid in response to immunogenic therapeutic acetaminophen (Zhang et al. 2017). Although there are several disadvantages, associated with

automated, multifunctional microfluidic organ-on-chip devices in the field of regenerative medicine, several attempts have been initiated to address the limitations.

5.8 Nanotechnology in Regenerative Medicine

The wide application of nanotechnology in recent times has opened a new horizon in regenerative medicine and related areas. Hence, the physiological changes of our body depend immensely on the nanoscale dimension and associated functionalization of the nanomaterials, it is very important to mimic the functional tissue to provide structural and functional support in regenerative medicine. Several biodegradable nanomaterials are getting huge interest in biosensor applications in the field of regenerative medicine. For example, Lee et al. micropatterned poly (ethylene glycol)-poly (acrylic acid) (PEG-PAA) hydrogel surface with biotin protein for the detection of streptavidin. Considering the application of hydrogels in tissue engineering, such micropatterned system can be used for biosensor application (W. Lee et al. 2008). Furthermore, various multifunctional nanomaterials have been widely used in biosensor applications for simultaneous monitoring as well as treatment. Such a multifunctional nanosystem can be used in stem cell transplantation alongside physiological monitoring (J. H. Lee et al. 2016). The technical advances in recent times have allowed systemic microfabrication on that multifunctional nanosystem to attain certain physicochemical properties required for regenerative therapy. Desai et al. microfabricated a smart biocapsule that has a homogenous pore size that allows molecules to pass with a diameter less than 6 nm whereas excludes molecules that have a diameter of more than 15 nm. Such a smart system can be advanced further by placing a small diagnostic device inside the biocapsule. Moreover, in a large bioprocess system, accurate control and systemic release of molecules can regulate the culture condition to improve tissue generation (Desai 2000).

5.9 Bioprocess Control and Therapeutic Applications in Regenerative Medicine

Biomanufacturing has taken an important role in the field of industrial process engineering for the last few decades. In the process of biomanufacturing, biological systems such as living organisms, animal tissues, and plants are generally used to produce important biomolecules. The manufacturing efficacy and commercial utilization in the field of biomanufacturing met a huge success after the establishment of different fabrication techniques such as 3D printing. Such rapid development in biomanufacturing can address many unmet problems in the field of regenerative medicine for instance organ fabrication. Biosensors can be used to check the quality parameters in such cases. The efficacy of biomanufacturing is very much dependent on the metabolites in the culture media. By regular sensing using enzyme-based biosensor devices, the appropriate measures can be taken in order to improve the

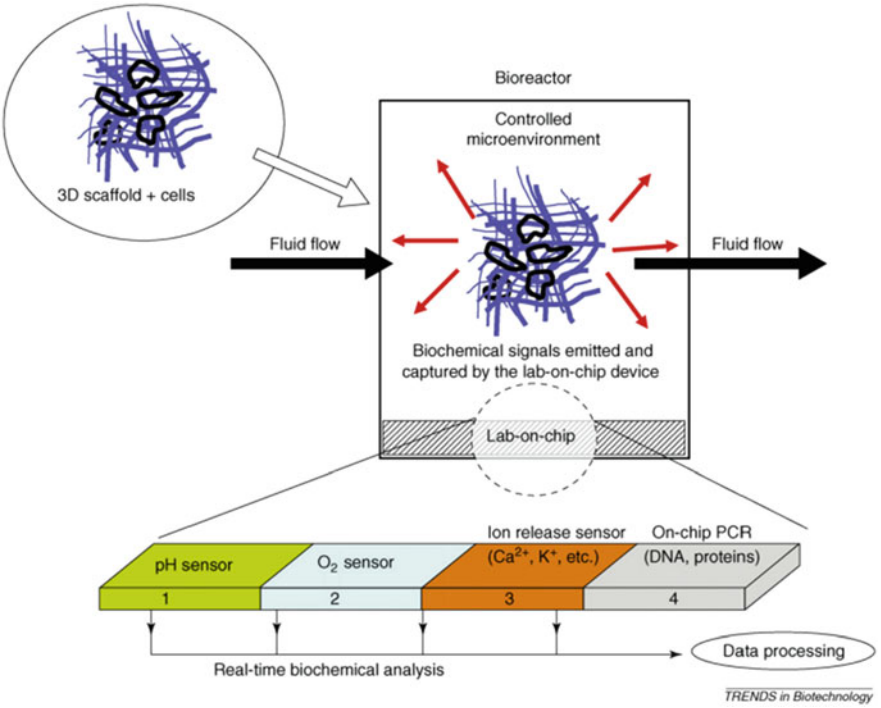


Fig. 5.2 Schematic representation of biomanufacturing in regenerative medicine. Reprinted with permission from Elsevier (Engel et al. 2008)

quality of the manufacturing process (Rogers and Church 2016). Further, the real-time monitoring of the production should also be checked to take necessary actions (Fig. 5.2).

With the development of an appropriate biosensor framework, metabolic products in the biomanufacturing industry can be examined, optimized, and regulated to have better process efficiency. In the field of regenerative medicine, therapeutic efficacy should also be sensed on regular basis. For instance, systemic administration of CD34-positive cells to immunocompromised mouse can influence neovascularization and enhance cardiovascular diseases like stroke. A relationship between neovascularization and the effect of CD-positive cells provides information about the therapeutic efficacy of different regenerative medicines to control angiogenesis (Yea et al. 2016). Thus by using of biosensing device the extracellular environment such as metabolic factors can be tracked to have a better understanding of the therapeutic control in regenerative medicine.

5.10 Conclusions

The recent advancement of nanotechnology and biosensors offers wide application in the biomedical field. Development in the fabrication techniques like 3D printing in addition to the integration of electronic components in biosensor development opened a new area in simple detection of the analyte of interest. The biosensor application has evolved in a wide range of biomedical field along with regenerative medicine. Smart sensing incorporated with responsive drug release allows personalized diagnosis and treatment. In this paper, we mentioned conventional and biosensor-related regenerative therapy. In stem cell research, Raman spectroscopy and electrochemical biosensors are the most reliable techniques. Although Raman spectroscopy allows label-free and nondestructive detection of analytes, a complex instrument setup is required. Moreover, a huge amount of time is required for each analyte detection. On the other hand, electrochemical biosensors enable rapid, real-time detection of the analyte of interest. In the field of regenerative medicine, the separation of differentiated and undifferentiated stem cells is much required. Simultaneous detection and drug delivery also reduce healthcare costs. Altogether, the combination of Raman spectroscopy, electrochemical biosensor, and advancement in nanotechnology has opened a new horizon in regenerative therapy. Although much work has been done, still their research progress and development are still in its infancy.

Acknowledgments Authors gratefully acknowledge the Start-Up Research Grant (SRG) funded by Science & Engineering Research Board (SERB) (SRG/2020/000712), Department of Science and Technology (DST) (Government of India, Ministry of Science and Technology, (Technology Development and Transfer, TDP/BDTD/12/2021/General), Indo-German Science & Technology Centre (IGSTC) (IGSTC/Call 2019/NOMIS/22/2020-21/164), and Institute Scheme for Innovative Research and Development (ISIRD) (IIT/SRIC/ISIRD/2019–2020/17), Indian Institute of Technology Kharagpur (IIT Kharagpur), India for the financial support.

References

Amato L, Heiskanen A, Caviglia C, Shah F, Zór K, Skolimowski M, Madou M, Gammelgaard L, Hansen R, Seiz EG, Ramos M, Moreno TR, Martínez-Serrano A, Keller SS, Emnéus J (2014) Pyrolysed 3D-carbon scaffolds induce spontaneous differentiation of human neural stem cells and facilitate real-time dopamine detection. Adv Funct Mater 24(44):7042–7052. https://doi.org/10.1002/adfm.201400812

An JH, Kim SU, Park MK, Choi JW (2015) Electrochemical detection of human mesenchymal stem cell differentiation on fabricated gold nano-dot cell chips. J Nanosci Nanotechnol 15(10): 7929–7934. https://doi.org/10.1166/jnn.2015.11225

Bagnaninchi PO, Drummond N (2011) Real-time label-free monitoring of adipose-derived stem cell differentiation with electric cell-substrate impedance sensing. Proc Natl Acad Sci U S A 108(16):6462–6467. https://doi.org/10.1073/pnas.1018260108

Carmichael SP, Shin J, Vaughan JW, Chandra PK, Holcomb JB, Atala AJ (2022) Regenerative medicine therapies for prevention of abdominal adhesions: a scoping review. J Surg Res 275: 252–264. https://doi.org/10.1016/J.JSS.2022.02.005

Chalklen T, Jing Q, Kar-Narayan S (2020) Biosensors based on mechanical and electrical detection techniques. Sensors 20(19):5605. https://doi.org/10.3390/S20195605

Cheng J, Amin D, Latona J, Heber-Katz E, Messersmith PB (2019) Supramolecular polymer hydrogels for drug-induced tissue regeneration. ACS Nano 13(5):5493–5501. https://doi.org/10.1021/ACSNANO.9B00281/SUPPL_FILE/NN9B00281_SI_001.PDF

Cho S, Gorjup E, Thielecke H (2009) Chip-based time-continuous monitoring of toxic effects on stem cell differentiation. Ann Anat 191(1):145–152. https://doi.org/10.1016/j.aanat.2008.08.005

Deng SJ, Bickett DM, Mitchell JL, Lambert MH, Blackburn RK, Carter HL, Neugebauer J, Pahel G, Weiner MP, Moss ML (2000) Substrate specificity of human collagenase 3 assessed using a phage-displayed peptide library. J Biol Chem 275(40):31422–31427. https://doi.org/10.1074/jbc.M004538200

Desai TA (2000). Micro-and nanoscale structures for tissue engineering constructs. In: Medical engineering & physics (vol 22). www.elsevier.com/locate/medengphy

Dutta G (2020) Electrochemical biosensors for rapid detection of malaria. Mater Sci Energy Technol 3:150–158. https://doi.org/10.1016/J.MSET.2019.10.003

Dutta G, Jallow AA, Paul D, Moschou D (2019) Label-free electrochemical detection of S. mutans exploiting commercially fabricated printed circuit board sensing electrodes. Micromachines 10(9):575. https://doi.org/10.3390/MI10090575

Dutta G, Kim S, Park S, Yang H (2014) Washing-free heterogeneous immunosensor using proximity-dependent electron mediation between an enzyme label and an electrode. Anal Chem 86(9):4589–4595. https://doi.org/10.1021/AC5006487/SUPPL_FILE/AC5006487_SI_001.PDF

Dutta G, Lillehoj PB (2017) An ultrasensitive enzyme-free electrochemical immunosensor based on redox cycling amplification using methylene blue. Analyst 142(18):3492–3499. https://doi.org/10.1039/C7AN00789B

Dutta G, Lillehoj PB (2018) Wash-free, label-free immunoassay for rapid electrochemical detection of PfHRP2 in whole blood samples. Sci Rep 8(1):1–8. https://doi.org/10.1038/s41598-018-35471-8

Dutta G, Regoutz A, Moschou D (2018) Commercially fabricated printed circuit board sensing electrodes for biomarker electrochemical detection: the importance of electrode surface characteristics in sensor performance. PRO 2:741. https://doi.org/10.3390/proceedings2130741

Dutta G, Regoutz A, Moschou D (2020) Enzyme-assisted glucose quantification for a painless Lab-on-PCB patch implementation. Biosens Bioelectron 167:112484. https://doi.org/10.1016/J.BIOS.2020.112484

Engel E, Michiardi A, Navarro M, Lacroix D, Planell JA (2008) Nanotechnology in regenerative medicine: the materials side. Trends Biotechnol 26(1):39–47. https://doi.org/10.1016/J.TIBTECH.2007.10.005

Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) THE DEVELOPMENT OF FIBROBLAST COLONIES IN MONOLAYER CULTURES OF GUINEA-PIG BONE MARROW AND SPLEEN CELLS. Cell Prolif 3(4):393–403. https://doi.org/10.1111/J.1365-2184.1970.TB00347.X

Furukawa Y, Shimada A, Kato K, Iwata H, Torimitsu K (2013) Monitoring neural stem cell differentiation using PEDOT-PSS based MEA. Biochim Biophys Acta General Subjects 1830(9):4329–4333. https://doi.org/10.1016/j.bbagen.2013.01.022

Georges PC, Janmey PA (2005) Cell type-specific response to growth on soft materials. J Appl Physiol (1985) 98(4):1547–1553. https://doi.org/10.1152/JAPPLPHYSIOL.01121.2004

Hawley RG, Ramezani A, Hawley TS (2006) Hematopoietic stem cells. Methods Enzymol 419:149–179

Jahani M, Rezazadeh D, Mohammadi P, Abdolmaleki A, Norooznezhad A, Mansouri K (2020) Regenerative medicine and angiogenesis; challenges and opportunities. Adv Pharm Bull 10(4):490–501. https://doi.org/10.34172/apb.2020.061

Janmey PA, Winer JP, Murray ME, Wen Q (2009) The hard life of soft cells. Cell Motil Cytoskeleton 66(8):597–605. https://doi.org/10.1002/CM.20382

Je HJ, Kim MG, Kwon HJ (2017) Bioluminescence assays for monitoring chondrogenic differentiation and cartilage regeneration. Sensors (Switzerland) 17(6). https://doi.org/10.3390/s17061306

Jeong HC, Choo SS, Kim KT, Hong KS, Moon SH, Cha HJ, Kim TH (2017) Conductive hybrid matrigel layer to enhance electrochemical signals of human embryonic stem cells. Sensors Actuators B Chem 242:224–230. https://doi.org/10.1016/j.snb.2016.11.045

Jiaul Haque AM, Kim J, Dutta G, Kim S, Yang H (2015) Redox cycling-amplified enzymatic Ag deposition and its application in the highly sensitive detection of creatine kinase-MB. Chem Commun 51(77):14493–14496. https://doi.org/10.1039/C5CC06117B

Jung M, El-Said WA, Choi JW (2011) Fabrication of gold nanodot arrays on a transparent substrate as a nanobioplatform for label-free visualization of living cells. Nanotechnology 22(23). https://doi.org/10.1088/0957-4484/22/23/235304

Kaur H, Sharma A (2015) Biosensors: recent advancements in tissue engineering and cancer diagnosis. Biosensors J 4(2):1–3. https://doi.org/10.4172/2090-4967.1000131

Ko KW, Park SY, Lee EH, Yoo YI, Kim DS, Kim JY, Kwon TG, Han DK (2021) Integrated bioactive scaffold with polydeoxyribonucleotide and stem-cell-derived extracellular vesicles for kidney regeneration. ACS Nano 15(4):7575–7585. https://doi.org/10.1021/ACSNANO.1C01098/SUPPL_FILE/NN1C01098_SI_001.PDF

Lee JH, Lee T, Choi JW (2016) Nano-biosensor for monitoring the neural differentiation of stem cells. Nano 6(12). https://doi.org/10.3390/NANO6120224

Lee R, Kim IS, Han N, Yun S, Park KI, Yoo KH (2014) Real-time discrimination between proliferation and neuronal and astroglial differentiation of human neural stem cells. Sci Rep 4:6319. https://doi.org/10.1038/srep06319

Lee W, Choi D, Lee Y, Kim DN, Park J, Koh WG (2008) Preparation of micropatterned hydrogel substrate via surface graft polymerization combined with photolithography for biosensor application. Sensors Actuators B Chem 129(2):841–849. https://doi.org/10.1016/j.snb.2007.09.085

Li Y-Q (2010) Master stem cell transcription factors and signaling regulation. Cell Reprogram 12(1):3–13

Lindner U, Kramer J, Rohwedel J, Schlenke P (2010) Mesenchymal stem or stromal cells: toward a better understanding of their biology? Transfus Med Hemother 37(2):75–83. https://doi.org/10.1159/000290897

Ling SH, Lam YC, Chian KS (2012) Continuous cell separation using dielectrophoresis through asymmetric and periodic microelectrode array. Anal Chem 84(15):6463–6470. https://doi.org/10.1021/AC300079Q/SUPPL_FILE/AC300079Q_SI_001.PDF

Ma J, Xue L, Zhang M, Li C, Xiang Y, Liu P, Li G (2019) An electrochemical sensor for Oct4 detection in human tissue based on target-induced steric hindrance effect on a tetrahedral DNA nanostructure. Biosens Bioelectron 127:194–199. https://doi.org/10.1016/J.BIOS.2018.12.029

Maleki M, Ghanbarvand F, Behvarz MR, Ejtemaei M, Ghadirkhomi E (2014) Comparison of mesenchymal stem cell markers in multiple human adult stem cells. Int J Stem Cells 7(2):118–126. https://doi.org/10.15283/ijsc.2014.7.2.118

Mandal M, Dutta N, Dutta G (2021) Aptamer-based biosensors and their implications in COVID-19 diagnosis. Anal Methods 13(45):5400–5417. https://doi.org/10.1039/D1AY01519B

Pelham RJ, Wang YL (1997) Cell locomotion and focal adhesions are regulated by substrate flexibility. Proc Natl Acad Sci U S A 94(25):13661–13665. https://doi.org/10.1073/PNAS.94.25.13661/ASSET/29B06AAA-EF44-464A-91B5-8ED8DEB779BC/ASSETS/GRAPHIC/PQ2571755004.JPEG

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284(5411):143–147. https://doi.org/10.1126/SCIENCE.284.5411.143/SUPPL_FILE/983855S5_THUMB.GIF

Pluripotent Stem Cells, iPs cells | Learn Science at Scitable. (n.d.). https://www.nature.com/scitable/topicpage/turning-somatic-cells-into-pluripotent-stem-cells-14431451/. Accessed 23 Mar 2022

Prakash SB, Abshire P (2008) Tracking cancer cell proliferation on a CMOS capacitance sensor chip. Biosens Bioelectron 23(10):1449–1457. https://doi.org/10.1016/j.bios.2007.12.015

R&D Systems Tools for Cell Biology Research™ (n.d.) NEUROSCIENCE FOCUS: NEURAL STEM CELLS Neural Stem Cells & Differentiation Markers. www.RnDSystems.com/StemCells

Rogers JK, Church GM (2016) Genetically encoded sensors enable real-time observation of metabolite production. Proc Natl Acad Sci U S A 113(9):2388–2393. https://doi.org/10.1073/pnas.1600375113

Sampogna G, Guraya SY, Forgione A (2015) Regenerative medicine: historical roots and potential strategies in modern medicine. J Microsc Ultrastruct 3(3):101–107. https://doi.org/10.1016/J.JMAU.2015.05.002

Smith M, Lindackers C, Mccarthy K, Kar-Narayan S, Smith M, Lindackers C, Mccarthy K, Kar-Narayan S (2019) Enhanced molecular alignment in poly-l-lactic acid nanotubes induced via melt-press template-wetting. Macromol Mater Eng 304(3):1800607. https://doi.org/10.1002/MAME.201800607

Sue MJ, Yeap SK, Omar AR, Tan SW (2014) Application of PCR-ELISA in molecular diagnosis. Biomed Res Int 2014:653014. https://doi.org/10.1155/2014/653014

Suhito IR, Angeline N, Choo SS, Woo HY, Paik T, Lee T, Kim TH (2018) Nanobiosensing platforms for real-time and non-invasive monitoring of stem cell pluripotency and differentiation. Sensors 18(9):2755. https://doi.org/10.3390/S18092755

Szulcek R, Bogaard HJ, van Nieuw Amerongen GP (2014) Electric cell-substrate impedance sensing for the quantification of endothelial proliferation, barrier function, and motility. J Vis Exp 85:51300. https://doi.org/10.3791/51300

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676. https://doi.org/10.1016/J.CELL.2006.07.024

Tarimeri N, Sezgintürk MK (2020) A high sensitive, reproducible and disposable immunosensor for analysis of SOX2. Electroanalysis 32(5):1065–1074. https://doi.org/10.1002/ELAN.201900557

Wang S, Qu X, Zhao RC (2012) Clinical applications of mesenchymal stem cells. J Hematol Oncol 5:19. https://doi.org/10.1186/1756-8722-5-19

Yadid M, Feiner R, Dvir T (2019) Gold nanoparticle-integrated scaffolds for tissue engineering and regenerative medicine. Nano Lett 19(4):2198–2206. https://doi.org/10.1021/ACS.NANOLETT.9B00472/ASSET/IMAGES/ACS.NANOLETT.9B00472.SOCIAL.JPEG_V03

Yea CH, Jeong HC, Moon SH, Lee MO, Kim KJ, Choi JW, Cha HJ (2016) In situ label-free quantification of human pluripotent stem cells with electrochemical potential. Biomaterials 75:250–259. https://doi.org/10.1016/j.biomaterials.2015.10.038

Zhang YS, Aleman J, Shin SR, Kilic T, Kim D, Shaegh SAM, Massa S, Riahi R, Chae S, Hu N, Avci H, Zhang W, Silvestri A, Nezhad AS, Manbohi A, de Ferrari F, Polini A, Calzone G, Shaikh N et al (2017) Multisensor-integrated organs-on-chips platform for automated and continual in situ monitoring of organoid behaviors. Proc Natl Acad Sci U S A 114(12):E2293–E2302. https://doi.org/10.1073/pnas.1612906114

Zhao W, Ji X, Zhang F, Li L, Ma L (2012) Embryonic stem cell markers. Molecules 17(6):6196–6236. https://doi.org/10.3390/molecules17066196

Acute and Chronic Wound Management: Assessment, Therapy and Monitoring Strategies

6

Anisha Kabir, Anwita Sarkar, and Ananya Barui

6.1 Introduction

Wound healing is a dynamic, interrelated and well-organized process dependent on highly regulated factors that work in synchrony to restore the structural and functional integrity of the injured tissue. The sequence of healing process works in a normal manner in the vast majority of wounds, however under underlying pathological conditions, like diabetes, it undergoes modification and leads to a delayed healing. Chronic wounds arising from the failed healing process possess a serious load on both the patient and the healthcare system. It has been estimated that nearly 6.5 million patients annually suffer from chronic wounds due to the increasing prevalence of diabetes. Treatment of chronic wounds nearly costs over US$25 billion per year to the medical system as a whole (Brem et al. 2007). Along with chronic wounds, acute and simple wounds also require proper care and attention. The process of wound healing is highly fascinating and gaining widespread interest both scientifically and commercially (Han and Ceilley 2017).

Treatment plans for both acute and chronic wounds involve invasive, non-invasive, external and internal techniques. Appropriate diagnosis, assessment and monitoring are the primary and the predominant approach to a wound care management plan. Assessment and monitoring of the healing process involve the evaluation of both the visual appearance and the detailed analysis of the internal microstructure of the wounds. Both invasive and non-invasive methods have evolved to evaluate the wound assessment rate. Several non-invasive optical imaging modalities have been currently adopted to evaluate the components of the healing wounds. Three conditions that are important to be considered during the

A. Kabir · A. Sarkar · A. Barui (✉)
Centre for Healthcare Science and Technology, Indian Institute of Engineering Science and Technology, Shibpur, Howrah, West Bengal, India
e-mail: ananyabarui.chest@faculty.iiests.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_6

use of an external aid for the wound healing purpose are the patient's safety, its effectiveness and convenience of use to treat the wound (MacNeil 2007). Non-invasive and minimally invasive treatment techniques are evolving at a tremendous pace due to their targeted therapy and non-contact nature. This chapter aims to provide an insight in the recent updates and advancements in the treatments, assessment, monitoring and management of chronic and acute wounds along with the underlying physiological, cellular and molecular aspects of the wounds.

6.2 Physiology of Wound Healing

The undertaking of the wound management plans and treatments requires a prior perception of the underlying physiological, cellular, biological and molecular aspects of the complex wound healing mechanism. The overall biological phenomenon of repairing a wound site is considered as an intricate physiological network of various processes that involve two of the most vital attributes of living organisms: repair and regeneration, which ultimately results in the restoration of the integrity of the tissue. Regeneration involves the replacement of injured damaged tissue with normal cells, whereas repair involves the re-establishment of the normal functioning and integrity of the tissue. The wound healing phenomenon can be physiologically classified into four different dynamic phases which entail haemostasis or coagulation, inflammatory phase, growth and proliferative phase and remodelling (Ellis et al. 2018) (Fig. 6.1).

Haemostasis: The first and the initial step of wound healing that occurs immediately post-trauma to the skin is the constriction of the injured ends of the blood vessels to control and minimize the blood loss. Any type of injury to the skin that penetrates deep into the dermis results in the traumatization of the blood vessels, which ultimately results in haemorrhage. Haemostasis and the formation of a wound matrix are the characteristics of the initial phase of wound healing.

A series of complex chain reactions occur upon immediate exposure of the blood to air, which results in the formation of a blood clot and is termed the coagulation cascade reaction. The blood clot formed is characterized by the formation of fibrin, fibronectin and vitronectin-rich provisional matrix that temporarily fills and closes the space created due to the wound and gradually desiccates to form a scab. This provides strength and protects the wound from further infection. As the process of healing proceeds, the scab formed gets lysed along with plasmin, bacteria and several inflammatory dead cells.

The peripheral vasoconstriction of the blood vessels results in a transient hypoxic condition of the surrounding tissues. This results in increased glycolysis, alteration in the pH levels and several other factors, which stimulate and activates the coagulation cascade reaction by activating and aggregating the platelets (Ruggeri 2006). These activated platelets promote the process of inflammation via the release of mitogens and chemoattractants, which modifies and increases the vascular permeability and tone. This facilitates cell migration and increased the diffusion of oxygen and nutrients for the newly formed cells.

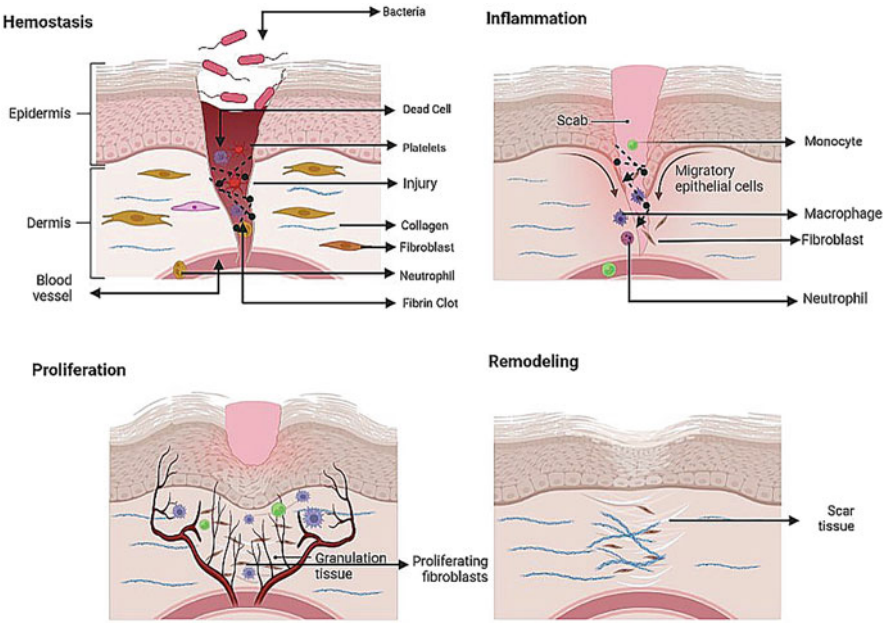


Fig. 6.1 The four phases of wound healing: haemostasis, inflammation, proliferation, remodelling; Created with BioRender.com

Inflammatory Phase: The inflammatory phase clinically termed the debridement phase is activated during the coagulation phase of wound healing. This phase of wound repair is designed to protect and prevent wounds from invading microorganisms. Secretions of inflammatory cells, growth factors, cytokines, macrophages and neutrophils function as the core of the early wound repair process. Several vasoactive mediators and chemoattractants produced by the coagulation process, injured stromal cells, mast cells and activated complement pathway recruit leukocytes at the site of trauma to initiate the process of rolling, adhesion and finally the migration of the inflammatory cells to the site of injury. Further, chemoattractants also stimulate the production of enzymes by activating neutrophils, accelerating their penetration deep into the dermis. Neutrophils are recruited and act as the first line of defence by removing debris and microorganisms through phagocytosis, followed by the production of enzymes and ROS. The process of neutrophil migration ceases after the destruction and clearance of the contaminants. The viable neutrophils die within 3–4 days and are engulfed and removed by the tissue macrophages (Singer and Clark 1999).

Macrophages perform an important role in healing wounds, by exerting inflammatory process, cytokine production and phagocytosis during the early phase of wound healing and later stimulating the proliferative phase to complete the formation of extracellular matrix (ECM). During the early phase, macrophages referred to as M1 macrophages express TNF-α (tumour necrosis factor-α) and interleukins,

which play a significant role in the recognition and killing of the pathogens. Later during the proliferative phase, it retrieves its fibrinolytic, anti-inflammatory phenotype, and is termed as the M2 macrophages. These macrophages actively signal to the dermal fibroblast and induce the process of re-epithelization and formation of the ECM. The process of inflammation halts through the universal pathway of apoptosis of the unneeded phagocytic cells that do not elicit any further inflammation (Mahdavian Delavary et al. 2011).

Growth and Proliferative Phase: The third phase of the wound healing clinically referred to as the wound repair phase is designed to protect the surface of the wound via the formation of a provisional matrix and a new epithelial cover to regain back the vascular integrity of the tissue. Formation of fibroblast, angiogenesis and epithelization are the predominant characteristics of the proliferative phase. After almost 2–7 days of the injury, migration of fibroblast and keratinocyte endothelial cells into the clot occur to form a granular tissue and replace the fibrin clot. The granular tissue formed is rich in fibronectin and hyaluronan and is characterized by a red, and granular appearance. The tissue is formed by the three elements: macrophages that protect the wound from invading microorganisms, promote angiogenesis and fibroblast formation, fibroblasts that induce the production of the ECM proteins, and new blood vessels to restore the vascular network and integrity of the tissue (Theoret 2016).

One of the key physiological processes involved in this phase is angiogenesis, that is, formation of new blood vessels and capillaries from the pre-existing ones to maintain the circulation of gases and nutrients through the newly formed cells and tissue structure. Angiogenesis occurs in response to the hypoxic condition of the surrounding tissues and is moderated by various angiogenic factors, like PDGF (platelet-derived growth factors), VGF (vascular growth factors), fibroblast growth factors, as well as several other cytokines and chemoattractants. In addition to the aforementioned metabolic phenomena, covering the denuded epithelial tissue is crucial for the successful closure of the surface of the wound (Liekens et al. 2001).

Remodelling: The final phase of wound healing also referred to as the maturation phase is characterized by the maturation of granulation tissue into scar tissue. Tensile strength of the tissue is enhanced by the random reorganization of the collagen fibres and increased cross-linking of the collagen molecule by the action of the enzyme lysyl oxidase. The initial scar tissue formed is replaced ECM, similar to the normal skin, and remodelling of ECM proteins occurs by the regulated actions of different classes of proteases.

6.2.1 Factors Affecting Wound Healing

The dynamic process of wound healing is related to multiple local and systemic factors that can lead to impairment of the process. Factors that have a direct effect on the wound properties can be called the local factors, while the overall individual health conditions fall under the systemic factors. The two most common and

important local factors that impact the process of healing involve low oxygen tension and infection of the underlying tissues (Guo and Dipietro 2010).

The most critical element that promotes wound contraction and thereby accelerates the process of healing is the level of oxygen. It promotes the process of re-epithelization, induces fibroblast formation, and thereby leads to a faster healing process. The production of various reactive oxygen species (ROS) in the polymorphonuclear leukocytes, which kills the pathogens and protects the wound from infection, is critically dependent on the oxygen level of the surrounding tissue. Disruption of vascularization, along with alteration in several factors of the surrounding wound tissues leads to temporary hypoxia. Although the fall in the oxygen level of the surrounding tissues triggers the process of healing, however, prolonged hypoxia leads to chronic and delayed wounds. Production of several growth factors and cytokines is triggered by hypoxic conditions. Cytokines like PDGF, VEGF (vascular endothelial growth factor), (transforming growth factor β) TGF-β and TNF-α (tumour necrosis factor-α) are produced and are considered as vital promoters of cell migration, re-epithelization and angiogenesis in wound healing (Rodriguez et al. 2008). Thus, in summary, hypoxia triggers the process of wound healing by releasing growth and factors and promoting angiogenesis, while a minimum oxygen level is necessary for sustaining the process of healing.

Infection of the wound is highly detrimental to the process of wound healing. Chronic and acute wounds are often colonized and contaminated with replicating and non-replicating microorganisms. Several microorganisms that are sequestered at the surface of the skin get access to the dermis upon injury of the skin. The removal of microorganisms requires the release of proinflammatory cytokines like TNF-α and interleukin-1 and often bacteria and endotoxins lead to prolonged inflammation. This chronic period of inflammation fails to heal the wound. Bacteria like *Staphylococcus*, *Pseudomonas aeruginosa* often form biofilms over the wounds, thus protecting them from the phagocytic activity of the invading neutrophils (Bjarnsholt et al. 2008). This ensures a delay in the healing process.

The systemic factors that impact and influence the healing process entails: age, stress, sex hormones, disease conditions like diabetes, jaundice, fibrosis, several hereditary disorders, nutrition, immunocompromised disease conditions like AIDS, cancer and medications. Age is considered a major factor in the delayed healing process. Several clinical studies at cellular and molecular levels have shown a delay in the healing process in aged individuals. Several factors that contribute to it are delayed T-cell infiltration, reduced phagocytic activities of the macrophages and neutrophils, delayed angiogenesis, collagen synthesis and a reduced secretion of growth factors (Swift et al. 2001). Sex hormones like oestrogen in females and androgens in males and their precursor molecules are known to have an important effect on the process of healing. Studies have shown that genes related to inflammation, regeneration, matrix production and protease inhibitors are regulated by the hormone oestrogen, and the genes for cutaneous wound healing are regulated by the hormone androgen (Hardman and Ashcroft 2008). Compromised wound healing is also related to stress. Stressors have a direct influence on the immune and endocrine systems and have been known to cause substantial delays in the process of healing.

Several medications like glucocorticoid steroids, chemotherapeutic drugs and non-steroidal anti-inflammatory drugs that are known to interfere with the process of inflammation have significant effects on the process of healing. Glucocorticoid steroids that are often used as anti-inflammatory agents are great suppressors of fibroblast and collagen synthesis. They are also known to inhibit hypoxia-inducible factor-1 (HIF-1) formation, a primary transcriptional factor required in oxygen homeostasis regulation during wound healing (Wagner et al. 2008; Hong et al. 2014). Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), widely used for the treatment of arthritis, causes reduced fibroblast production, reduced wound contraction and delayed epithelialization, thus inhibiting the healing process. Several chemotherapeutic drugs like bevacizumab, a fragment of an antibody that neutralizes VEGF, were found to be the inhibitor of the process of angiogenesis. Thus, single or multiple factors affect individual phases, contributing to impaired wound healing (Guo and Dipietro 2010).

6.3 Challenges Faced in the Wound Healing Procedure

Management of chronic wounds is a challenge to healthcare professionals, requiring the utilization of a huge number of resources in the process worldwide. In the present times, the key challenge to the treatment of wounds and the primary component of comprehensive wound care management are overcoming the factors that are the primary mediators of the impairment of wounds. Failure of wounds to heal after almost 4 weeks of proper care and management requires the reassessment of the underlying pathology of tissue and the undertaking of advanced therapeutic strategies. Chronic lower extremity ulcers like leg and foot ulcers often require advanced therapies like the use of tissue-engineered skin grafts, in combination with other processes like phototherapy and negative pressure wound therapy. These treatments alone are reported to cost about 2–3% of the healthcare budgets in the countries (Frykberg and Banks 2015). Antibiotics and antiseptics promote the process of healing in wound care management; however, the formation of antimicrobial-resistant biofilms over the wounds is a predominant challenge in wound care management.

The prolonged persistence of the proinflammatory phase and the cytokine cascade of the wound healing process stimulate the production and release of more protease. Although in acute wounds, the elevated level of protease is managed by their inhibitors; however in chronic wounds, the level of protease often exceeds their inhibitors leading to the destruction of the matrix formed. This inhibits its progress toward the proliferative phase, lengthening the inflammatory phase by attracting more inflammatory cells (McCarty and Percival 2013). Although the production of ROS destroys the microorganisms, however in the case of chronic wounds prolonged hypoxic condition and the inflammatory phase increase the production of ROS, which ultimately destroys the extracellular matrix (ECM) proteins (Schreml et al. 2010). Moreover, chronic wounds are often characterized by senescent cells

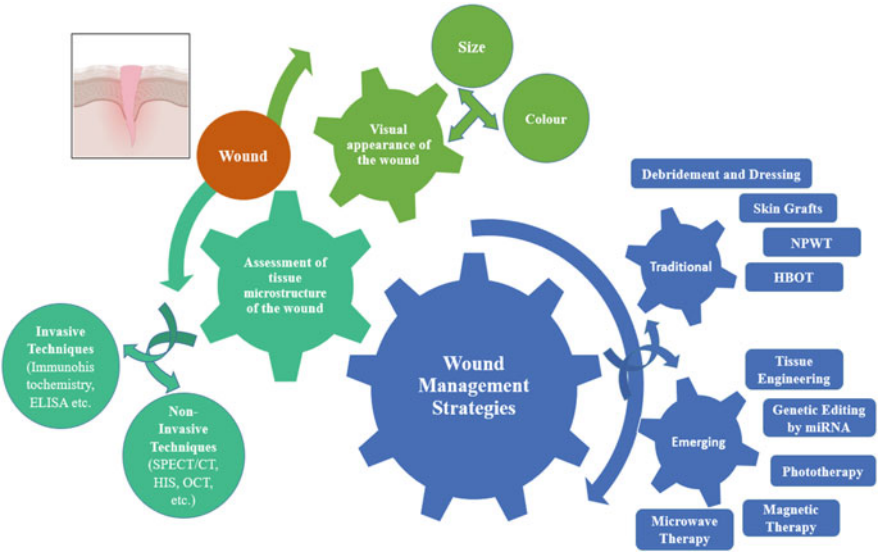


Fig. 6.2 The various steps involved in devising an effective wound management strategy

with reduced proliferative capacity and hence unresponsive to the healing signals and factors (Schultz et al. 2003).

An appropriate diagnosis for addressing the underlying etiopathogenesis of the wound is another significant challenge in the wound healing process. Proper care and management of the wound are associated with the proper and appropriate wound diagnosis and assessment (Fig. 6.2). Parallel to the management of the etiological factors of the wound, the preparation of an appropriate wound bed is also critical for the surrounding tissues. Thus, along with proper wound management, holistic or clinical treatment of the patient must also commence alongside.

6.4 Therapeutic Strategies

6.4.1 Traditional Methods

6.4.1.1 Debridement and Dressing

The most common method to treat a wounded area is to sterilize the place, remove dead tissues, or debride the area and externally apply a wound dressing to close the open wound site for its proper healing. This helps to shield the internal area from the outside environment, which enables faster healing. Traditionally used dressing materials like cotton and wool have given way to more advanced dressings, which are incorporated with therapeutic materials to aid in faster healing. The re-epithelization phase requires a moist environment, therefore dressings made of hydrocolloid, hydrogels etc. provide better results than the traditional materials

(Miller and Nanchahal 2005). Debridement of the wounds can either be achieved by dressings that help in enzymatic reactions for fibrin degradation in the wounds (Mulder et al. 1993) or surgical procedures for the removal of dead tissue to inhibit pathogenic infection and expose healthy tissues for regeneration. Natural biological enzymes from the maggot larvae have also successfully been used for the treatment of necrotic wound tissues (Thomas et al. 1999). Wound dressings are also imbibed with a number of antibiotics like tetracyclines (Anjum et al. 2016), quinolones (Ye et al. 2018) and cephalosporins (Rădulescu et al. 2016). They help reduce the bacterial load in the wounded area by either disruption of the bacterial cell wall synthesis or inhibiting protein or nuclei acid formation by disrupting the various pathways involved. With the growing number of antibiotic-resistant strains of bacteria emerging, various natural substitutes are being tried to be implemented for their antimicrobial properties (Das and Horton 2016). Essential oils (Aumeeruddy-Elalfi et al. 2016), honey (Scagnelli 2016) etc., have been successfully shown to have sufficient antibacterial properties, which may be exploited alone, or in combination with other therapeutic methods, like nanotechnology to effectively treat wounds without causing antibiotic resistance. Chronic wounds, where the healing is impeded by a lack of growth factors, may be provided externally with growth factors such as Rh-PDGF (recombinant human PDGF), TGF-β (Miller and Nanchahal 2005) to accelerate the healing process.

6.4.1.2 Skin Grafts

The use of skin grafts has been an established treatment mode for various skin injuries. Depending on their composition, they may be either epidermal, consisting of only the epidermal layer, full-thickness comprising of both the epidermal and the dermal layers, or split-thickness, made up of the epidermal and a partial dermal layer. While split-thickness grafts provide better results at large injuries with poor circulation, full-thickness grafts are more preferable for exposed body areas that require a consideration of aesthetic healing due to lesser contraction (Sun et al. 2014). Major cells used in the epithelial grafts consist of keratinocytes, which help enhance the cell proliferation in the wounds for effective healing (Kanapathy et al. 2017).

Based on their source, skin grafts may be classified into autografts, allografts and xenografts. Autografts are obtained from a different body part of the same patient whose wound is being treated (Janeway et al. 2001). While an obvious advantage is the lesser chances of rejection by the patient body, it is a painful procedure and often for large injuries unsuitable due to the limited source (Rowan et al. 2015). Allografts are obtained from either cadavers, or other living human beings, which can help in angiogenesis and vascularization, along with promoting immune cell production in the wound area of patients to assist in healing. However, the cost factor is a point of consideration while going for allogeneic grafts. Xenografts are skin grafts obtained from a different species, mainly porcine skin (Halim et al. 2010), which may be used as a temporary wound covering to help in skin regeneration in wounds. However, due to species differences, there is an issue of graft rejection, which in major parts can be solved by their genetic modification (Yamamoto et al. 2018). Another rich source of collagen and growth factors which have been effectively used in skin grafts

is amniotic membrane obtained from the placenta of various donors. Amnion membrane dressing used on burn patients has shown an adequate re-epithelization rate leading to faster wound healing with lesser pain (Eskandarlou et al. 2016).

6.4.1.3 Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy, as the name insinuates, is a therapeutic method, which includes the inhalation of 100% oxygen by the patient in an artificially created hyperbaric atmospheric chamber, that is, where the pressure is higher than the sea-level pressure (Howell et al. 2018). One of the major hindrances that compromise the healing potential in wounds leading to their chronic nature is the hypoxic environment created in it, which aids in the growth of pathogen (Mustoe 2004). Collagen formation and deposition, immune response and angiogenesis all require oxygen, and hence using the hyperbaric oxygen therapy may lead to an increase in the above, leading to wound repair (Hopf et al. 2005). A study measuring transcutaneous oxygen tension in diabetic patients with lower extremity wounds showed positive improvement of the wounds following this therapy (Fife et al. 2002). However, hyperbaric oxygen therapy might not be a suitable method for all; therefore, the wound aetiology along with patient-specific factors needs to be considered before its application. Apart from being used solely for treatment purposes of various wounds, hyperbaric therapy provides a synergistic effect when coupled with other healing treatments, such as debridement and the use of proper dressing, or skin grafts. It has been extensively used in the treatment of necrotizing fasciitis to lessen the healing time and increase the patient survival rate (Jallali et al. 2005). It has shown promising results in the treatment of foot ulcers in diabetic patients with combination to other wound care management therapies (Duzgun et al. 2008). However, a recent study on the effects of this therapy on acute injuries in rabbits did not have any significant improvement on the wound healing capacity, as otherwise stated in literature (Tlapák et al. 2021). Therefore, further evaluations need to be performed for its efficient use in wound management.

6.4.1.4 Negative Pressure Wound Therapy (NPWT)

The use of mechanical forces to modulate injuries and tissue regeneration has been a well-studied area of research for quite a while. Negative pressure wound therapy (NPWT) refers to the use of negative pressure in a controlled environment for increasing the wound healing rate. Even before its official approval to be used as a clinical therapy, the use of the core principle of NPWT dates back centuries. Its current major application is in the treatment of acute surgical wounds, along with burn patients and chronic wounds. NPWT has been shown to drastically reduce the number of times, the wound dressing needs to be changed, along with improvement of circulation in patients, thus it is the most effective therapy method for acute level burn patients as an immediate treatment (Banwell and Musgrave 2004). Due to its reports of being able to increase vascularization in the wound beds, NPWT is further used to prepare the wound beds of patients who would receive grafts (Saaiq et al. 2010). This led to a decrease in the graft rejection rate in burn patients (Scherer et al. 2002). It has shown major success in the treatment of wounds in the upper

extremities (Shine et al. 2019), which makes it a viable treatment option as a follow-up therapy for reconstruction surgeries. The traditional method requires the presence of a gauze or foam below the permeable membrane to provide suction pressure to the wounds, which along with being slightly uncomfortable has a tendency to be infected with pathogens in the process. To overcome this problem, a study group has developed a NPWT delivery method using a single layer dressing without the requirement of the gauze, which has successfully been shown to have improved wound healing potential (Nuutila et al. 2019). In comparison to treatments using standard dressing, studies on surgical injuries treated using NPWT have shown a lesser incidence of surgical site infection (Masters et al. 2021). Though limited by its high cost and requirement of learning to properly dress the wounds for this therapy, its advantages make this therapy extremely prevalent in clinical therapies for wound management, particularly in soft tissue and burn injuries. In light of the lesser number of studies with NPWT as compared to other therapies, more research studies need to be conducted to fully understand its mechanism of action at the molecular level to better optimize different treatment procedures for different types of wounds.

6.4.2 Advanced and Emerging Methods

In the recent years, more and more therapeutic methods are being developed to give way to better wound healing properties with minimal scarring. In this regard, non-invasive to minimally invasive methods are gaining more popularity due to their innate nature of targeting the wound area without much requirement of a surgical procedure.

6.4.2.1 Tissue Engineered Grafting

Skin grafts of natural origin have been used since the premodern times to treat skin injuries. However, owing to the inadequacy of donor skin graft source for more serious injuries, or its unavailability due to some pathological conditions, tissue-engineered grafts present a suitable alternative. Tissue engineering of the skin refers to the process of growing the skin cells, or the keratinocytes, at a rate faster than the normal in vivo process, and using it at the wounded area to promote healing. As an initial treatment process, though the wound can be covered with an engineered graft from synthetic materials, for the continuation of the regeneration process, angiogenesis and vascularization are essential; hence, living keratinocyte cells are required.

Various factors that are required for the proper design of a tissue-engineered scaffold are choosing suitable types of cells, materials for the scaffold, and the different types of nutrients required for their growth. Biodegradable and bioresorbable materials have proven to be more effective than synthetic materials for the construction of the scaffold, which enables angiogenesis for the proper development of the vascular system of the engineered grafts (Olson et al. 2011). Major biomaterials used for clinical engineering of grafts are agarose (Garzón et al. 2013), collagen (Meuli et al. 2019), hyaluronic acid (Galassi et al. 2000) etc. Among these, the most frequently used collagen is combined with other biomaterials such as

fibrin and hyaluronic acid. The skin surface is made up of the epidermal and the dermal layer, therefore tissue-engineered grafts may be a substitute for each individual layer, or a combination of the two. They may also be acellular, or cellular, based on the presence of cells. Examples of acellular grafts available commercially include Alloderm® (Gordley et al. 2009), Biobrane® and Integra® (Shevchenko et al. 2010). Cellular grafts consist of cells that may be autologous, or allogeneic in origin. They have been seen to provide better results as shown by a study using keratinocyte seeded hyaluronic acid membrane grafts on full-thickness wounds in nude mice wound models (Horch et al. 2019) In the recent times, newer combinations of biomaterials are being tested for better skin regeneration capabilities to increase their efficiency. Keratinocytes seeded on a fibrin-agarose scaffold were tested in vivo on nude mice models, which showed promising results (Carriel et al. 2012).

Though tissue-engineered constructs have considerably improved over the years, further studies need to be done on the development of a complete scaffold that would be functional with fully-developed blood vessels, nerves and proper pigmentation to include the aesthetic look of the graft. Further, in the recent times, 3D-bioprinted skin substitutes may provide a more effective solution (Ishack and Lipner 2020). However, the cost of implementation also needs to be optimized for covering a wider patient pool.

6.4.2.2 Genetic Editing (miRNA)

Human body consists of millions of coding and non-coding RNAs that serve a variety of functions to maintain normal physiological functions. The coding RNAs, as the name suggests, code for a variety of proteins responsible for different biological functions. The non-coding RNAs can further be divided into long and short non-coding RNAs, based on the number of nucleotides. They have been found to have important roles in posttranscriptional regulation of different proteins and signalling pathways. miRNAs (miRs) are a type of short non-coding RNAs of about 22–25 nucleotides long, which have a major role in the regulation of gene expression by degrading mRNAs, and thus preventing the protein formation. Initially transcribed by RNA pol 2 in the nucleus, the miR is processed and exported to the cytoplasm where it is further processed by RNase enzymes. The final product forms the RNA-induced silencing complex (RISC), which then helps bind to the 3′ untranslated region of complementary target mRNA strands for disruption in translation and their ultimate degradation. miR-based gene silencing has led way to an increasing interest for its potential use in the tissue repair mechanism for the treatment of chronic wounds. Chronic wounds are a result of either direct characteristics of the wound site, or an indirect result of the overall health of an individual. Few of the major markers of chronic wounds are loss of re-epithelization, reduced mitogenesis and migration potential in keratinocytes and skin fibroblast cells. In this regard, miRs have been found to be either upregulated or downregulated, which points to their critical role in various stages of tissue repair response mechanism during the different phases of wound healing.

The inflammatory phase requires a fine balance of pro- and anti-inflammatory signalling mechanisms, which may be upset due to an irregularity in miR biogenesis.

An in-depth study of over 200 miRs revealed that several of them, like miR-132, 146a/b and 155 were found to be regulated by the presence of proinflammatory cytokines and endotoxins in the cell, a typical response during inflammation (Taganov et al. 2006). This helps them in regulating the expression patterns of various genes like interleukin-1 receptor-associated kinase 1 (IRAK), Src homology 2 domain-containing inositol 5-phosphatase (SHIP1) and cyclooxygenase-2 (COX2), which are implicated in the immune response of the cell. Several miRs have also been found to have anti-inflammatory roles. miR-223 has a negative regulatory effect on the transcription factor Mef2c for myeloid progenitor formation and granulocyte differentiation, thus regulating the inflammatory response (Johnnidis et al. 2008). miR-203 is present in abundance in the keratinocytes of inflamed skin and represses the formation of proinflammatory cytokines TNFα and IL24 to inhibit immune responses required for wound healing (Primo et al. 2012).

One of the key events required for proper proliferation and angiogenesis phase is the ample formation of keratinocyte and their migration. Post-transcriptional control studies of miR-198 host gene follistatin-like 1 (FSTL1) showed a downregulation of miR-198 levels by TGFβ in keratinocytes of wounded areas, which helped form FSTL1 protein (Sundaram et al. 2013). miR-198 upregulation leads to impaired keratinocyte proliferation and prevents wound closure (Wang et al. 2015). Other miRs implicated in the keratinocyte migration for wound healing by regulation of various signalling pathways include the miR-99 family members, which act by regulating AKT/mTOR signalling pathway to aid in wound healing (Jin et al. 2013), and miR-4516 which reduces the keratinocyte motility by fibronectin/integrin alpha9 signalling pathway regulation (Chowdhari et al. 2017). Studies of wounded diabetic mouse model have revealed a myriad of miRs that aid in the angiogenesis process by targeting various signalling pathways involved in wound closure and re-epithelization, like miR-26a (Jiang et al. 2020), miR-135a-3p (Icli et al. 2019b), miR-615-5p (Icli et al. 2019a) and miR-4674 (Icli et al. 2020).

Collagen deposition during the remodelling phase of wound healing has been found to be aided by miR-29 expression in systemic sclerosis patients (Maurer et al. 2010). miR-192 and miR-29b/c also have been found to help in the scar-free healing process by enhancing collagen expression by targeting TGF-β, SMADs and SMAD-interacting protein 1 (SIP1) (Kato et al. 2007). A list of the different miRNAs is provided in Table 6.1 which play important roles in different phases of wound healing.

In view of the role of miRs in the wound healing process, it is evident that changes in their expression levels are key to an impaired wound healing process. A comparison of the miR expression profile of normal and chronic wound healing revealed major changes in the upregulation and downregulation patterns on many miRs (Banerjee et al. 2011), which point to their possible use as therapeutic agents to aid in the healing process. Though proper delivery vehicles need to be incorporated for this nucleic acid therapeutic treatment, these molecules can be detected as biomarkers in the serum, which make the process of safe optimization and dose determination easier than other molecules for similar therapeutic purposes, thus making it a potential treatment to promote healing of chronic wounds. However,

Table 6.1 A list of miRNAs used alone, or in combination with other methods in wound healing

miR	Function in wound healing	Reference
miR-132	Decreased chemokine production; suppressed NF-κB pathway. Helps in proliferation	Li et al. (2015)
miR-146 a/b	Endotoxin-responsive; targets NF-κB pathway	Taganov et al. (2006)
miR-155	Inhibition reduces inflammation in wounds; promotes healing	Ye et al. (2017)
miR-223	Negative regulatory effect on immune response; its downregulation enhances cell proliferation and nerve regeneration.	Johnnidis et al. (2008); Zhang et al. (2021)
miR-203	Repress formation of proinflammatory cytokines; inhibit immune responses	Primo et al. (2012)
miR-198	Upregulation leads to impaired keratinocyte proliferation	Sundaram et al. (2013)
miR-99	Regulating AKT/mTOR signalling pathway—keratinocyte migration	Jin et al. (2013)
miR-4516	Reduces keratinocyte motility by fibronectin/integrin alpha9 signalling pathway regulation	Chowdhari et al. (2017)
miR-26a	Inhibits healing by reduced keratinocyte migration	Jiang et al. (2020)
miR-135a-3p	Targets p38 signal pathway to reduce angiogenesis	Icli et al. (2019b)
miR-615-5p	Angiogenesis and granulation	Icli et al. (2019a)
miR-4674	Wound closure and angiogenesis	Icli et al. (2020)
miR-29	Inhibits TGF-β1/Smad/CTGF pathway; lesser scar formation	Guo et al. (2017)
miR-192	Enhances collagen expression; promotes scar-free healing	Kato et al. (2007)

accumulation in the liver and kidney, leading to their toxicity and ultimate damage, is a potential risk factor involved in this process. Therefore, a careful consideration of other health implications needs to be taken into account to be able to put this treatment at a larger scale.

6.4.2.3 Phototreatment

Since the demonstration of the biostimulatory role of low-level laser light on cells as early as in the 1970s by Mester et al. (Mester et al. 1971), various light-based therapeutic methods have been explored for their potential use for chronic wound treatment. The traditional treatment method using antibiotics for wound dressing has led to an increase in multidrug-resistant bacterial infection in the wound site, leading to their chronic nature. The non-invasive and effective germicidal activity of ultraviolet light on the wound site bioburden, in addition to their lesser adverse effect on the host tissues, has led way to their potential use in wound treatment.

Phototherapy is the utilization of polarized light for wound treatment by the proliferation of keratinocytes via triggering the cellular and humoral defence mechanisms (Fenyö 1984). Studies on the effects of treatments using low-level laser (660 nm) in the early phase rat pleurisy model as a coherent light source revealed their anti-inflammatory effect by a dose-dependent modulation of immune response molecules like interleukins and TNF-alpha. (Boschi et al. 2008) Thus, it is progressively being used in the treatment of chronic wounds and burn patients to minimize pain, promote tissue regeneration and wound healing. Additionally, studies revealed their role in increasing human gingival fibroblast proliferative capacity with a small exposure time (Almeida-Lopes et al. 2001). Treatments of slow-healing wounds using polarized polychromatic non-coherent light source have also shown significant results by increasing macrophages and neutrophils, thereby increasing bacterial phagocytosis in the wound site (Monstrey et al. 2002). The basic principle of photodynamic therapy is that photon molecules combine with several photosensitive non-toxic dyes specific to microbial cells in the wounded area to effectively increase reactive oxygen species (ROS) production, and promote their death. A comparative study to understand the more efficient method of wound treatment using polarized light as opposed to a low-level laser light source in burn patients revealed that the former had better results owing to their biostimulatory capacity to produce a cascade reaction that helps in a better healing process (Mowafy et al. 2021).

Studies of the effect of varying intensities of light emitting diodes (LEDs) showed an increase in cell growth of mouse fibroblasts and human epithelial cells in vitro, while reducing the wound size, pain and healing time in vivo (Whelan et al. 2004). Following this study, it can be hypothesized that a combined exposure to varied wavelengths would result in an enhancement of the healing process. Studies with diabetic ulcer rats treated with 660 nm and 890 nm combined LED radiation proved the same by boosting the healing process by increased ulcer granulation (Minatel et al. 2009). A recent study using a combination treatment of 630 and 940 nm LED therapy in pressure injury patients also exhibited an improvement in arterial and venous circulation resulting in an accelerated healing (Baracho et al. 2021). Further, cutaneous open wound treatment using phototherapy combined with photodynamic therapy may be a viable treatment option and be a major improvement over the utilization of the individual treatment procedures (Sampaio et al. 2021).

Near-infrared (NIR) activated nanomaterials composed of either inorganic molecules or polymers have also gained popularity for their use in wound treatment by producing local hyperthermia in the wound site for killing pathogens. Yang et al. have designed a NIR-triggerable hollow $Cu_{2-X}S$ nano-homojunction (nano-HJ) platform with covalently attached hyaluronan for their specific binding to wound sites to promote cutaneous wound healing (Gao et al. 2021). This has opened up vast possibilities for the use of nano-homojunction platforms as delivery vehicles for treatment in difficult-to-access areas in our body. Copper sulphide nanodots with antimicrobial peptides have recently been designed and successfully used in another study as a NIR-activable antimicrobial platform for wound treatment (Wang et al. 2022). Addition of collagen to this mix indicated further improvement in the healing.

Recently, a wireless LED patch design of adequate flexibility with an Internet of Thing (IoT) healthcare platform has been proposed for wound healing (Phan et al. 2021). Though the wavelength and exposure time need to be further optimized, it can be considered to be an important contribution in the biomedical field for improving the ease of access and use, along with the improved quality of cost-effective and non-invasive wound healing treatment procedures. Thus, the non-invasive nature of phototherapy provides a viable option for its use in the treatment of various wounds.

However, with the large number of positive results gained using phototherapy, more in-depth studies need to focus on the risks involved in their clinical use. Blue light irradiation studies in a scratch wound healing model were found to reduce keratinocyte migration and proliferation processes, which are important for the re-epithelization phase (Denzinger et al. 2021). Thus, a critical evaluation of the wavelength, dosage and exposure time needs to be optimized for phototherapy studies for their efficient clinical use.

6.4.2.4 Magnetic Therapy

An interesting technique in alternative medicine is the use of magnetic field for the improvement of the circulation and hence the promotion of wound healing. Electromagnetic therapies have been effectively used in bone healing since the 1950s for the treatment of fractures (Sharrard 1990), osteotomies (Fredericks et al. 2000) etc. Studies of their use in soft tissue healing started more than a decade back which showcased the improvement in healing of cutaneous wounds by their exposure to a low-power, static magnetic field (Henry et al. 2008). Cell migration, one of the key players in wound healing, is aided by the cells moving toward the injury site following the cellular cytoskeletal fibres, or the concentration gradient by sensing the changes in the internal electromagnetic fields. Understanding their effects and their potential manipulation may thus be a potential therapeutic measure in regenerative medicine. Diabetic patients, treated with the static magnetic field, have shown a significant improvement in anti-inflammatory gene expression via the JAK/STAT pathway, angiogenesis and re-epithelization, which led to faster wound closure (Shang et al. 2019).

Magnetic therapy can also be used to externally manipulate iron-oxide-based extracellular nanovesicles as delivery agents used in regenerative medicine. Human mesenchymal stem cell (MSC)-derived exosomes loaded nanoparticles in a clinical skin injury model have shown promising results for effective external navigation using the magnet to the in vivo injury site to promote angiogenesis and cell proliferation (Li et al. 2020b). Dynamic magnetic field may also be used in this regard. Extremely low-frequency electromagnetic force (ELF-EMF) exposure of chronic ulcers showed anti-inflammatory and angiogenic effects, with increased collagen formation, hinting at their multitude role in the various wound healing phases (Costin et al. 2012). They further increase ROS production (Calcabrini et al. 2017), cytokine release and upregulate matrix metalloproteinase-9 (MMP-9) expression (Ayuk et al. 2016) to help in cell migration and phagocytosis of pathogens in the wound area via the Atk/ERK pathway (Patruno et al. 2018).

Since the establishment of its role in wound healing, magnetic therapy is seen as a promising addition to traditional treatment procedures to aid in wound healing. However, more in-depth studies need to be conducted on the various factors like exposure type (local or overall), exposure duration and wound type that affect the modulation of the healing process using the magnetic field. Further, for the effective inclusion of this therapy as a mainstream treatment procedure, a more effective method of delivery with portable equipment needs to be developed for its more accessible use.

6.4.2.5 Microwave Therapy

A drawback of the use of light in a therapeutic wound management method is its inability to penetrate deep into the tissues. Various forms of electromagnetic radiations at a low frequency have been used in the medical field for a long time due to their therapeutic effects on the biological tissues. The neuromuscular system utilizes electrical signals to perform different biological functions, and hence electromagnetic radiation may be utilized to control them. Of them, the use of microwaves has emerged as a beneficial option for the treatment of various diseases due to their higher thermal ability, along with better penetration into tissues. Microwaves have a frequency range of 300 GHz to 300 MHz, with wavelengths ranging from a meter to a millimetre, on the electromagnetic spectral scale. Due to their ability to produce physiological changes, or localized heating effect to destroy diseased tissues with a minimally invasive procedure, they have long been used for tissue ablation purposes in the field of oncology. Their frequencies can be modulated to suit the required purpose, for example, a lower frequency range is used for ablation of a large amount of tissue, whereas a higher frequency range on a smaller surface area produces better results. Early studies in the mid-90s have demonstrated the physiological effect of microwaves on wound healing on the immune system by aiding in the process of WBC accumulation and phagocytosis of pathogens in the wound site, leading to faster wound closure than the control groups (Korpan et al. 1994). Further, at specific frequencies, they cause an increase in blood flow and tissue metabolism at the injury site to promote their healing (Wyper and McNiven 1976). A recent study with burn model rats revealed that short nanosecond-pulsed microwave application of low frequency at the burn site promotes faster healing (Samoylova 2020). With proper optimization, this may be applied to the human burn patients to promote wound healing with less scarring and a minimally invasive procedure. Hydrogel dressing prepared using microwave-inducible materials provides an interesting solution for targeted transdermal wound treatment with minimal invasion. A novel levofloxacin-loaded hydrogel dressing prepared using polymerization of microwave-triggerable material has recently been successfully designed with optimal delivery to the wound site, along with bactericidal properties (Gao et al. 2022). This has opened up newer avenues for developing novel biomaterial drug delivery systems which may externally be triggered for treatment of hard-to-reach wound sites.

Microwave therapy is emerging as a viable option for wound treatment due to its non-invasive nature, along with its ability for precise targeting in the deep tissues.

With miniaturization of treatment devices for ease of access, further studies need to be conducted for optimal design of portable devices to help with treatment of various types of wounds.

6.4.2.6 Nanotechnology for Wound Therapy

Nanoparticles provide an excellent method of drug delivery owing to their small size, coupled with their ability to be surface modified to reach specific targets. Therefore, it is of no surprise that they are extensively used to optimize the drug delivery process to wound sites. Along with their targeting ability, use of various biomaterials for their construction leads to further added advantages. A common example is the use of silver or zinc nanoparticles for their antibacterial properties and acceleration of wound healing. In this regard, various studies have been conducted by using different forms of nanoparticles to provide a better alternative to already available methods. Utilizing their targeting abilities, studies conducted using nanoparticle-coated stem cell provided selective targeting of the nanocarrier-coated cell toward the wound site (Liu et al. 2016). Carboxymethyl chitosan nanoparticles loaded with bioactive peptide were shown to mediate wound healing without any scarring, owing to a controlled release of the drug, along with overcoming the enzymatic environment inside the cell (Sun et al. 2018). Utilizing their antibacterial properties, various types of hydrogel dressings have been developed to treat burn wounds or chronic wounds. A collagen dressing with zinc oxide nanoparticles and plant essential oil was synthesized that gave a faster healing rate, coupled with adequate biocompatibility and low toxicity rate (Balaure et al. 2019). Silver nanoparticles have been a common choice as a drug delivery vehicle and have been commercially manufactured on hydrogel dressings to accelerate healing (Fong et al. 2005). Addition of nanoparticles to polymers such as gelatin or chitosan are a preferred model of wound healing dressing due to their ability to prevent sepsis in the wound area along with re-epithelization abilities (Hamdan et al. 2017). A study conducted using chitosan-based hydrogels, in which silver nanoparticles and calendula extract were added as an alternative to traditional drugs, showed satisfactory results to treat diabetic wound patient model (Rodríguez-Acosta et al. 2021). Injectable europium oxide nanorod (Eu_2O_3 NRs) reinforced nanocomposite hydrogel dressing has recently been developed, which displays impressive anti-inflammatory capacity by TNF-alpha and interleukin inhibition, and enhanced angiogenic potential (Luo et al. 2021). In another study, a novel polymer film combined with modified chitosan–curcumin nanoparticles was tested for skin tissue regeneration on burn patients, which showed a significantly better regenerative capability due to their synergistic effect than the individual treatments (Basit et al. 2021). Drug-loaded nanoparticles may also be used with a combination of other therapies, such as photodynamic therapy, to overcome the poor solubility issues associated with various drugs. Pulsed photomodulation of curcumin-loaded iron-oxide nanoparticles was seen to provide better wound closure abilities, along with lowering the bacterial load on the wound site, than the control groups, or only laser, or only drugs groups (Moradi et al. 2019). Thus, these properties may further be

studied and developed to exploit in creating appropriate wound therapies to treat chronic injury patients with poor natural healing capacities.

6.5 Assessment and Monitoring Wound Healing

The assessment of the wound begins during the initial and first encounter with the patient, which includes the understanding of the general appearance of both the wound and the patient. Visual inspection of the wound involving, depth, size, odour and general appearance provides immediate information about certain features of the wound. This further helps in the evaluation and treatment of the wound. Determination of the depth of penetration of the wound and tracking of several underlying associated disease conditions like osteomyelitis is possible with probing the wound (Grayson et al. 1995). Other than visual observation, evaluation of the detailed information of the microstructure and components of skin, undetectable to the visual inspection is crucial for the clinicians to assess the intensity of the injury and its healing ability, thereby developing therapeutic actions.

6.5.1 Invasive Assessment Techniques

The common invasive ancillary techniques that are utilized for the assessment and monitoring of the components of healing wound are special stains, immunohistochemistry, and enzyme-linked immunosorbent assay (ELISA). Masson's trichrome staining provides a standardized system to analyse and demonstrate the collagen content of the healing wound (Lee et al. 2012; Pişkin et al. 2014). Several immunohistochemical markers have been utilized to exhibit constituents of a healing wound. These include the use of CD31 for angiogenesis, antiloricrin for epithelial differentiation, and various antibodies against cytokine ligands and receptors. Surgical wound assessment techniques that include wound drains and wound scoring have been utilized for the quantitative and qualitative analysis of the healing wounds. Multiple stages of the healing process involving the components of the skin are evaluated for the quantitative analysis of the healing wounds. Histological parameters, which include epidermal closure, differentiation, migration, granulation tissue formation, inflammation dermal closure, re-epithelization and collagen deposition, are evaluated to provide insights into the defects of healing stages (Gupta and Kumar 2015).

6.5.2 Non-invasive Assessment Techniques

Imaging the structural alterations of the underlying wound tissues to monitor the anatomic, physiologic and mechanical progress of the healing of the wound is crucial for the management of the process. The common imaging modalities that are used to monitor various wound parameters involve CT (computed tomography),

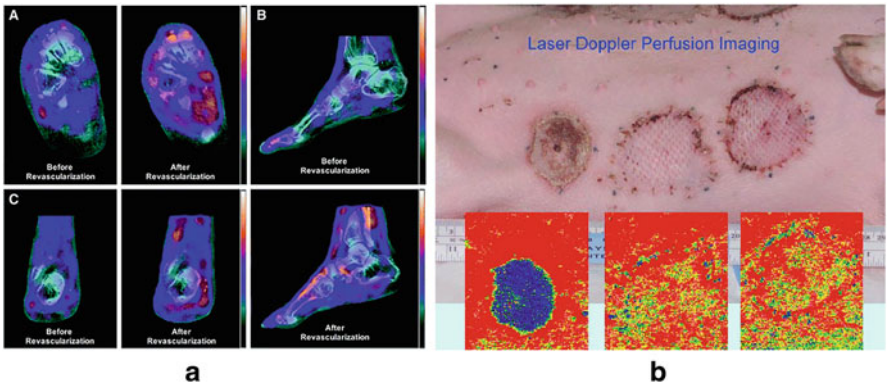


Fig. 6.3 (**a**) The SPECT/CT perfusion imaging in a critical limb ischaemia patient before and after revascularization. (A) Axial, (B) sagittal, and (C) coronal views of the foot by fused SPECT/CT demonstrate increased radiotracer uptake and better microvascular perfusion. [Reproduced from (Chou et al. 2020)]. (**b**) Laser Doppler perfusion imaging of full-thickness sulfur mustard injuries in a weanling pig model after 8 days of surgery. [Reproduced/adapted from (Graham et al. 2005)]

MRI (magnetic resonance imaging), THz (TetraHertz) spectroscopy, ultrasound imaging, SPECT/CT (single photon emission/computed tomography) (Mukherjee et al. 2017). The primary objective of the imaging databases is to improve the evaluation of wound pathogenesis and develop an effective wound treatment plan (Fig. 6.3). The joint and bone pathology of the surrounding wounded region can be assessed by a CT scan. CT angiography is utilized to obtain three-dimensional visualization of the peripheral vasculature of diabetic foot ulcers (Elsayed, Elsayed et al. 2018). SPECT/CT uses a SPECT gamma scanner along with traditional CT to produce highly sensitive, high-resolution images of the regional microvasculature of the wounded tissues to evaluate the peripheral artery diseases in limb ischaemia (Chou et al. 2020). Due to the enhanced sensitivity and resolution of MRI over CT, it is preferred both as an imaging modality and a good surgical aid in osteomyelitis of chronic wounds (Cohen et al. 2019). THz spectroscopy is utilized to detect the tissue hydration gradient of normal and injured tissue due to its unique penetrability.

Various optical non-invasive imaging techniques that have shown potential in imaging of the wound include HSI (hyperspectral imaging), OCT (optical coherence tomography), thermal imaging, laser Doppler imaging (LDI), spatial frequency domain imaging, NIR imaging spectroscopy and fluorescence imaging. These tools enable the gathering of the broad spectrum of wound parameters, which includes structural and chemical components of the tissue, oxygen and moisture level, skin blood flow, collagen deposition and re-establishment and infection (Li et al. 2020a). Digital camera imaging is a widely used non-invasive imaging method for recording the size and depth of the wound. HSI is another optical imaging modality utilized to quantify the oxygen, moisture and haemoglobin content of the wound (Lu and Fei 2014). On the other hand, thermal imaging is utilized to evaluate the depth of burn in case of burn wounds. Thermal imaging or thermography is also used to quantify the thermal diffusivity of the wounded tissue and thereby reveals the extent and stage of the healing process. The skin blood flow of the wound

Table 6.2 Different types of imaging techniques currently used for non-invasive wound assessment

Imaging mode	Uses in wound assessment	Image produced using
CT	Wounds in bones and various joints; 3D vasculature imaging of wound area	X-rays
SPECT/CT	Regional microvasculature of the wounded tissues	Gamma rays
OCT	Wound dimension, epidermal migration, vascular structures and effects, epithelization; high resolution but lesser penetration rate	Near IR rays
MRI	Highly sensitive imaging; used as surgical aid; highly penetrative	Strong magnetic field and radio waves
THz	Tissue hydration gradient detection	Terahertz waves
Ultrasound	Blood flow speed; high resolution; cost-effective; fast, real-time imaging	Ultrasound waves
Digital camera imaging	Wound size and depth measurement; poor specificity	Digital camera with 3.0 megapixels or higher resolution
HSI	Oxygen, moisture, haemoglobin content quantification of the wound; poor penetration rate	Light source (halogen lamps or light-emitting diodes)
Thermography	Thermal diffusivity of the wounded tissue; gives an idea about the phase of the wound; burn depth; poor specificity and accuracy	IR radiation measurement using IR camera
LDI	Skin blood flow measurement; less accurate	Laser beam
NIR imaging	Oxygen, haemoglobin, moisture content quantification; burn wound depth measurement; high resolution but poor specificity	Near IR rays
Spatial frequency domain	Saturation of oxygen measured; vessel structures; autofluorescence detection; time-consuming	Incoherent monochromatic light

is quantified using the unique imaging modality known as laser Doppler imaging. LDI evaluates the variation in the wavelengths of the reflected and scattered radiation after confronting the tissue and the moving RBCs (red blood cells). NIR imaging spectroscopy also quantifies the oxygen, haemoglobin, moisture content of the wound by measuring the maximum light absorbing capacities of the different components of the wound tissues. Although digital camera imaging, NIR spectroscopy and thermal imaging provide an easier and simple imaging modality, however due to their poor specificity, HSI and OCT are currently widely used imaging technique to image the internal microstructure of the tissue. A brief summary of the various types of available imaging techniques for wound assessment has been given in Table 6.2.

6.5.2.1 OCT Imaging

Optical coherence tomography (OCT) is a non-invasive diagnostic tool that has been widely and currently used in the clinical diagnosis and monitoring of the structural changes of the wounds, which include dimensions, migration of the epidermis, vasodilation and vasoconstriction, and epithelization during the process of healing (Li et al. 2020a). OCT utilizes the principle of low coherence interferometry to obtain high-resolution images of the internal network and microstructure of the tissue. OCT angiography has great potential in the quantification of the area and contraction rate of the wound (Deegan et al. 2018). PS-OCT referred to as a polarization-sensitive OCT is widely used in the determination of the functional insights of the tissues, specifically wherein the density and orientation of the collagen are significantly altered. PS-OCT can also be used to determine the birefringence of the tissue and the retardation of the phase of collagen deposition in the ECM (extracellular matrix). Multi-functional PS-OCT is used to evaluate and compute the phase retardation and the relative orientation of the axis of the tissue collagen fibres during the healing process (Park et al. 2018). Currently, the PS-OCT is utilized to monitor the optic axis, for evaluating the collagen content correlated with a particular volume of collagen orientation in the fibrotic and scar tissue (Fig. 6.4). Fourier domain-optical coherence tomography (FD-OCT), namely spectral-domain OCT (SD-OCT) and swept source OCT (SS-OCT) are utilized for the histological evaluation and clinical assessment of the wound healing process. FD-OCT images the surface and sub-surface of the wound and reveals the internal modifications of the tissue microstructure crucial for the monitoring and management of the wound. OCT employs the use of backscattered near-infrared photons from the tissues and reconstructs the image of the internal microstructure using interferometry. However, OCT images suffer a loss of intensity with depth, which limits the application of OCT to only monitoring of the cutaneous wounds, and

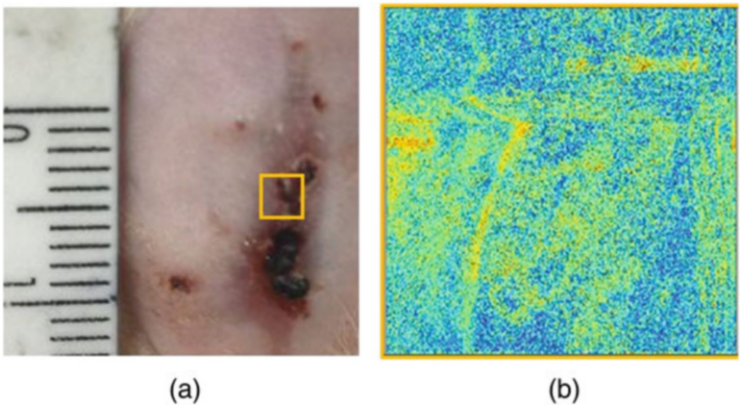


Fig. 6.4 (**a**) PS OCT imaging of scab region (indicated by square) of a wound. (**b**) En face birefringence map calculated from the PS-OCT image. [Reproduced from (Sowa et al. 2016)]

hence correction of the attenuation loss is highly crucial to effectively visualize and differentiate the stages of the healing process (Ghosh et al. 2021).

6.5.2.2 High-Frequency Ultrasound Imaging

Ultrasound imaging is widely considered a good non-invasive technique for the evaluation of chronic wounds due to its higher safety, higher spatial resolution, low cost and lower operating time compared to other imaging modalities. Ultrasound utilizes acoustic pulses to generate two-dimensional images of the tissue cross-sections. Regions with small density variations like the scar tissues appear dark in ultrasound, while regions with significant density variations appear as bright images (Li et al. 2020a). High-frequency ultrasound (HFU) skin scanners with a frequency greater than 20 MHz have been used in the clinical assessments of the dermal burns, imaging of skin microstructure, visualization of the modifications of the wound structure, evaluation of the mechanical properties, and assessment of the healing status of both acute and chronic wounds. High-resolution images with shallow depth of penetration are obtained by HFU, which further can be employed by the clinicians to develop therapeutic actions. HFU imaging is mainly employed for the evaluation and assessment of pressure ulcers, monitoring the wound status and healing progress of the underlying tissues as it does not interfere with the healing process. 2D-B mode HFU non-invasively images the surface and sub-surface of the wounds and reveals the depth and volume of the scar tissue, blood clot, content of collagen, irregularity and inhomogeneity of the tissue, and granulation tissue formation (Mohafez et al. 2018).

6.5.3 Wound Healing Models and Quantitative Analysis of the Wounds

The evaluation of the process and standardization of the quantitative analysis of the healing wounds require the establishment of several healing models. Quantitative analysis involves the evaluation of various wound parameters, like the length and width of the wound, its projected surface area, perimeter and volume. The most commonly selected technique for the delineation of the wound perimeter is the tracing method using acetate or polyurethane films. Computer-assisted planimetry is the most standardized technique utilized the clinicians to delineate the surface area and the perimeter of the wound. The simplest technique for the evaluation of the wound depth involves the use of a sterile blunt-tipped rod. The mathematical formula to assess the volume (V) of the wound was developed by Kundin using wound surface area (A) as: $V = A*D*0.327$, where A = length (L)* Width (W)* 0.785 (Kundin 1985). Alginate moulds are employed to evaluate the volume of the wound by weighing or the displacement of water. Stereophotogrammetry evaluates the wound depth by viewing the wound from two different angles and also allows the measurement of the surface area, contour and perimeter of the wound. (Langemo et al. 2001). Standardized photography evaluates the various parameters of the healing wound non-invasively.

Several wound healing models both 2D and 3D have been established to understand the healing process and generate healing products required to heal different types of wounds. 2D scratch assay, in which a wound is created by scratching a layer of confluent cells on a substrate to study the migration of the cells, is a well-established model. A mathematical model established by Lemo et al. provides a standardized model to determine the contraction of the wound and scoring of the healing process (Lemo et al. 2010). This model is based on five parameters that involve L (length of the re-epithelialization zone), S (distance between the borders of the wound), D (depth of the wound), T (thickness of the connective tissue) and N (thickness of the natural dermis on both sides of the wound). Three indices namely SCI (superficial contraction index), the DCI (deep contraction index) and the WCI (wound contraction index) are determined from the given parameters, which allow the measurement of the wound contraction. Karamichos et al. developed a three-dimensional wound healing model to study the external culture condition effects on implanted fibroblast cells (Karamichos et al. 2009). The results obtained from varying the different culture components and parameters may be utilized in future to optimize the culture conditions for increased cell growth. Another 3D model by Zhou J Chen et al. closely resembles the wound bed and its environment, allowing to observe the fibroblast migration rates on varied cell types, along with the effects of growth factors in this environment (Chen et al. 2014). Further modifications on this model by variations of different cell types and factors would lead to a better understanding of the in vivo process. Thus, these models help to understand the underlying healing process in an environment that closely imitates the wound environment in vivo.

6.6 Conclusion

Development of more potent healing strategies requires a thorough knowledge of the wound microenvironment and the molecular interactions taking place within it. Undertaking of the treatment plans and management of the wound healing phenomenon involve the prior understanding of the wound aetiology and the complex physiology of the process that constitutes haemostasis, inflammation, proliferation and remodelling. Multiple factors affect the process that include oxygen, infection of the underlying tissues and systemic factors like underlying pathological conditions like diabetes. The traditional treatment plans involve debridement and dressing of the wounds, skin graft therapy and hyperbaric oxygen therapy. Advanced non-invasive treatment techniques, tissue engineering, genetic editing and photo treatments provide targeted healing wounds with minimal scarring. The limitation of light source as a therapeutic agent in penetrating deep inside the wounded tissue is replaced by microwave and magnetic therapy. Assessment and monitoring of the healing wounds utilizing both invasive and non-invasive modalities have provided a better management approach of the healing process. Several wound healing models have been established to provide both the qualitative and quantitative analyses of the healing wounds.

References

Almeida-Lopes L et al (2001) Comparison of the low level laser therapy effects on cultured human gingival fibroblasts proliferation using different irradiance and same fluence. Lasers Surg Med 29(2):179–184

Anjum S, Abha Arora MS, Alam, and Bhuvanesh Gupta. (2016) Development of antimicrobial and scar preventive chitosan hydrogel wound dressings. Int J Pharm 508(1):92–101. https://www.sciencedirect.com/science/article/pii/S0378517316303817

Aumeeruddy-Elalfi Z, Gurib-Fakim A, Fawzi Mahomoodally M (2016) Chemical composition, antimicrobial and antibiotic potentiating activity of essential oils from 10 tropical medicinal plants from Mauritius. J Herbal Med 6(2):88–95

Ayuk SM, Abrahamse H, Houreld NN (2016) The role of matrix metalloproteinases in diabetic wound healing in relation to photobiomodulation. J Diabetes Res 2016:2897656. https://pubmed.ncbi.nlm.nih.gov/27314046/. Accessed 7 Jan 2022

Balaure PC et al (2019) In vitro and in vivo studies of novel fabricated bioactive dressings based on collagen and zinc oxide 3D scaffolds. Int J Pharm 557:199–207

Banerjee J, Chan YC, Sen CK (2011) MicroRNAs in skin and wound healing. Physiol Genomics 43(10):543–556

Banwell PE, Musgrave M (2004) Topical negative pressure therapy: mechanisms and indications. Int Wound J 1(2):95–106. https://doi.org/10.1111/j.1742-4801.2004.00031.x

Baracho V d S et al (2021) Phototherapy (Cluster Multi-Diode 630 Nm and 940 Nm) on the healing of pressure injury: a pilot study. J Vasc Nurs 39(3):67–75

Basit HM et al (2021) microwave enabled physically cross linked sodium alginate and pectin film and their application in combination with modified chitosan-curcumin nanoparticles. A novel strategy for 2nd degree burns wound healing in animals. Polymers 13(16):2716. https://www.mdpi.com/2073-4360/13/16/2716/htm. Accessed 7 Jan 2022

Bjarnsholt T et al (2008) Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen 16(1):2–10

Boschi ES et al (2008) Anti-inflammatory effects of low-level laser therapy (660 nm) in the early phase in carrageenan-induced pleurisy in rat. Lasers Surg Med 40(7):500–508. https://pubmed.ncbi.nlm.nih.gov/18727002/. Accessed 7 Jan 2022

Brem H et al (2007) Molecular markers in patients with chronic wounds to guide surgical debridement. Mol Med (Cambridge, Mass) 13(1–2):30–39

Calcabrini C et al (2017) Effect of extremely low-frequency electromagnetic fields on antioxidant activity in the human keratinocyte cell line NCTC 2544. Biotechnol Appl Biochem 64(3):415–422

Carriel V et al (2012) Epithelial and stromal developmental patterns in a novel substitute of the human skin generated with fibrin-agarose biomaterials. Cells Tissues Organs 196(1):1–12. https://pubmed.ncbi.nlm.nih.gov/22146480/. Accessed 7 Jan 2022

Chen ZJ, Yang JP, Wu BM, Tawil B (2014) A novel three-dimensional wound healing model. J Dev Biol 2(4):198–209. https://www.mdpi.com/2221-3759/2/4/198

Chou T-H et al (2020) SPECT/CT imaging: a noninvasive approach for evaluating serial changes in angiosome foot perfusion in critical limb ischemia. Adv Wound Care 9(3):103–110

Chowdhari S, Sardana K, Saini N (2017) MiR-4516, a microRNA downregulated in psoriasis inhibits keratinocyte motility by targeting fibronectin/integrin A9 signaling. Biochim Biophys Acta Mol basis Dis 1863(12):3142–3152

Cohen M, Cerniglia B, Gorbachova T, Horrow J (2019) Added value of MRI to X-ray in guiding the extent of surgical resection in diabetic forefoot osteomyelitis: a review of pathologically proven, surgically treated cases. Skelet Radiol 48(3):405–411

Costin G-E, Birlea SA, Norris DA (2012) Trends in wound repair: cellular and molecular basis of regenerative therapy using electromagnetic fields. Curr Mol Med 12(1):14–26

Das P, Horton R (2016) Antibiotics: achieving the balance between access and excess. Lancet 387(10014):102–104. http://www.thelancet.com/article/S0140673615007291/fulltext. Accessed 7 Jan 2022

Deegan AJ et al (2018) Optical coherence tomography angiography monitors human cutaneous wound healing over time. Quant Imaging Med Surg 8(2):135–150

Mahdavian Delavary B et al (2011) Macrophages in skin injury and repair. Immunobiology 216(7): 753–762

Denzinger M et al (2021) Does phototherapy promote wound healing? Limitations of blue light irradiation. Wounds 33(4):91–98

Duzgun AP et al (2008) Effect of hyperbaric oxygen therapy on healing of diabetic foot ulcers. J Foot Ankle Surg 47(6):515–519

Ellis S, Lin EJ, Tartar D (2018) Immunology of wound healing. Curr Dermatol Rep 7(4):350–358. https://link.springer.com/article/10.1007/s13671-018-0234-9. Accessed 7 Jan 2022

Elsayed EE, Zytoon AA, Eltelwany AM (2018) Role of computed tomography angiography and color doppler ultrasonography in the evaluation of diabetic foot. Menoufia Med J 31(2):508. http://www.mmj.eg.net/article.asp?issn=1110-2098;year=2018;volume=31;issue=2;spage=508;epage=513;aulast=Elsayed. Accessed 7 Jan 2022

Eskandarlou M, Azimi M, Rabiee S, Rabiee MAS (2016) The healing effect of amniotic membrane in burn patients. World J Plastic Surg 5(1):39–44

Fenyö M (1984) Theoretical and experimental basis of biostimulation by laser irradiation. Opt Laser Technol 16(4):209–215

Fife CE et al (2002) The predictive value of transcutaneous oxygen tension measurement in diabetic lower extremity ulcers treated with hyperbaric oxygen therapy: a retrospective analysis of 1144 patients. Wound Repair Regen 10(4):198–207. https://onlinelibrary.wiley.com/doi/full/10.1046/j.1524-475X.2002.10402.x. Accessed 7 Jan 2022

Fong J, Wood F, Fowler B (2005) A silver coated dressing reduces the incidence of early burn wound cellulitis and associated costs of inpatient treatment: comparative patient care audits. Burns 31(5):562–567

Fredericks DC et al (2000) Effects of pulsed electromagnetic fields on bone healing in a rabbit tibial osteotomy model. J Orthop Trauma 14(2):93–100

Frykberg RG, Banks J (2015) Challenges in the treatment of chronic wounds. Adv Wound Care 4(9):560–582

Galassi G et al (2000) In vitro reconstructed dermis implanted in human wounds: degradation studies of the HA-based supporting scaffold. Biomaterials 21(21):2183–2191. https://pubmed.ncbi.nlm.nih.gov/10985492/. Accessed 7 Jan 2022

Gao X et al (2021) Engineering of a hollow-structured Cu2−XS nano-homojunction platform for near infrared-triggered infected wound healing and cancer therapy. Adv Funct Mater 31(52): 2106700. https://onlinelibrary.wiley.com/doi/full/10.1002/adfm.202106700. Accessed 7 Jan 2022

Gao Y et al (2022) Microwave-triggered ionic liquid-based hydrogel dressing with excellent hyperthermia and transdermal drug delivery performance. Chem Eng J 429:131590

Garzón I et al (2013) Wharton's jelly stem cells: a novel cell source for oral mucosa and skin epithelia regeneration. Stem Cells Transl Med 2(8):625–632

Ghosh B, Mandal M, Mitra P, Chatterjee J (2021) Attenuation corrected-optical coherence tomography for quantitative assessment of skin wound healing and scar morphology. J Biophotonics 14(4):e202000357

Gordley K, Cole P, Hicks J, Hollier L (2009) A comparative, long term assessment of soft tissue substitutes: AlloDerm, Enduragen, and Dermamatrix. J Plast Reconstr Aesthet Surg 62(6): 849–850

Graham JS et al (2005) Wound healing of cutaneous sulfur mustard injuries: strategies for the development of improved therapies. J Burns Wounds 4:e1

Grayson ML et al (1995) Probing to bone in infected pedal ulcers. a clinical sign of underlying osteomyelitis in diabetic patients. JAMA 273(9):721–723

Guo S, Dipietro LA (2010) Factors affecting wound healing. J Dent Res 89(3):219–229
Guo J et al (2017) MiR-29b promotes skin wound healing and reduces excessive scar formation by inhibition of the TGF-B1/Smad/CTGF signaling pathway. Can J Physiol Pharmacol 95(4): 437–442
Gupta A, Kumar P (2015) Assessment of the histological state of the healing wound. Plast Aesthet Res 2:239–242
Halim AS, Khoo TL, Yussof SJM (2010) Biologic and synthetic skin substitutes: an overview. Indian J Plastic Surg 43(Suppl):S23–S28
Hamdan S et al (2017) Nanotechnology-driven therapeutic interventions in wound healing: potential uses and applications. ACS Central Sci 3(3):163–175. https://pubmed.ncbi.nlm.nih.gov/28386594/. Accessed 7 Jan 2022
Han G, Ceilley R (2017) Chronic wound healing: a review of current management and treatments. Adv Ther 34(3):599–610
Hardman MJ, Ashcroft GS (2008) Estrogen, not intrinsic aging, is the major regulator of delayed human wound healing in the elderly. Genome Biol 9(5):R80
Henry SL, Concannon MJ, Yee GJ (2008) The effect of magnetic fields on wound healing: experimental study and review of the literature. Eplasty 8:e40
Hong WX et al (2014) The role of hypoxia-inducible factor in wound healing. Adv Wound Care 3(5):390
Hopf HW et al (2005) Hyperoxia and angiogenesis. Wound Repair Regen 13(6):558–564. https://onlinelibrary.wiley.com/doi/full/10.1111/j.1524-475X.2005.00078.x. Accessed 7 Jan 2022
Horch RE et al (2019) Keratinocyte monolayers on hyaluronic acid membranes as 'upside-down' grafts reconstitute full-thickness wounds. Med Sci Monit 25:6702–6710. https://pubmed.ncbi.nlm.nih.gov/31490908/. Accessed 7 Jan 2022
Howell RS et al (2018) Hyperbaric oxygen therapy: indications, contraindications, and use at a tertiary care center. AORN J 107(4):442–453. https://onlinelibrary.wiley.com/doi/full/10.1002/aorn.12097. Accessed 7 Jan 2022
Icli B, Winona W, Ozdemir D, Li H, Cheng HS et al (2019a) MicroRNA-615-5p regulates angiogenesis and tissue repair by targeting AKT/ENOS (Protein Kinase B/Endothelial Nitric Oxide Synthase) signaling in endothelial cells. Arterioscler Thromb Vasc Biol 39(7):1458–1474
Icli B, Winona W, Ozdemir D, Li H, Haemmig S et al (2019b) MicroRNA-135a-3p regulates angiogenesis and tissue repair by targeting p38 signaling in endothelial cells. FASEB J 33(4): 5599–5614. https://onlinelibrary.wiley.com/doi/full/10.1096/fj.201802063RR. Accessed 7 Jan 2022
Icli B et al (2020) MiR-4674 regulates angiogenesis in tissue injury by targeting P38K signaling in endothelial cells. Am J Physiol Cell Physiol 318(3):C524–C535
Ishack S, Lipner SR (2020) A review of 3-dimensional skin bioprinting techniques: applications, approaches, and trends. Dermatol Surg 46(12):1500–1505
Jallali N, Withey S, Butler PE (2005) Hyperbaric oxygen as adjuvant therapy in the management of necrotizing fasciitis. Am J Surg 189(4):462–466
Janeway CA Jr, Travers P, Walport M, Shlomchik MJ (2001) Immunobiology. Immunobiology (14102):1–10. https://www.ncbi.nlm.nih.gov/books/NBK10757/. Accessed 7 Jan 2022
Jiang Z et al (2020) MicroRNA-26a inhibits wound healing through decreased keratinocytes migration by regulating ITGA5 through PI3K/AKT signaling pathway. Biosci Rep 40(9): BSR20201361. https://doi.org/10.1042/BSR20201361
Jin Y et al (2013) MicroRNA-99 family targets AKT/MTOR signaling pathway in dermal wound healing. PLoS One 8(5):e64434. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0064434. Accessed 7 Jan 2022
Johnnidis JB et al (2008) Regulation of progenitor cell proliferation and granulocyte function by MicroRNA-223. Nature 451(7182):1125–1129
Kanapathy M et al (2017) Epidermal grafting for wound healing: a review on the harvesting systems, the ultrastructure of the graft and the mechanism of wound healing. Int Wound J 14(1):16–23

Karamichos D, Lakshman N, Matthew Petroll W (2009) An Experimental model for assessing fibroblast migration in 3-D collagen matrices. Cell Motil Cytoskeleton 66(1):1

Kato M et al (2007) MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. Proc Natl Acad Sci U S A 104(9):3432–3437

Korpan NN, Resch KL, Kokoschinegg P (1994) Continuous microwave enhances the healing process of septic and aseptic wounds in rabbits. J Surg Res 57(6):667–671

Kundin JI (1985) Designing and developing a new measuring instrument. Perioper Nurs Q 1(4): 40–45

Langemo DK et al (2001) Comparison of 2 wound volume measurement methods. Adv Skin Wound Care 14(4):190–196

Lee Y-H et al (2012) Antioxidant Sol-Gel improves cutaneous wound healing in streptozotocin-induced diabetic rats. Exp Diabetes Res 2012:504693

Lemo N, Marignac G, Reyes-Gomez E, Lilin T, Crosaz O, Ehrenfest DMD (2010) Cutaneous reepithelialization and wound contraction after skin biopsies in rabbits: a mathematical model for healing and remodelling index. Vet Arh 80:637–652

Li D et al (2015) MicroRNA-132 Enhances Transition from Inflammation to Proliferation during Wound Healing. J Clin Invest 125(8):3008–3026

Li S et al (2020a) Imaging in chronic wound diagnostics. Adv Wound Care 9(5):245–263

Li X et al (2020b) Magnetic targeting enhances the cutaneous wound healing effects of human mesenchymal stem cell-derived iron oxide exosomes. J Nanobiotechnol 18(1):113. https://doi.org/10.1186/s12951-020-00670-x

Liekens S, De Clercq E, Neyts J (2001) Angiogenesis: regulators and clinical applications. Biochem Pharmacol 61(3):253–270

Liu ZJ et al (2016) Directing and potentiating stem cell-mediated angiogenesis and tissue repair by cell surface E-selectin coating. PLoS One 11(4):e0154053. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0154053. Accessed 7 Jan 2022

Lu G, Fei B (2014) Medical hyperspectral imaging: a review. J Biomed Opt 19(1):10901

Luo M et al (2021) Injectable Self-healing anti-inflammatory europium oxide-based dressing with high angiogenesis for improving wound healing and skin regeneration. Chem Eng J 412:128471

MacNeil S (2007) Progress and opportunities for tissue-engineered skin. Nature 445(7130): 874–880

Masters J, Cook J, Achten J, Costa ML (2021) A feasibility study of standard dressings versus negative-pressure wound therapy in the treatment of adult patients having surgical incisions for hip fractures: the WHISH randomized controlled trial. Bone Joint J 103-B(4):755–761. https://online.boneandjoint.org.uk/doi/abs/10.1302/0301-620X.103B4.BJJ-2020-1603.R1. Accessed 7 Jan 2022

Maurer B et al (2010) MicroRNA-29, a key regulator of collagen expression in systemic sclerosis. Arthritis Rheum 62(6):1733–1743

McCarty SM, Percival SL (2013) Proteases and delayed wound healing. Adv Wound Care 2(8): 438–447

Mester E, Spiry T, Szende B, Tota JG (1971) Effect of laser rays on wound healing. Am J Surg 122(4):532–535

Meuli M et al (2019) A cultured autologous dermo-epidermal skin substitute for full-thickness skin defects: a Phase I, open, prospective clinical trial in children. Plast Reconstr Surg 144(1): 188–198

Miller M-C, Nanchahal J (2005) Advances in the modulation of cutaneous wound healing and scarring. BioDrugs 19(6):363–381. https://doi.org/10.2165/00063030-200519060-00004

Minatel DG, Marco AC, Frade SCF, Enwemeka CS (2009) Phototherapy promotes healing of chronic diabetic leg ulcers that failed to respond to other therapies. Lasers Surg Med 41(6): 433–441. https://pubmed.ncbi.nlm.nih.gov/19588536/. Accessed 7 Jan 2022

Mohafez H et al (2018) Quantitative assessment of wound healing using high-frequency ultrasound image analysis. Skin Res Technol 24(1):45–53

Monstrey S et al (2002) The effect of polarized light on wound healing. Eur J Plast Surg 24(8): 377–382. https://doi.org/10.1007/s00238-001-0305-0
Moradi A et al (2019) An improvement in acute wound healing in mice by the combined application of photobiomodulation and curcumin-loaded iron particles. Lasers Med Sci 34(4):779–791
Mowafy ZME, Ibrahim ISM, Ibrahim MB, Elshahawy AMMM (2021) Wound surface area and colony count of various modes of phototherapy. Egypt J Hosp Med 85(2):3524–3529. https://ejhm.journals.ekb.eg/article_200581.html. Accessed 7 Jan 2022
Mukherjee R, Tewary S, Routray A (2017) Diagnostic and prognostic utility of non-invasive multimodal imaging in chronic wound monitoring: a systematic review. J Med Syst 41(3):46
Mulder G et al (1993) Fibrin cuff lysis in chronic venous ulcers treated with a hydrocolloid dressing. Int J Dermatol 32(4):304–306
Mustoe T (2004) Understanding chronic wounds: a unifying hypothesis on their pathogenesis and implications for therapy. Am J Surg 187(5A):65S–70S
Nuutila K et al (2019) Novel negative pressure wound therapy device without foam or gauze is effective at −50 MmHg. Wound Repair Regen 27(2):162–169
Olson JL, Atala A, Yoo JJ (2011) Tissue engineering: current strategies and future directions. Chonnam Med J 47(1):1–13
Park KS et al (2018) Multifunctional in vivo imaging for monitoring wound healing using swept-source polarization-sensitive optical coherence tomography. Lasers Surg Med 50(3):213–221
Patruno A et al (2018) Extremely low-frequency electromagnetic fields accelerates wound healing modulating MMP-9 and inflammatory cytokines. Cell Prolif 51(2):e12432
Phan DT et al (2021) A flexible, and wireless LED therapy patch for skin wound photomedicine with IoT-connected healthcare application. Flex Printed Electr 6(4):045002. https://iopscience.iop.org/article/10.1088/2058-8585/ac2c50. Accessed 7 Jan 2022
Pişkin A et al (2014) The beneficial effects of Momordica charantia (bitter gourd) on wound healing of rabbit skin. J Dermatolog Treat 25(4):350–357
Primo MN, Bak RO, Schibler B, Mikkelsen JG (2012) Regulation of pro-inflammatory cytokines TNFα and IL24 by microRNA-203 in primary keratinocytes. Cytokine 60(3):741–748
Rădulescu M et al (2016) Fabrication, characterization, and evaluation of bionanocomposites based on natural polymers and antibiotics for wound healing applications. Molecules (Basel, Switzerland) 21(6)
Rodriguez PG, Felix FN, Woodley DT, Shim EK (2008) The role of oxygen in wound healing: a review of the literature. Dermatol Surg 34(9):1159–1169
Rodríguez-Acosta H et al (2021) Chronic wound healing by controlled release of chitosan hydrogels loaded with silver nanoparticles and calendula extract. J Tissue Viability 31(1): 173–179
Rowan MP et al (2015) Burn wound healing and treatment: review and advancements. Crit Care 19: 243
Ruggeri ZM (2006) Platelet interactions with vessel wall components during thrombogenesis. Blood Cells Mol Dis 36(2):145–147
Saaiq M, Hameed-Ud-Din MIK, Chaudhery SM (2010) Vacuum-assisted closure therapy as a pretreatment for split thickness skin grafts. J Coll Physicians Surg Pak 20(10):675–679
Samoylova AV (2020) Dynamics of burn wound healing in rats irradiated by nanosecond microwave pulses. Biomed J Sci Tech Res 32(2)
Sampaio ABA et al (2021) Combination of photodynamic therapy and phototherapy for the treatment of cutaneous open wounds in dogs-case reports. Veterinary Arch 91(5):559–564
Scagnelli AM (2016) Therapeutic review: manuka honey. J Exotic Pet Med 25(2):168–171
Scherer LA et al (2002) The vacuum assisted closure device: a method of securing skin grafts and improving graft survival. Arch Surg (Chicago, Ill: 1960) 137(8):930–934
Schreml S et al (2010) Oxygen in acute and chronic wound healing. Br J Dermatol 163(2):257–268
Schultz GS et al (2003) Wound bed preparation: a systematic approach to wound management. Wound Repair Regen 11(Suppl 1):S1–S28
Shang W et al (2019) Static magnetic field accelerates diabetic wound healing by facilitating resolution of inflammation. J Diabetes Res 2019:5641271

Sharrard WJ (1990) A double-blind trial of pulsed electromagnetic fields for delayed union of tibial fractures. J Bone Joint Surg Br 72(3):347–355
Shevchenko RV, James SL, Elizabeth James S (2010) A review of tissue-engineered skin bioconstructs available for skin reconstruction. J R Soc Interface 7(43):229–258
Shine J et al (2019) Negative pressure wound therapy as a definitive treatment for upper extremity wound defects: a systematic review. Int Wound J 16(4):960–967. https://onlinelibrary.wiley.com/doi/full/10.1111/iwj.13128. Accessed 7 Jan 2022
Singer AJ, Clark RA (1999) Cutaneous wound healing. N Engl J Med 341(10):738–746
Sowa MG, Kuo W-C, Ko AC, Armstrong DG (2016) Review of near-infrared methods for wound assessment. J Biomed Opt 21(9):1–17. https://doi.org/10.1117/1.JBO.21.9.091304
Sun BK, Siprashvili Z, Khavari PA (2014) Advances in skin grafting and treatment of cutaneous wounds. Science (New York, NY) 346(6212):941–945
Sun T et al (2018) Carboxymethyl chitosan nanoparticles loaded with bioactive peptide OH-CATH30 benefit nonscar wound healing. Int J Nanomedicine 13:5771–5786
Sundaram GM et al (2013) 'See-saw' expression of microRNA-198 and FSTL1 from a single transcript in wound healing. Nature 495(7439):103–106
Swift ME, Burns AL, Gray KL, DiPietro LA (2001) Age-related alterations in the inflammatory response to dermal injury. J Invest Dermatol 117(5):1027–1035
Taganov KD, Boldin MP, Chang K-J, Baltimore D (2006) NF-KappaB-dependent induction of microRNA MiR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A 103(33):12481–12486
Theoret C (2016) Physiology of wound healing. In: Equine wound management. Wiley, pp 1–13. https://onlinelibrary.wiley.com/doi/abs/10.1002/9781118999219.ch1
Thomas S, Andrews A, Jones M, Church J (1999) Maggots are useful in treating infected or necrotic wounds. BMJ (Clinical research ed) 318(7186):807–808
Tlapák J et al (2021) THE EFFECT OF HYPERBARIC OXYGEN THERAPY ON ACUTE WOUND HEALING IN RABBITS: AN EXPERIMENTAL STUDY AND HISTOPATHOLOGICAL ANALYSIS. Milit Med Sci Lett 90(1):2–11. http://mmsl.cz/doi/10.31482/mmsl.2021.001.html. Accessed 7 Jan 2022
Wagner AE et al (2008) Dexamethasone impairs hypoxia-inducible factor-1 function. Biochem Biophys Res Commun 372(2):336–340
Wang J et al (2015) MiR-198 represses the proliferation of HaCaT cells by targeting cyclin D2. Int J Mol Sci 16(8):17018–17028. https://www.mdpi.com/1422-0067/16/8/17018/htm . Accessed 7 Jan 2022
Wang X et al (2022) Nanodot-doped peptide hydrogels for antibacterial phototherapy and wound healing. Biomater Sci 10:654–664. https://pubs.rsc.org/en/content/articlehtml/2022/bm/d1bm01533h. Accessed 7 Jan 2022
Whelan HT et al (2004) Effect of NASA light-emitting diode irradiation on wound healing. J Clin Laser Med Surg 19(6):305–314. https://www.liebertpub.com/doi/abs/10.1089/104454701753342758. Accessed 7 Jan 2022
Wyper DJ, McNiven DR (1976) The effect of microwave therapy upon muscle blood flow in man. Br J Sports Med 10(1):19–21. https://pubmed.ncbi.nlm.nih.gov/963368/. Accessed 7 Jan 2022
Yamamoto T et al (2018) Skin xenotransplantation: historical review and clinical potential. Burns 44(7):1738–1749. https://pubmed.ncbi.nlm.nih.gov/29602717/. Accessed 7 Jan 2022
Ye J et al (2017) MicroRNA-155 inhibition promoted wound healing in diabetic rats. Int J Low Extrem Wounds 16(2):74–84
Ye S et al (2018) Flexible amoxicillin-grafted bacterial cellulose sponges for wound dressing: in vitro and in vivo evaluation. ACS Appl Mater Interfaces 10(6):5862–5870. https://pubs.acs.org/doi/full/10.1021/acsami.7b16680. Accessed 7 Jan 2022
Zhang Y et al (2021) Transcriptional network analysis reveals the role of MiR-223-5p during diabetic corneal epithelial regeneration. Front Mol Biosci 8:737472. https://pubmed.ncbi.nlm.nih.gov/34513931

Stem Cells and Therapies in Cardiac Regeneration

7

Harshavardhan Renikunta, Rituparna Chakrabarti, Sushmitha Duddu, Anindita Bhattacharya, Nishant Chakravorty, and Praphulla Chandra Shukla

7.1 Cardiac Regeneration

Cardiovascular disease's (CVD) burden remains the most significant cause of deaths in the world, especially ischemic heart disease (IHD) plays a crucial role, with an annual average of 17.3 million deaths that are accountable due to (IHD) (Mendis et al. 2011). The major reason associated with the high mortality rate of heart disease is the inadequate regeneration of the heart. Most heart diseases are at least in part due to the decrease in the number of functional cardiomyocytes (Becker et al. 2011). The heart is a vital organ in the circulatory system and comprises four chambers; the upper two chambers are atria while the lower two are the ventricles. Oxygen-poor blood from the body is received by the right atrium (RA) and pumped into the right ventricle (RV), and then RV pumps the oxygen-poor blood to the lungs. The left atrium (LA) receives the oxygen-rich blood from the lungs and pumps the blood to the left ventricle (LV). Then LV pumps the oxygenated rich blood to the rest of the body. The body requires nutrients and oxygen for the process of life (Young 2010). Any obstruction of blood flow and oxygen in a part of the body leads to a condition called Ischemia. IHD is also referred to coronary heart disease (CHD), or coronary artery disease (CAD) a condition that results from the blockade of the coronary arteries due to build-up of plaques in the arteries that leads to the death of heart muscle cells, which is termed as heart attack or myocardial infarction (Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria 2010). Myocardial infarction remains the most common cause of heart failure

H. Renikunta
Department of Cardiology, Charité Universitätsmedizin Berlin, Berlin, Germany

R. Chakrabarti · S. Duddu · A. Bhattacharya · N. Chakravorty · P. C. Shukla (✉)
School of Medical Science and Technology, Indian Institute of Technology Kharagpur, Kharagpur, West Bengal, India
e-mail: pcshukla@smst.iitkgp.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_7

(HF). Despite progress made over the last decades, heart failure remains the most significant health issue worldwide (Writing Group Members et al. 2016).

7.1.1 Cardiomyocyte Loss During Myocardial Infarction

Myocardial infarction is due to the blockade of any coronary arteries of the heart. The coronary arteries supply oxygen-rich blood and metabolites to the cardiac muscle, and their blockage leads to the death of cardiomyocytes and scar formation. Most commonly, the blockage is due to the accumulation of cholesterol plaques and fats in the coronary arteries. The gradual deposition of the plaques leads to their rupture, followed by the rapid accumulation of clotting factors at the site of the rupture. This accumulation can suddenly obstruct the blood flow in the respective coronary arteries, resulting in the obstruction of blood flow depriving the heart muscle of the vital oxygenated blood supply (Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria 2010). There are approximately four billion cardiomyocytes that build the left ventricle. One billion cardiomyocytes may be wiped out within a few hours post-myocardial infarction (Murry et al. 2006). In this regard, it is of utmost important to prevent the loss of cardiomyocytes, as the damaged myocardium is irreparable therefore, new therapies are needed to stimulate adult human cardiomyocyte proliferation to remuscularize the heart. Not only the loss of CMs but also, MI leads to several events that include a strong inflammatory response, infiltration of neutrophils, local hypoxia, and formation of a fibrous tissue, with subsequent scar formation, which ultimately compromises the heart function (Cahill and Kharbanda 2017). Adult heart was believed to be a post-mitotic organ, recent studies have described that a few cardiomyocytes also undergo mitosis in the diseased hearts in an attempt to regenerate the myocardium (Kajstura et al. 1998; Rumyantsev 1977; Beltrami et al. 2001). It has also been shown that in adolescence, a negligible count of cardiomyocytes within the human heart undergoes cell cycle re-entry (Bergmann et al. 2009). However, even if cardiomyocyte proliferation does occur, the rate of proliferation is too low to repair the injured heart and the competency of human cardiomyocyte proliferation still largely remains unknown; therefore, novel regenerative strategies to increase cell cycling of human cardiomyocytes with subsequent regeneration of the heart muscle are urgently needed.

7.1.2 Cell Cycle and Cardiomyocyte Proliferation

Cardiomyocyte cell cycle activity diminishes during the postnatal heart growth due to the downregulation of promitotic factors such as cyclins and their dependent kinases, which play a crucial role in cardiac regeneration (Minami et al. 2012). The fact that the proliferation of cardiomyocytes occurs during the early development and after birth, and they lose the capacity to regenerate, makes them an interesting model to study, especially the cell cycle and its regulation which play a crucial role

in cardiomyocyte proliferation. The events that occur in a cell leading to its duplication and division are called the cell cycle. There are four discrete phases in the mammalian cell cycle (G1, S, G2, and M). The G1 (Gap) phase is where the cell prepares to divide, to perform this step, the cell enters the S phase (synthesis), where the DNA replication occurs, later the cell moves into G2 (gap) phase where the genetic material is organized, and the cell prepares to divide. The later phase is the M-phase (Mitosis) where the cell divides into two daughter cells. The M-mitosis phase comprises the division of the nucleus, which is Karyokinesis, and the division of the cytoplasm, which is cytokinesis. Mitosis is composed of five different phases which include prophase, prometaphase, metaphase, anaphase, and telophase. Cytokinesis is the final step in the cell cycle to achieve cell division (Srivastava and Ivey 2006; Choi et al. 2012).

The mammalian fetal heart grows by cardiomyocyte proliferation. Shortly after birth, cardiomyocytes enter a terminal state of cell cycle whereby they binucleate via cytokinesis failure and subsequently downregulate cell cycle activators (e.g., cyclins A and D) and upregulate cell cycle inhibitors (e.g., p21CIP1 and p27KIP1) (Beltrami et al. 2001). Consistent with this, the postnatal heart grows by cardiomyocyte cell enlargement (i.e., hypertrophy) rather than proliferation. Although somewhat debated, at best, adult mammalian cardiomyocytes have little capacity to proliferate in response to injury, a deficiency that underlies the poor regenerative ability of human hearts after MI (Choi et al. 2013). Even if proliferation does occur, the rate of proliferation is too low to repair the injured heart (Beltrami et al. 2001). This has led to the dogma that adult cardiomyocytes are terminally differentiated cells (i.e., permanently withdrawn from a proliferative state) (Kajstura et al. 1998). However, recently, a number of reports have challenged this dogma. Based on C14 level in the atmosphere, it was shown that ~1% of adult human cardiomyocytes undergo DNA synthesis in a year (Bergmann et al. 2009). Furthermore, it has been suggested that preexisting cardiomyocytes are the dominant source of cardiomyocyte replacement in normal myocardial homeostasis as well as after myocardial injury in mice (Senyo et al. 2013). Additionally, recent work has shown that a small percentage of adult cardiomyocytes can be induced to proliferate after injury. For instance, in vitro data suggest that treatment with the mitogen FGF1 and pharmacological inhibition of the stress kinase p38 induce adult cardiomyocyte proliferation (Engel et al. 2006). Collectively, these results challenge the dogma of terminal differentiation, potentiating novel cardiac regenerative strategies via cardiomyocyte proliferation. During the last two decades, many other molecules have been studied to determine their ability to induce fetal and neonatal cardiomyocyte proliferation. This includes overexpression of cell cycle regulators (e.g., cyclin D, cyclin A, cyclin B, Cdk2), overexpression of transcription factors (e.g., c-Myc, E2F2), knockout of cell cycle inhibitors (e.g., p27KIP1, retinoblastoma protein), external application of growth factors (e.g., IGF-1, FGF2, TWEAK), and external application of kinase inhibitors (e.g., BIO, a pharmacological inhibitor of GSK3-beta) (Kikuchi and Poss 2012). Although many of these mitogenic factors are capable of inducing cell cycle activation in cardiomyocytes, they exhibit a limited ability to promote proliferation in neonatal cardiomyocytes, and to date they show no ability to promote proliferation

in adult cardiomyocytes. Thus, while works by Engel and co-workers and Bergmann and co-workers challenge the dogma that all adult cardiomyocytes are terminally differentiated and, at least a subpopulation, may be capable of proliferation, factors capable of promoting the robust proliferation required for regeneration have yet to be elucidated. In contrast to mammals, zebrafish regenerate heart muscle after trauma by inducing the proliferation of remaining cardiomyocytes, providing a model for identifying manipulations to induce regeneration in mammalian hearts (Choi et al. 2013). One strategy for reversing IHD is cardiac regenerative therapy. It is a multidisciplinary research area that employs experts in physiology, stem cell biology, developmental biology, biomaterial science, and tissue engineering with the goal of succeeding in regenerative medicine and preventing or reversing heart failure (Kikuchi and Poss 2012). Tissue engineering has emerged, aiming to regenerate an insulted organ using biomaterials. Biomaterials combine cells with matrices and can release bioactive molecules, for example, to enhance vascularization and proliferation of cardiomyocytes (Engel et al. 2006). Such biomaterials can improve the functioning of the heart, but the vital factor is tolerance by the host immune system (Patra et al. 2012).

For cell replacement therapies, there are several potential sources of cardiomyocytes, which are either endogenous or exogenous stem cells. Exogenous stem cells include bone marrow-derived stem cells, hematopoietic stem cells, mesenchymal stem cells, endothelial progenitor cells, embryonic stem cells (ESCs), and induced pluripotent stem cells. These stem cells possess varying capacities to differentiate into cardiomyocytes. Among the sources of the cardiomyocytes, pluripotent stem cells were identified to undergo differentiation into cardiomyocytes (Reinecke et al. 2008), with an efficiency of up to 98% in cytokine- and serum-free, xeno-free, defined medium (Minami et al. 2012).

7.1.3 Stem Cells as a Model for Cardiac Regeneration

It has been demonstrated that embryonic stem cells (ESCs) and progenitor cells with ESC-like characteristics have the ability to differentiate into cardiomyocytes both in vitro and in vivo. Thus, ESCs and progenitor cells have been considered to be a potential source of donor cardiomyocytes for therapeutic purposes in diseased hearts (Srivastava and Ivey 2006). Usage of stem cells holds a great potential for generating replacement cells for the heart muscle, valve, vessel, and conduction cells. Along with the stem cells, the recently discovered multipotent progenitor cells in the heart, the understanding of the developmental processes with respect to pluripotent embryonic stem cells could assist in the generation of the specific cell types required for the treatment of heart disease (Srivastava and Ivey 2006). ESC differentiation into cardiomyocytes can be modulated resulting in a high differentiation rate. This makes ESCs very attractive for cell therapy approaches. However, ESCs have also significant disadvantages, not only from an ethical point of view. They may be causing teratoma development and will be rejected by the host if the immune system is not suppressed. This is the main reason for the non-usage of ESCs in clinical cell

therapy studies (Choi et al. 2012). Thus, there is a great need to test other stem cells for their suitability in cardiac regenerative therapies. For example, recent publications have suggested that autologously induced pluripotent stem cells (iPSC) are tolerated by the host immune system (Araki et al. 2013). Stem cells have unique properties and thus they have individual advantages and disadvantages regarding cardiac therapy. These properties have to be considered in order to determine the most suitable cell for enhancing cell survival and longevity of engraftment post-transplantation. Other factors such as the injecting method and dosage of the stem cells must be established in order to increase the stability and efficiency for clinical applications.

Stem cells are often considered to be "master cells" because they are a class of undifferentiated cells that can differentiate into a specific cell type. Stem cells "self-renew" by a process called asymmetric cell division—that is when cell division results in one daughter cell that resembles the mother cell and a second daughter cell that forms a specialized cell type (a "committed progenitor"). These distinctive abilities of stem cells are predicted to offer new potential treatments for chronic diseases such as diabetes and heart diseases (Lakshmipathy and Verfaillie 2005). Stem cells can be utilized to understand diverse developmental mechanisms like cell differentiation, maturation, and early human lineage commitment. Also, they enable the analyses of disease genes and the establishment of in vitro disease models to test treatment strategies (e.g., cell damage, diabetes, cardiac failure, and neurological disorders) (Thomas et al. 1959; Weissman 2000). Stem cells are separated into subgroups of cells dependent on their potency and the ability of cells to differentiate into other cell types (Lakshmipathy and Verfaillie 2005).

I. Totipotent cells—Totipotent cells are those that have the ability to differentiate into every cell type. Examples include the fertilized egg, which has the capability to give rise to all embryonic and extra-embryonic tissues.
II. Multipotent—Multipotency is the ability of the cells to produce closely related types of cells. Examples are the cells derived from ectoderm, mesoderm, and endoderm (the three embryonic germ layers). These cells can undergo repeated differentiation to form particular cells, tissues, and organs (Lakshmipathy and Verfaillie 2005).
III. Pluripotent cells—ESCs, isolated from the inner cell mass of the blastocyst of an early embryo, can give rise to mesoderm, endoderm, and ectoderm but not to extra-embryonic tissues (Lakshmipathy and Verfaillie 2005).

7.1.3.1 Multipotent Stem Cells

Fetal Stem Cells

The source of fetal stem cells is the fetus or the umbilical cord blood, amniotic fluid, Wharton"s jelly, the amniotic membrane, and the placenta (Hemberger et al. 2008). Usage of the fetus itself as a source of stem cells has several ethical issues but extraembryonic structures can be considered a suitable source of stem cells (Pappa and Anagnou 2009).

Adult Stem Cells

Adult stem cells have limited self-renewal and differentiation capacity. They are found in differentiated tissues of the cell types into which they differentiate. Although adult stem cells possess limited differentiation and self-renewal capability, they are more suited for therapeutic purposes, as patients" own stem cells can be utilized eliminating the problem of immune rejection. Also, adult stem cells are easy to isolate with limited ethical reservations (Choumerianou et al. 2008).

Cardiac Stem Cells

In the heart, several types of cells with stem cell characteristics have been discovered. These cell types include cells expressing stem cell factor receptor (c-Kit) (Bearzi et al. 2007), or stem cell antigen-1 (Sca-1) on their cell surface (Oh et al. 2003). Cells expressing the homeodomain transcription factor islet-1 (Isl-1) (Laugwitz et al. 2005), side population cells (SP), and cells that have the ability to grow in cardiospheres have also been discovered (Messina et al. 2004).

7.1.3.2 Pluripotent Stem Cells

Embryonic Stem Cells

ESCs represent a versatile biological system. These cells have been established as a permanent cell line from the early embryo and have led to several advances in the fields of cell and developmental biology (Wobus and Boheler 2005). The term "embryonic stem cell" was first used in 1981 to differentiate between embryo-derived pluripotent cells from teratocarcinoma-derived pluripotent embryonal carcinoma (EC) cells (Martin 1981). The first isolation of ES cells was successfully carried out from mouse inner cell mass (ICM) in 1981 (Evans and Kaufman 1981), and in 1994 Bongso and co-workers reported the isolation of human ICM cells and their continued culture in vitro (Bongso et al. 1994). The trophoblast is the outer cell mass, while the embryoblast is the ICM, which is the main source of totipotent embryonic stem cells. These embryonic stem cells compared to adult stem cells can differentiate into more than 220 cell types in the adult human body and are easy to culture. The major disadvantage of using embryonic stem cells is with respect to the ethical issues related to the destruction of an embryo after cell extraction. Embryonic stem cells are only pluripotent but not totipotent as they do not have the capability to form extra-embryonic membranes or the placenta (Sreenivas et al. 2011). Transplantation experiments were conducted with cardiomyocytes produced from human embryonic stem cells (hES) utilizing athymic rats with healing infarcts 4 days after ischemia or reperfusion injury. These initial experiments were unsuccessful and exhibited poor cell survival. To increase cell survival, combination approaches were followed where donor cells are pretreated with reagents to block cell death mediated by ischemia, inflammation, apoptosis. This pretreatment was found to markedly increase the survival rate of the donor cardiomyocytes after transplanting into infarcted tissue. The progressive decrease typically was also found to be decreased following 4 weeks of infarction (Rubart and Field 2007). The engrafted human cardiomyocytes attenuated ventricular dilation and preserved regional and

global contractile function after myocardial infarction compared to controls receiving noncardiac hES cell derivatives or vehicle (Laflamme et al. 2007).

Induced Pluripotent Stem Cells

In 2006, Yamanaka's group for the first time generated induced pluripotent stem (iPS) cells. The iPS were generated by forced expression of four transcription factors, namely octamer binding transcription factor 4 (Oct-3/4), SRY-box 2 (SOX2), V-myc myelocytomatosis viral oncogene homolog (c-Myc), and Krüppel-like factor (Klf4) in mouse embryonic or adult fibroblasts (Takahashi and Yamanaka 2006). These iPS cells express similar properties to embryonic stem cells (hESCs) (Evans and Kaufman 1981) and can be differentiated into a variety of cells including cardiomyocytes (Takahashi and Yamanaka 2006; Yoshida and Yamanaka 2017). Since the past decade, the method is widely applied in the generation of human-induced pluripotent stem cells (Takahashi et al. 2007; Thomson et al. 1998), and their differentiation into functional cardiomyocytes (hiPSC-CMs) (Ieda et al. 2010; Burridge et al. 2012; Burridge et al. 2014), that are currently being significantly improved to overcome hurdles in achieving cardiac regeneration (Tohyama and Fukuda 2016) (Fig. 7.1). During the last years, by using myocardial ischemic reperfusion model in non-human primates (pig-tailed macaque), the research groups of Laflamme and Murray delivered one billion human embryonic stem cell-derived cardiomyocytes (hESCs-CMs) via intra-myocardial injections in the infarcted heart (Chong et al. 2014). The infarcted monkey heart was remuscularized and cardiac function was improved upon delivery of hESC-CMs. However, during the 3-month period, a large number of cardiomyocytes that were engrafted remained immature resulting in non-lethal ventricular arrhythmias (Chong et al. 2014). Similarly, the group from Ikeda, injected induced pluripotent stem cell-derived cardiomyocytes in primates resulted in improved cardiac functionality and contractility (Shiba et al. 2016). HiPSC-derived cardiomyocytes were also used in several applications. As the hiPSC-derived cardiomyocytes remain mostly immature, recent studies focused on the maturation of cardiomyocytes derived from hiPSCs. As it takes several years in vivo for the cardiomyocytes to mature (Vreeker et al. 2014), recent studies described about long-term culturing of hiPSC-CMs (Kamakura et al. 2013), which resulted in mature phenotype in the alignment of myofibril density, with visible sarcomeric structures, a larger cell size, and the presence of the cardiac maturation genes (e.g., MYH7). Altogether, these studies suggest that the cardiac maturation process is still achievable (Lundy et al. 2013; Lewandowski et al. 2018).

However, several experiments are still needed to fully understand the maturation and integrity of hiPSC-CMs and resolve the safety and feasibility of transplanted cardiomyocytes derived from hESCs and hiPSCs (Ahmed et al. 2020). However, hiPSC-CMs can also be utilized as a model system in various fields. Such as screening of chemical libraries (Magadum et al. 2017), microRNAs (Eulalio et al., 2012); Diez-Cunado et al. 2018, and in drug discovery (Del Alamo et al. 2016), to assess the effect of drug toxicity (Kussauer et al. 2019; McKeithan et al. 2020). Recently, hiPSCs were also used in precision medicine by utilizing patient-specific human iPSC-derived cardiomyocytes (Karakikes et al. 2015), in understanding and

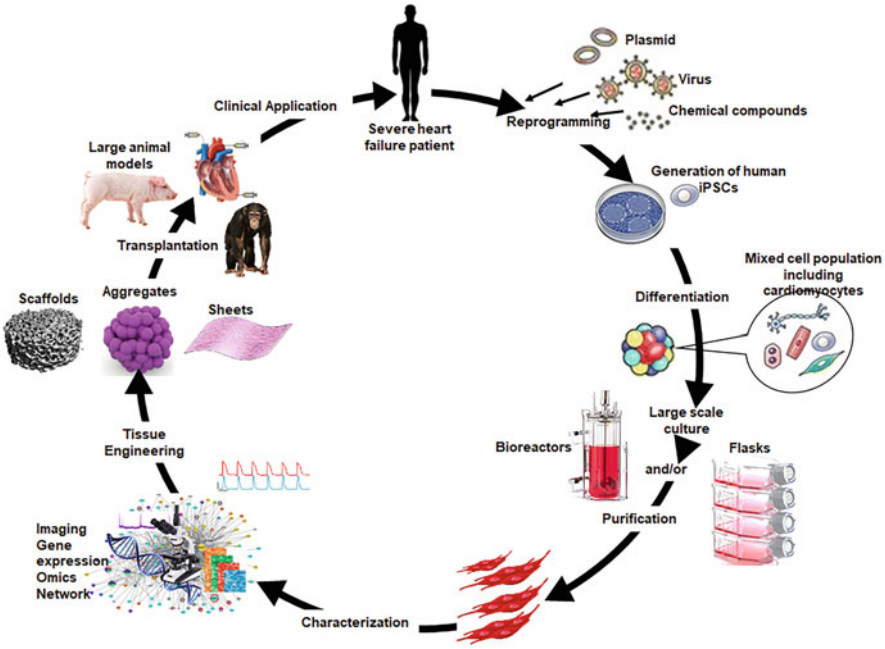


Fig. 7.1 Hurdles to overcome cardiac regenerative therapy. (1) Improving the efficiency of cardiomyocyte differentiation. (2) Cultivating cardiomyocytes in large-scale systems, (3) Purification of cardiomyocytes in large-scale systems (4) characterization of electrophysiological properties, (5) Utilizing the state of art techniques to improve tissue engineering and (6) improving the feasibility and safety of using cardiomyocytes in large animal models. Figure Adopted from Shugo T et al., 2016 (Tohyama and Fukuda 2016)

to delve mechanisms involved in cardiac diseases (Kamakura et al. 2013; Paik et al. 2020), and in recreating new models for translational cardiac regenerative medicine (Harris et al. 2013; Cambria et al. 2017).

7.2 Noncoding RNAs and Cardiac Regeneration

Noncoding RNAs among them microRNAs (miRNAs) are a class of small noncoding RNAs, with an average of 20–23 nucleotides in length, which interfere with messenger RNAs (mRNA) at the post-transcriptional level. This results in degradation or translational repression of the targeted mRNAs and thus occupies a central role in gene regulation (Bartel 2004). In the year 1993, the groups from Ambros and Ruvkun discovered the first microRNA, as lin-4 in *Caenorhabditis elegans* (nematode) (Lee et al. 1993; Wightman et al. 1993). Horvitz and his team previously showed that Lin-4 is a gene that controls the temporal development events in *C. elegans* (Horvitz and Sulston 1980). Later after a few years, Ambros and Ruvkun team discovered that Lin-4 is rather a small noncoding RNA, and not a protein-

coding RNA (Lee et al. 2004). This discovery made an important revolution in the field of molecular biology. Most of the miRNAs are transcribed into primary miRNAs (pri-miRNAs), which undergo multistep biogenesis and can be processed into precursor miRNAs (pre-miRNAs) and later into mature miRNAs (Ha and Kim 2014; Gebert and MacRae 2019; O'Brien et al. 2018). The microRNA fundamental biogenesis factors such as Drosha (Chong et al. 2010) and Dicer (Bernstein et al. 2003) are involved in pre-miR splicing. Deletions in these factors are lethal in embryos of mouse (Gebert and MacRae 2019; Burger and Gullerova 2015). The pri-miRNAs are transcribed by RNA polymerase II, which is processed in the nucleus, which is then cleaved by RNase III enzyme Drosha and the DiGeorge Syndrome Critical Region 8 (DGCR8) is a protein, which is known as Pasha, that is partner of Drosha to form pre-miRNAs, which are short hairpin RNAs, consisting of around 70 nucleotides (Lee et al. 2003; Denli et al. 2004). The pre-miRNA is then exported from the nucleus to the cytoplasm with the help of Ran-GTP/Exportin 5 mechanism (Lund et al. 2004), and the pre-miRNA is processed by Dicer, to generate a 22-nucleotide mature miRNA (Du and Zamore 2005). The gene silencing induced by microRNAs is processed with the association of a multiprotein complex called RNA-induced silencing complex (RISC) (Gebert and MacRae 2019). The mature miRNAs enter into Argonaute containing (RISC), within this complex the protein expression is repressed by the microRNAs, by interacting with 3′--untranslated regions (Bartel 2004). The 3′ untranslated region (3′UTR) of the target mRNA is the most common binding sites of the microRNA. However, functional binding sites can also be found in the coding region (Forman et al. 2008), and 5′-untranslated region (5′UTR) (Lytle et al. 2007; Appasani 2008). MiRNAs are involved in various cellular and biological process, which are important for cell differentiation, cell death, and cell proliferation (Gebert and MacRae 2019; Hata 2013). Intriguingly, one miRNA may target hundreds of mRNAs mostly involved in the inhibition of a common biological pathway such as hypertrophy or proliferation or apoptosis. By repression of these mRNAs, the output of a pathway can be profoundly altered. In recent years, several microRNAs have been tested in animal models in cardiomyocyte hypotrophy, and apoptosis and several microRNAs were identified in regulating cardiomyocyte proliferation, which involves in cardiac regeneration, and can be used in treating several cardiovascular diseases. Of interest, miRNAs can be therapeutically manipulated and therefore hold exciting opportunities for future clinical therapies (Olson 2014; van Rooij and Kauppinen 2014).

MiRNAs are required for modulation of the proliferative capacity of cardiomyocytes, as cardiac deletion of enzymes that are involved in the biogenesis and processing of miRNAs resulted in dilatation of the heart and premature lethality (Chen et al. 2008; Rao et al. 2009). Several studies have shown that artificial inhibition or upregulation of miRNAs improves LV-function after myocardial infarction (Eulalio et al. 2012; Borden et al. 2019; Chen et al. 2013; Gao et al. 2019; Porrello et al. 2011a, 2013; Tian et al. 2015; Wahlquist et al. 2014; Lesizza et al. 2017). This miRNA-induced regenerative potential is not only observed in animals during the neonatal phase (Porrello et al. 2011a), where the cardiac

regenerative potential is preserved (Porrello et al. 2011b), but also in the postnatal/adult phase (Porrello et al. 2013), where the regenerative response is absent. An important modulating role of miRNAs is further supported by the observation that miRNA-levels change in a spatiotemporal manner during the short phase where the regenerative cardiac potential is preserved in postnatal murine hearts (Bergmann et al. 2009). As an example, miR-195, a member of the miR-15-family, is highly upregulated in mouse hearts between days 1 and 10 after birth. Delivery of artificial anti-miRs targeting miR-15-family members in neonatal mice increased cardiomyocyte proliferation by deinhibition of cell cycle genes (Porrello et al. 2011a, 2013). They further investigated the impact of miR-15 on cardiac regeneration after cardiac injury in postnatal mice. Postnatal myocardial infarction at day 1 resulted in an extensive infarcted area. However, functional recovery was observed at day 21 (Porrello et al. 2013). Furthermore, pretreatment of adult mice with anti-miR-15 improved cardiac function after induction of myocardial infarction in adult mice (Porrello et al. 2013). Another study investigated the miR-302-367 cluster in hearts that suppresses contributors of the Hippo-signaling pathway. This recently discovered conserved pathway regulates cardiomyocyte proliferation (Tian et al. 2015). Transgenic cardiac or systemic transient overexpression of the miR-302-367 enhanced cardiomyocyte proliferation and improved cardiac function in a mouse myocardial infarction model (Tian et al. 2015). In recent reports, the research group from Giacca performed a high throughput screening of human microRNA library, and identified microRNA-mimics (miR-199 and miR-590), as potential regulators of mouse and rat cardiomyocyte proliferation in vitro, in vivo. Later using a myocardial infarction model in 8–12 weeks old mice, they generated AAV9 (adeno associated virus) vectors that express miR-199 or miR-590, and injected these at the peri-infarct area post-myocardial infarction. Post 60 days, administration with one of the vectors displayed a significant improvement in the cardiac function in the adult mice (Eulalio et al. 2012). In a very recent study, the same group injected AAV-mediated microRNA mimics (miR-199 and miR-590) in a pig model (Gabisonia et al. 2019), also resulted in functional recovery, post-injury, and increased cardiomyocyte proliferation (Gabisonia et al. 2019; Sadek and Olson 2020). All these studies (Eulalio et al. 2012; Chen et al. 2013; Tian et al. 2015; Gabisonia et al. 2019) indicate that cell-cycle reentry of cardiomyocytes can be induced by the administration of pro-proliferative miRNAs. Most of these miRNAs modulate an important conserved signaling pathway, which regulates tissue and organ size, termed the Hippo kinase pathway (Tian et al. 2015; Xin et al. 2013; Torrini et al. 2019). In the heart, inhibition of Hippo pathway augments cardiomyocyte proliferation. Interestingly, Hippo deficiency reverses systolic heart failure after myocardial infarction (Leach et al. 2017). Therefore, targeting this pathway using miRNA therapeutics is a promising strategy to treat heart failure after myocardial infarction. Although strong evidence for the use of miRNAs as a regenerative application in murine cardiomyocytes exists (Eulalio et al. 2012; Borden et al. 2019; Tian et al. 2015), the support for a miRNA-induced proliferative potential in human cardiomyocytes is scarce.

References

Ahmed RE, Anzai T, Chanthra N, Uosaki H (2020) A brief review of current maturation methods for human induced pluripotent stem cells-derived cardiomyocytes. Front Cell Dev Biol 8:178

Appasani K (2008) MicroRNAs: from basic science to disease biology

Araki R, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S et al (2013) Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature 494(7435):100–104

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116(2): 281–297

Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A et al (2007) Human cardiac stem cells. Proc Natl Acad Sci U S A 104(35):14068–14073

Becker AM, Rubart M, Field LJ (2011) Inducing embryonic stem cells to become cardiomyocytes. In: Cohen IS, Gaudette GR (eds) Regenerating the heart: stem cells and the cardiovascular system. Humana Press, Totowa, NJ, pp 7–24

Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R et al (2001) Evidence that human cardiac myocytes divide after myocardial infarction. N Engl J Med 344(23):1750–1757

Bergmann O, Bhardwaj RD, Bernard S, Zdunek S, Barnabe-Heider F, Walsh S et al (2009) Evidence for cardiomyocyte renewal in humans. Science 324(5923):98–102

Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ et al (2003) Dicer is essential for mouse development. Nat Genet 35(3):215–217

Bongso A, Fong CY, Ng SC, Ratnam S (1994) Isolation and culture of inner cell mass cells from human blastocysts. Hum Reprod 9(11):2110–2117

Borden A, Kurian J, Nickoloff E, Yang Y, Troupes C, Ibetti J et al (2019) Transient introduction of miR-294 in the heart promotes cardiomyocyte cell cycle reentry after injury. Circ Res

Burger K, Gullerova M (2015) Swiss army knives: non-canonical functions of nuclear Drosha and Dicer. Nat Rev Mol Cell Biol 16(7):417–430

Burridge PW, Keller G, Gold JD, Wu JC (2012) Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. Cell Stem Cell 10(1):16–28

Burridge PW, Matsa E, Shukla P, Lin ZC, Churko JM, Ebert AD et al (2014) Chemically defined generation of human cardiomyocytes. Nat Methods 11(8):855–860

Cahill TJ, Kharbanda RK (2017) Heart failure after myocardial infarction in the era of primary percutaneous coronary intervention: mechanisms, incidence and identification of patients at risk. World J Cardiol 9(5):407–415

Cambria E, Pasqualini FS, Wolint P, Gunter J, Steiger J, Bopp A et al (2017) Translational cardiac stem cell therapy: advancing from first-generation to next-generation cell types. NPJ Regen Med 2:17

Chen JF, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z et al (2008) Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci U S A 105(6):2111–2116

Chen J, Huang ZP, Seok HY, Ding J, Kataoka M, Zhang Z et al (2013) mir-17-92 cluster is required for and sufficient to induce cardiomyocyte proliferation in postnatal and adult hearts. Circ Res 112(12):1557–1566

Choi SH, Jung SY, Kwon SM, Baek SH (2012) Perspectives on stem cell therapy for cardiac regeneration. Advances and challenges. Circ J 76(6):1307–1312

Choi WY, Gemberling M, Wang J, Holdway JE, Shen MC, Karlstrom RO et al (2013) In vivo monitoring of cardiomyocyte proliferation to identify chemical modifiers of heart regeneration. Development 140(3):660–666

Chong MM, Zhang G, Cheloufi S, Neubert TA, Hannon GJ, Littman DR (2010) Canonical and alternate functions of the microRNA biogenesis machinery. Genes Dev 24(17):1951–1960

Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ et al (2014) Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. Nature 510(7504):273–277

Choumerianou DM, Dimitriou H, Kalmanti M (2008) Stem cells: promises versus limitations. Tissue Eng Part B Rev 14(1):53–60

Del Alamo JC, Lemons D, Serrano R, Savchenko A, Cerignoli F, Bodmer R et al (2016) High throughput physiological screening of iPSC-derived cardiomyocytes for drug development. Biochim Biophys Acta 1863(7 Pt B):1717–1727

Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ (2004) Processing of primary microRNAs by the Microprocessor complex. Nature 432(7014):231–235

Diez-Cunado M, Wei K, Bushway PJ, Maurya MR, Perera R, Subramaniam S et al (2018) miRNAs that Induce Human Cardiomyocyte Proliferation Converge on the Hippo Pathway. Cell Rep 23(7):2168–2174

Du T, Zamore PD (2005) microPrimer: the biogenesis and function of microRNA. Development 132(21):4645–4652

Engel FB, Hsieh PC, Lee RT, Keating MT (2006) FGF1/p38 MAP kinase inhibitor therapy induces cardiomyocyte mitosis, reduces scarring, and rescues function after myocardial infarction. Proc Natl Acad Sci U S A 103(42):15546–15551

Eulalio A, Mano M, Dal Ferro M, Zentilin L, Sinagra G, Zacchigna S et al (2012) Functional screening identifies miRNAs inducing cardiac regeneration. Nature 492(7429):376–381

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292(5819):154–156

Forman JJ, Legesse-Miller A, Coller HA (2008) A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc Natl Acad Sci U S A 105(39):14879–14884

Gabisonia K, Prosdocimo G, Aquaro GD, Carlucci L, Zentilin L, Secco I et al (2019) MicroRNA therapy stimulates uncontrolled cardiac repair after myocardial infarction in pigs. Nature 569(7756):418–422

Gao F, Kataoka M, Liu N, Liang T, Huang ZP, Gu F et al (2019) Therapeutic role of miR-19a/19b in cardiac regeneration and protection from myocardial infarction. Nat Commun 10(1):1802

Gebert LFR, MacRae IJ (2019) Regulation of microRNA function in animals. Nat Rev Mol Cell Biol 20(1):21–37

Ha M, Kim VN (2014) Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol 15(8):509–524

Harris K, Aylott M, Cui Y, Louttit JB, McMahon NC, Sridhar A (2013) Comparison of electrophysiological data from human-induced pluripotent stem cell-derived cardiomyocytes to functional preclinical safety assays. Toxicol Sci 134(2):412–426

Hata A (2013) Functions of microRNAs in cardiovascular biology and disease. Annu Rev Physiol 75:69–93

Hemberger M, Yang W, Natale D, Brown TL, Dunk C, Gargett CE et al (2008) Stem cells from fetal membranes—a workshop report. Placenta 29(Suppl A):S17–S19

Horvitz HR, Sulston JE (1980) Isolation and genetic characterization of cell-lineage mutants of the nematode Caenorhabditis elegans. Genetics 96(2):435–454

Ieda M, Fu JD, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG et al (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142(3): 375–386

Institute of Medicine (US) Committee on Social Security Cardiovascular Disability Criteria (2010) Cardiovascular disability: updating the social security listings. Washington, DC

Kajstura J, Leri A, Finato N, Di Loreto C, Beltrami CA, Anversa P (1998) Myocyte proliferation in end-stage cardiac failure in humans. Proc Natl Acad Sci U S A 95(15):8801–8805

Kamakura T, Makiyama T, Sasaki K, Yoshida Y, Wuriyanghai Y, Chen J et al (2013) Ultrastructural maturation of human-induced pluripotent stem cell-derived cardiomyocytes in a long-term culture. Circ J 77(5):1307–1314

Karakikes I, Ameen M, Termglinchan V, Wu JC (2015) Human induced pluripotent stem cell-derived cardiomyocytes: insights into molecular, cellular, and functional phenotypes. Circ Res 117(1):80–88

Kikuchi K, Poss KD (2012) Cardiac regenerative capacity and mechanisms. Annu Rev Cell Dev Biol 28:719–741

Kussauer S, David R, Lemcke H (2019) hiPSCs derived cardiac cells for drug and toxicity screening and disease modeling: what micro- electrode-array analyses can tell us. Cell 8(11)

Laflamme MA, Chen KY, Naumova AV, Muskheli V, Fugate JA, Dupras SK et al (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25(9):1015–1024

Lakshmipathy U, Verfaillie C (2005) Stem cell plasticity. Blood Rev 19(1):29–38

Laugwitz KL, Moretti A, Lam J, Gruber P, Chen Y, Woodard S et al (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433(7026):647–653

Leach JP, Heallen T, Zhang M, Rahmani M, Morikawa Y, Hill MC et al (2017) Hippo pathway deficiency reverses systolic heart failure after infarction. Nature 550(7675):260–264

Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75(5):843–854

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J et al (2003) The nuclear RNase III Drosha initiates microRNA processing. Nature 425(6956):415–419

Lee R, Feinbaum R, Ambros V (2004) A short history of a short RNA. Cell 116(2 Suppl):S89–S92, 1 p following S6

Lesizza P, Prosdocimo G, Martinelli V, Sinagra G, Zacchigna S, Giacca M (2017) Single-dose intracardiac injection of pro-regenerative microRNAs improves cardiac function after myocardial infarction. Circ Res 120(8):1298–1304

Lewandowski J, Rozwadowska N, Kolanowski TJ, Malcher A, Zimna A, Rugowska A et al (2018) The impact of in vitro cell culture duration on the maturation of human cardiomyocytes derived from induced pluripotent stem cells of myogenic origin. Cell Transplant 27(7):1047–1067

Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U (2004) Nuclear export of microRNA precursors. Science (New York, NY) 303(5654):95–98

Lundy SD, Zhu WZ, Regnier M, Laflamme MA (2013) Structural and functional maturation of cardiomyocytes derived from human pluripotent stem cells. Stem Cells Dev 22(14):1991–2002

Lytle JR, Yario TA, Steitz JA (2007) Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci U S A 104(23):9667–9672

Magadum A, Ding Y, He L, Kim T, Vasudevarao MD, Long Q et al (2017) Live cell screening platform identifies PPARdelta as a regulator of cardiomyocyte proliferation and cardiac repair. Cell Res 27(8):1002–1019

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78(12):7634–7638

McKeithan WL, Feyen DAM, Bruyneel AAN, Okolotowicz KJ, Ryan DA, Sampson KJ et al (2020) Reengineering an antiarrhythmic drug using patient hiPSC cardiomyocytes to improve therapeutic potential and reduce toxicity. Cell Stem Cell 27(5):813–21 e6

Mendis S, Puska P, Norrving B, World Health O, World Heart F, World Stroke O. Global atlas on cardiovascular disease prevention and control/edited by: Shanthi Mendis ... [et al.] Geneva: World Health Organization; 2011

Messina E, De Angelis L, Frati G, Morrone S, Chimenti S, Fiordaliso F et al (2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95(9):911–921

Minami I, Yamada K, Otsuji TG, Yamamoto T, Shen Y, Otsuka S et al (2012) A small molecule that promotes cardiac differentiation of human pluripotent stem cells under defined, cytokine- and xeno-free conditions. Cell Rep 2(5):1448–1460

Murry CE, Reinecke H, Pabon LM (2006) Regeneration gaps: observations on stem cells and cardiac repair. J Am Coll Cardiol 47(9):1777–1785

Young DB (2010) Control of cardiac output. Colloquium series on integrated systems physiology: from molecule to function to disease. San Rafael, CA

Writing Group Members, Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ et al (2016) Heart disease and stroke statistics-2016 update: a report from the American Heart Association. Circulation 133(4):e38–e360

Rumyantsev PP (1977) Interrelations of the proliferation and differentiation processes during cardiact myogenesis and regeneration. Int Rev Cytol 51:186–273
Srivastava D, Ivey KN (2006) Potential of stem-cell-based therapies for heart disease. Nature 441(7097):1097–1099
Senyo SE, Steinhauser ML, Pizzimenti CL, Yang VK, Cai L, Wang M et al (2013) Mammalian heart renewal by pre-existing cardiomyocytes. Nature 493(7432):433–436
Patra C, Talukdar S, Novoyatleva T, Velagala SR, Muhlfeld C, Kundu B et al (2012) Silk protein fibroin from Antheraea mylitta for cardiac tissue engineering. Biomaterials 33(9):2673–2680
Reinecke H, Minami E, Zhu WZ, Laflamme MA (2008) Cardiogenic differentiation and transdifferentiation of progenitor cells. Circ Res 103(10):1058–1071
Thomas ED, Lochte HL Jr, Cannon JH, Sahler OD, Ferrebee JW (1959) Supralethal whole body irradiation and isologous marrow transplantation in man. J Clin Invest 38:1709–1716
Weissman IL (2000) Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science 287(5457):1442–1446
Pappa KI, Anagnou NP (2009) Novel sources of fetal stem cells: where do they fit on the developmental continuum? Regen Med 4(3):423–433
Oh H, Bradfute SB, Gallardo TD, Nakamura T, Gaussin V, Mishina Y et al (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci U S A 100(21):12313–12318
Wobus AM, Boheler KR (2005) Embryonic stem cells: prospects for developmental biology and cell therapy. Physiol Rev 85(2):635–678
Sreenivas SD, Rao AS, Satyavani SS, Reddy BH, Vasudevan S (2011) Where will the stem cells lead us? Prospects for dentistry in the 21 century. J Indian Soc Periodontol 15(3):199–204
Rubart M, Field LJ (2007) ES cells for troubled hearts. Nat Biotechnol 25(9):993–994
Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676
Yoshida Y, Yamanaka S (2017) Induced pluripotent stem cells 10 years later: for cardiac applications. Circ Res 120(12):1958–1968
Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131(5):861–872
Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS et al (1998) Embryonic stem cell lines derived from human blastocysts. Science (New York, NY) 282(5391):1145–1147
Tohyama S, Fukuda K (2016) Future treatment of heart failure using human iPSC-derived cardiomyocytes. In: Nakanishi T, Markwald RR, Baldwin HS, Keller BB, Srivastava D, Yamagishi H (eds) Etiology and morphogenesis of congenital heart disease: from gene function and cellular interaction to morphology. Springer, Tokyo, pp 25–31
Shiba Y, Gomibuchi T, Seto T, Wada Y, Ichimura H, Tanaka Y et al (2016) Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature 538(7625): 388–391
Vreeker A, van Stuijvenberg L, Hund TJ, Mohler PJ, Nikkels PG, van Veen TA (2014) Assembly of the cardiac intercalated disk during pre- and postnatal development of the human heart. PLoS One 9(4):e94722
Paik DT, Chandy M, Wu JC (2020) Patient and disease-specific induced pluripotent stem cells for discovery of personalized cardiovascular drugs and therapeutics. Pharmacol Rev 72(1):320–342
Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75(5):855–862
O'Brien J, Hayder H, Zayed Y, Peng C (2018) Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol (Lausanne) 9:402
Olson EN (2014) MicroRNAs as therapeutic targets and biomarkers of cardiovascular disease. Sci Transl Med 6(239):239ps3
van Rooij E, Kauppinen S (2014) Development of microRNA therapeutics is coming of age. EMBO Mol Med 6(7):851–864

Rao PK, Toyama Y, Chiang HR, Gupta S, Bauer M, Medvid R et al (2009) Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res 105(6):585–594

Porrello ER, Johnson BA, Aurora AB, Simpson E, Nam YJ, Matkovich SJ et al (2011a) MiR-15 family regulates postnatal mitotic arrest of cardiomyocytes. Circ Res 109(6):670–679

Porrello ER, Mahmoud AI, Simpson E, Johnson BA, Grinsfelder D, Canseco D et al (2013) Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. Proc Natl Acad Sci U S A 110(1):187–192

Tian Y, Liu Y, Wang T, Zhou N, Kong J, Chen L et al (2015) A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. Sci Transl Med 7(279): 279ra38

Wahlquist C, Jeong D, Rojas-Munoz A, Kho C, Lee A, Mitsuyama S et al (2014) Inhibition of miR-25 improves cardiac contractility in the failing heart. Nature 508(7497):531–535

Porrello ER, Mahmoud AI, Simpson E, Hill JA, Richardson JA, Olson EN et al (2011b) Transient regenerative potential of the neonatal mouse heart. Science (New York, NY) 331(6020): 1078–1080

Sadek H, Olson EN (2020) Toward the goal of human heart regeneration. Cell Stem Cell 26(1): 7–16

Xin M, Kim Y, Sutherland LB, Murakami M, Qi X, McAnally J et al (2013) Hippo pathway effector Yap promotes cardiac regeneration. Proc Natl Acad Sci U S A 110(34):13839–13844

Torrini C, Cubero RJ, Dirkx E, Braga L, Ali H, Prosdocimo G et al (2019) Common regulatory pathways mediate activity of microRNAs inducing cardiomyocyte proliferation. Cell Rep 27(9): 2759–71.e5

Hydrogel-Based Tissue-Mimics for Vascular Regeneration and Tumor Angiogenesis

8

Sushmita Bist, Abhinaba Banerjee, Indira Priyadarshani Patra, Sruthi Rayadurgam Jayaprakash, Rajat Sureka, and Shantanu Pradhan

Abbreviations

2D	Two-dimensional
2P	Two-photon
3D	Three-dimensional
ADSC	Adipose-derived stromal cell
Ang2	Angiopoietin-2
BBB	Blood-brain barrier
bFGF	Basic fibroblast derived factor
BM	Basement membrane
BMP-2	Bone morphogenetic protein-2
CAD	Computer-aided design
DA	Diacrylate
DexMA	Methacrylated dextran
DMA	Dimethacrylate
DMD	Digital mirror device

Sushmita Bist and Abhinaba Banerjee contributed equally to this work.

S. Bist · I. P. Patra · S. R. Jayaprakash · R. Sureka · S. Pradhan (✉)
Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India
e-mail: spradhan@iitm.ac.in

A. Banerjee
Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai, India

Department of Chemical Engineering, Indian Institute of Technology Madras, Chennai, India

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_8

ECFC	Endothelial colony forming cell
ECM	Extracellular matrix
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
FN	Fibronectin
GAG	Glycosaminoglycan
HA	Hyaluronic acid
HIF-1α	Hypoxia-inducible factor-1α
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cell
HVP	Human vascular pericyte
IPN	Interpenetrating polymer network
LSEC	Liver sinusoid endothelial cell
MeHA	Methacrylated hyaluronic acid
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem/stromal cell
NHDF/HNDF	Normal human dermal fibroblasts
PCL	Poly(ε-caprolactone)
PDGF-BB	Platelet derived growth factor BB
PDMS	Poly(dimethylsiloxane)
PEG	Poly(ethylene glycol)
PEGDA	Poly(ethylene glycol diacrylate)
PLGA	Poly(lactic-*co*-glycolic acid)
PVA	Polyvinyl alcohol
RGD	Arginine-glycine-aspartate
RHAMM	Receptor for HA-mediated motility
RPE	Retinal pigment epithelial
SMC	Smooth muscle cell
TEC	Tumor-(associated) endothelial cell
TEVG	Tissue-engineered vascular grafts
TME	Tumor microenvironment
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor

8.1 Introduction

In recent decades, there has been an increasing demand for large-volume tissue-engineered organs and synthetic biomaterial-based constructs for use in regenerative medicine applications. One of the fundamental requirements of such organs or constructs is the presence of stable and functional vascular network which can

provide the required supply of oxygen and nutrients as well as metabolic waste clearance in a continuous and consistent manner. The proper function of the vascular network is essential in maintaining long-term cell viability, and overall structure and function in a wide range of organ- and tissue-mimics (Auger et al. 2013).

However, generation of such vascular networks has been beset with several challenges. A wide range of biomaterials have been employed so far for synthetic vascularized tissues and more are under development. Also, vascular cells used for tissue engineering applications are obtained from a wide variety of sources, making it difficult to control and compare experimental conditions for further translation and replication. Given the strict requirements of vascular cells in terms of matrix microenvironment conducive to vascular network formation, it is important to match the right biomaterial properties and culture conditions with the vascular cell types used for specific applications (Novosel et al. 2011; Chang and Niklason 2017).

In addition to tissue engineering applications, in vitro modeling of various diseases and pathologies has also gained prominence. Particularly, modeling of cancer has been an attractive research problem, owing to the ability to recapitulate the native tumor microenvironment (TME) using various biomaterial-based approaches (Pradhan et al. 2016). One of the most influential components of the TME is the tumor-associated endothelial cells (TECs), which constitute the leaky and tortuous tumor vasculature adjacent to growing tumors. TECs actively participate in tumor angiogenesis and promote tumor growth, progression, and metastasis via a wide range of mechanisms (Dudley 2012). When creating in vitro tumor models, it is important to incorporate TECs along with cancer cells, so that the wide diversity of intercellular and cell-matrix crosstalk can be accurately captured in in vitro engineered systems. These vascularized tumor models can provide high-quality predictive information regarding candidate drug efficacy and accelerate the anti-cancer drug screening process.

In recent years, numerous biomaterials (including natural, synthetic, and semi-synthetic hydrogels) have been implemented for the generation of vascularized matrices and scaffolds for both regenerative medicine and disease modeling applications (Chang and Niklason 2017). Hydrogels are an attractive material of choice owing to their versatility, user programmability, ease of fabrication, availability of wide range of biochemical and mechanical properties that fits diverse requirements of various physiological and pathological tissues. Hydrogels have been used for both two-dimensional (2D) and three-dimensional (3D) culture of vascular cells by modifying the matrix properties to suit vascular growth, morphogenesis, and function. Additionally, hydrogels have also been used to model the processes of vasculogenesis (formation of blood vessels de novo) and angiogenesis (formation of new blood vessels from pre-existing ones) within 3D matrices (Rouwkema and Khademhosseini 2016). An important advantage of using hydrogel matrices for vascular studies is the ability to monitor and control the process of vascular morphogenesis in a dynamic, user-controllable fashion simply by altering the material properties in a spatiotemporal manner. These matrices can also be used to obtain vascular networks from a wide variety of cell sources (including human induced pluripotent stem cells, patient-derived adipose stromal cells, and others). By

controlling the matrix properties, including adhesivity, porosity, stiffness, alignment, and matrix compliance, the evolution of the vascular networks can be controlled to obtain patent, perfusable, and stable branched microchannels that closely human capillary networks (Nerem and Seliktar 2001; Stegemann et al. 2007). Overall, hydrogels have an important role to play in modeling vascular morphogenesis and function to mimic various physiological and diseased states.

In this chapter, an overview of the microenvironmental considerations for recreating vascular networks for regenerative medicine and tissue engineering applications is provided. Further, special considerations of the TME that are required to mimic tumor angiogenesis are described. Multiple hydrogel-based strategies employed for these applications, especially modifications of biochemical and biophysical properties in hydrogel matrices that induce vascular differentiation, morphogenesis, and stabilization, are also described. Various biofabrication strategies (ranging from chemical and molecular modifications to large-scale volumetric matrix modifications) that have been implemented to mimic the vasculogenesis and angiogenesis processes are described at length. Finally, the current challenges pertaining to tissue vascularization and modeling of vascularized tumors are analyzed and perspectives for future studies are discussed. We anticipate the readers to obtain a fundamental understanding of the basic requirements to recreate vascular networks for various applications, various material-based strategies and biofabrication technologies available to recreate these networks, and finally apply these principles to further translate these biomaterial-based vascularization techniques towards clinical implementation.

8.2 Microenvironmental Considerations for Vascular Regeneration

In order to design engineered vascular networks using hydrogel-based scaffolds, it is necessary to understand the basic vascular structure found across various parts of the human body as well as the surrounding tissue niches and associated microenvironments served by these networks. A typical large blood vessel consists of three layers: the innermost layer, tunica intima; the middle layer, tunica media; and the outermost layer, tunica adventitia.

Tunica intima is composed of a monolayer of endothelial cells (ECs), whose cellular, molecular, and genetic characteristics vary from organ to organ. The monolayer is surrounded by a thin and protein-rich basement membrane (BM) primarily composed of collagen type IV and laminin which forms the endothelium. The endothelium forms a selective but permeable barrier between the blood and the surrounding tissues, primarily through the tight intercellular junction and adhesion molecules between the ECs (e.g., VE-Cadherin, CD31, CD144 amongst others). A healthy and stable endothelium maintains vascular homeostasis by regulating vascular tone, blood pressure, inflammation, and angiogenesis by secretion of multiple cytokines and signaling molecules, and permeability to nutrients, oxygen, and metabolites (James and Allen 2018; Charbonier et al. 2019; Pradhan

et al. 2020). All vascularized tissue-engineered models must incorporate ECs as they are an indispensable component of blood vessels. Additionally, organ-specific recapitulation of engineered tissues requires incorporation of organ-specific ECs (Marcu et al. 2018). For example, in case of liver regeneration, recreating hepatic sinusoids requires fabrication of a fenestrated endothelium made of liver sinusoid endothelial cells (LSECs), while in case of retinal tissue regeneration, choroidal ECs are required to form the perfusable network underlying the retinal pigment epithelial (RPE) cells.

The tunica media is composed of pericytes or smooth muscle cells (SMCs) that surround the endothelium and are held together by a supportive layer of ECM proteins primarily composed of collagen type I and elastin. The medial layer provides structural support to the endothelium and maintain vascular quiescence, stability, and integrity. Disruption of the medial layer is often associated with increased vascular sprouting and angiogenesis, particularly in cases of wound healing and tumor growth. The tight wrapping of pericytes around microvascular networks is particularly critical for selectively permeable vascular barriers in the body, for example, the blood-brain barrier (BBB) that closely regulates the exchange of nutrients, metabolites, and toxins in the brain niche. Considering the physical forces involved with flow of blood, nutrients, and culture media within the endothelium, the SMCs in the medial layer are particularly responsible for vasoconstriction and vasodilation, especially in the larger blood vessels (James and Allen 2018; Charbonier et al. 2019; Pradhan et al. 2020). Although many tissue-engineered vascularized constructs operate solely on encapsulated ECs, incorporation of SMCs, pericytes, or differentiated stromal/stem cells along with ECs has been shown to increase microvascular network stability, patency, and permeability. However, similar to ECs, the proper selection of organ-specific perivascular cells is also important, especially when fabricating organ-specific vascular networks.

The tunica adventitia is composed of a heterogenous population of cells including fibroblasts, myofibroblasts, macrophages, mesenchymal stem/stromal cells (MSCs) amongst others surrounded by a thicker ECM layer composed of collagen types I and III and elastin. Similar to the medial layer, the adventitial layer, provides additional mechanical stability to large blood vessels and regulates vasomodulation in response to fluidic pressures within the endothelium. Disruption of the tunica adventitia can lead to serious vascular damage and may require greater involvement of external microenvironmental factors (immune cell infiltration, inflammation, and deposition of ECM proteins) as a regenerative strategy for vascular restabilization and repair (James and Allen 2018; Charbonier et al. 2019; Pradhan et al. 2020). Majority of tissue-engineered constructs primarily recapitulate microvascular networks and hence incorporation of mural cells comprising the adventitia is generally overlooked. However, several studies have explored the incorporation of fibroblasts, MSCs and even SMCs to stabilize rudimentary vascular networks. In addition to maintaining cell-cell contact with ECs, these stromal cells also secrete a multitude of pro-angiogenic and/or pro-vasculogenic factors which help regulate the growth, spreading, and quiescence of underlying ECs in a context-dependent manner.

Regulating the biophysical and biochemical interactions of these stromal cells with ECs is key to maintaining stable vasculature in engineered tissues (Fig. 8.1).

In addition to different cell types constituting the vasculature, it is important to consider the role of the ECM surrounding the cells as well as various hemodynamic variables which exert physical forces on both the cellular and acellular components in the vascular niche. The composition and microarchitecture of the ECM present in the basement membrane (BM) and the surrounding ECM vary with location in the body, age of the individual, physiological state, and other broader environmental factors. Particularly, in younger individuals, the basement membrane is composed of collagen IV which progressively gets replaced with collagen I with aging. Similarly, the production of elastin is also reduced drastically with age, thereby making blood vessels much stiffer, inability to modulate vascular tone, more prone to inflammation, and susceptible to damage under milder physical forces. The porosity and mechanical compliance of the BM and the permeability of the endothelium (resulting from the tight EC junctions) are also dependent upon the proteomic composition. With increasing collagen I deposition due to vascular aging and misalignment in deposited fibrillar proteins, the BM can become more porous and the EC junctions can weaken over time to lose their phenotypic plasticity. This can cause the vascular endothelium to become leaky and more permeable (James and Allen 2018; Charbonier et al. 2019; Pradhan et al. 2020). Hence, when designing synthetic scaffolds for vascularized tissue, it is important to maintain the appropriate stiffness, matrix compliance, porosity, and fibrillar alignment so that rudimentary blood vessel segments can interconnect with each other into an integrated fluidic network that has uniform coverage of the entire scaffold or matrix.

Among the hemodynamic factors influencing vascular regeneration, the most important are fluid shear stress, circumferential strain, cyclic stretch, and fluid pressure. Considering the pulsatile nature of blood flow (originating in the heart and propagating to the capillaries) and the wide range of shear stress values existing across the spectrum of blood vessels, it is necessary to study the effect of these factors in the context of vascular regeneration. Physiological levels of shear stress are necessary to maintain vascular patency, EC junction adhesion strength, and an overall healthy endothelium. In addition, the fluid pressure level and a consistent level of pulsatile flow should also be maintained to ensure physiological levels of circumferential strain and cyclic stretch. The biophysical forces acting on the endothelium also ensures optimum secretion of pro-vasculogenic biochemical signaling to ensure proper endothelial structure and function (James and Allen 2018; Charbonier et al. 2019; Pradhan et al. 2020). Some of these factors include vascular endothelial growth factor (VEGF), basic fibroblast derived factor (bFGF), angiopoietin-2 (Ang2), platelet derived growth factor BB (PDGF-BB) and others. When designing vascularized scaffolds, it is important to incorporate these hemodynamic factors on 2D substrates or within the 3D volumes, which shape the initial spreading and connectivity of ECs and ensure proper tubulogenesis and lumenogenesis to form vascular networks.

In addition to soluble factor signaling, local oxygen concentration is one of the key determinants of vascularization. Hypoxic microenvironments promote

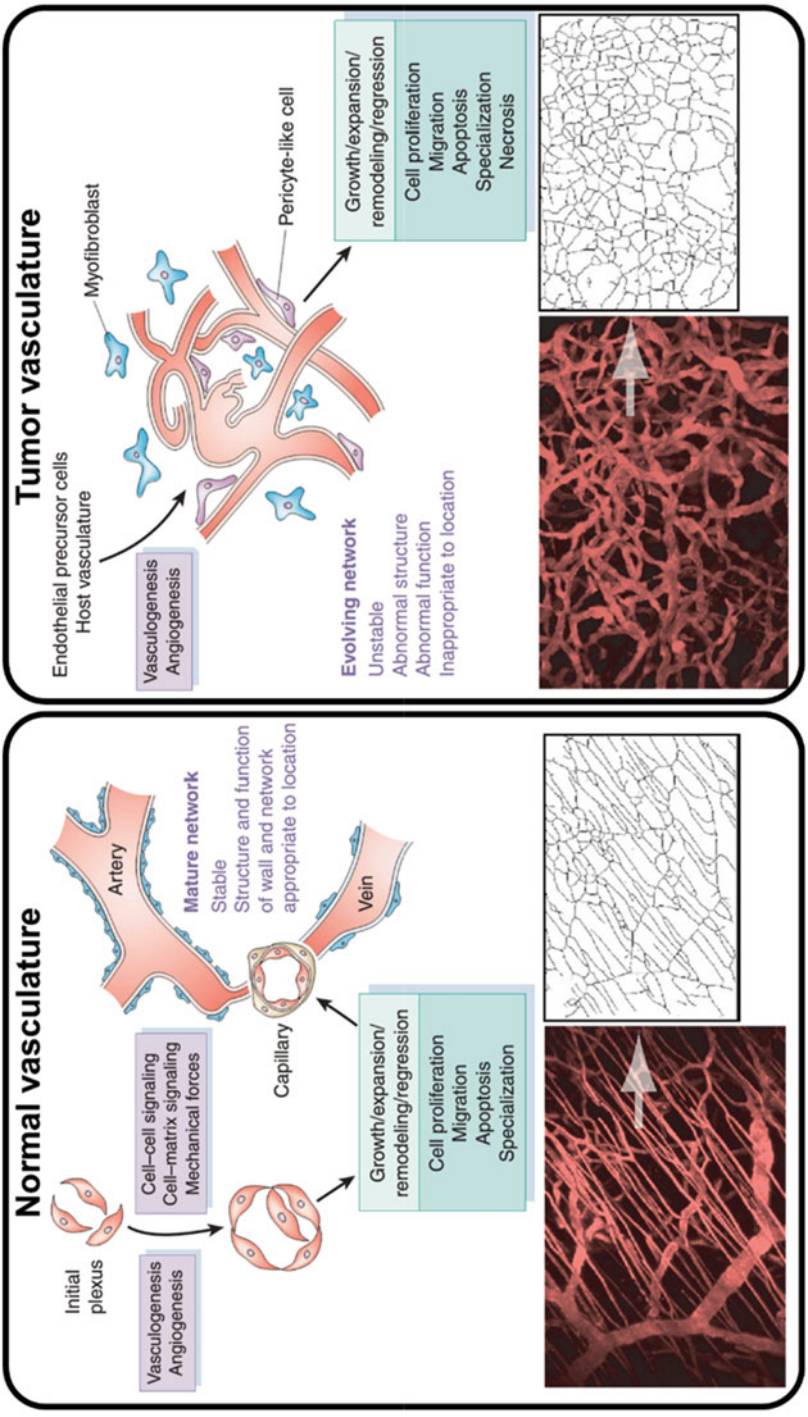


Fig. 8.1 Morphological differences between normal and tumor vascular networks. Normal vasculature is characterized by an initial cell plexus that is regulated by cell-cell and cell matrix interactions and appropriate mechanical signaling processes that help form ordered networks with

upregulation in expression of key pro-angiogenic genes including hypoxia-inducible factor-1α (HIF-1α), vascular endothelial growth factor-A (VEGFA), and others. This leads to promotion of angiogenic sprouting, vessel branching, and rapid vascularization (Krock et al. 2011). This principle has often been used for wound healing and reparative angiogenesis. On the flip side, this process is also hijacked by diseased cells, including cancer, to promote excess and undesired vessel formation, which leads to further complications. Physiological oxygen concentration helps in maintaining stable microvascular networks. Hence, the local oxygen concentration must be carefully monitored and dynamically controlled to ensure that rudimentary vascular segments undergoing anastomosis are gradually stabilized and form patent and long-lasting microvascular networks.

Overall, when fabricating designer-scaffolds for vascularization and tissue regeneration, the initial matrix microenvironmental conditions must be made amenable not only to the functional cells of interest but also the organ-specific vascular cells that would eventually form the vasculature. This includes control of cellular components (ratio of different cell types, density of cells in the matrix volume, inclusion of supportive mural cells, etc.), culture conditions (composition of media, growth factors, hormones, etc.), extracellular components (ECM composition, stiffness, adhesivity, porosity, alignment, etc.), hemodynamic forces (shear stress, interstitial fluid pressure, diffusion, matrix perfusability, cyclic load, etc.), amongst others.

8.3 Microenvironmental Considerations for Tumor Angiogenesis

The tumor microenvironment is a unique and complex milieu of a wide range of cellular, extracellular, and biochemical factors that work in an intertwined fashion to promote the growth and invasion of the central tumor mass. A growing tumor mass generally consists of an outer proliferating cell layer, a middle quiescent layer, and an inner necrotic core of cells densely packed with each other. Due to limitations in oxygen diffusion through this densely packed mass, majority of cancer cells undergo hypoxia and secrete pro-angiogenic factors to promote tumor angiogenesis from surrounding healthy vasculature (Harris 2002).

However, tumor angiogenesis leads to rapid angiogenic sprouting, without sufficient time for recruitment of supportive mural cells for vessel stabilization. Hence,

Fig. 8.1 (continued) structurally and functionally stable lumens. Tumor vasculature owing to recruitment of multiple cell types and imbalance of various signaling factors results in a disordered, tortuous, and leaky network. Images of blood vessels from intravital microscopy and corresponding skeletonized images demonstrate the differences between the organization and structure of the vascular networks. Adapted with permission from (Jain 2003)

the resulting vascular network is disorganized, tortuous, immature, and hyperpermeable. Although normal blood vessels have unidirectional flow, tumor blood vessel exhibit chaotic loops and irregular branches, leading to disrupted and bidirectional flow. The discontinuous endothelial lining and poor EC junctions lead to leaky vessels and deposition of vascular components in the extravascular space. At the cellular level, TECs are significantly different in their genetic and molecular characteristics from normal ECs, which also lead to differences at the hierarchical tissue level (Dudley 2012) (Fig. 8.1).

Designing this complex pathological state within tissue-engineered constructs is both crucial and challenging at the same time. 3D models of vascularized tumors are important testbeds for screening the efficacy of candidate drugs and therapeutic agents. Tumor cells cultured alone and those in co-culture with endothelial cells (or even better, tumor-associated endothelial cells, TECs) have been shown to have distinct response profiles to drugs. The presence of supportive vascular or stromal cells in the TME also confers additional chemoresistance to tumor cells and enables survival and future relapse. Hence, it is important to incorporate vascular cells and/or stromal cells to accurately recapitulate the native microenvironmental conditions of tumors. In that regard, employing hydrogels as ECM-mimics is an advantageous strategy owing to the ability to independently tune the biochemical and mechanical properties to suit multiple cell types and modularly assemble them into integrated complex synthetic tissues for co-culture studies. For example, it is known that the tumor ECM is stiffer than adjacent normal ECM due to altered secretion and deposition of matrix proteins. Hence, hydrogels with higher stiffness can be used to culture tumor cells or tumor spheroids to form a central 3D tumor mass. Adjacent to this mass, hydrogels encapsulating endothelial cells in a softer matrix can be used for co-culturing with cancer cells. The combined hydrogel assembly can be used as an integrated vascularized tumor-mimic for drug-testing applications.

Recapitulating the complex architecture of tumor microvasculature is technologically challenging owing to various factors. Obtaining TECs from reliable and consistent sources is not guaranteed and isolation of TECs is also associated with inter-patient variability. Encapsulation of ECs in co-culture with cancer cells within 3D hydrogel matrices is one common strategy employed in many studies. However, the ability of the ECs to reassemble into tumor vessel-like networks depends completely on the culture and matrix conditions and the ability of tumor cells to secrete pro-angiogenic factors. This variability has led to some engineering innovations where tumor vessel-like microchannels are prefabricated within 3D hydrogel volumes or within microfluidic devices and later seeded with ECs which assume the morphology and structure of these microchannels (Michna et al. 2018). Although most studies use normal endothelial cells like human umbilical vein endothelial cells (HUVECs) for cancer co-culture studies, the use of tumor-specific ECs or patient-derived ECs is also gaining prominence.

In general, hydrogel-based vascularized tumor models provide a reasonable approach for modeling the complexity of native tumors and can be designed from a bottom-up approach to match the characteristics of the complex milieu. Considering the wide range of intra-tumor and inter-tumor heterogeneity, it is necessary to

standardize the practice of fabricating vascularized tumors, especially for discovery of targets against tumor angiogenesis and the development of anti-angiogenic drugs. Alternate strategies for normalization of tumor blood vessels are also being explored for more efficient delivery of drugs and therapeutics that can reach the tumor mass effectively.

8.4 Hydrogel-Based Models for Vascular Regeneration and Tumor Angiogenesis

As discussed earlier, hydrogels provide a diverse and dynamic set of matrix microenvironmental conditions to mimic native ECM that can promote vascular growth, long-term maintenance, integration with host vasculature for tissue engineering, and regenerative medicine applications. By tuning the chemical building blocks of polymer chains, constitutive functional groups, and the degree of crosslinking, higher order macromolecular properties of hydrogel-based scaffolds (including viscoelasticity, adhesivity, hydrophilicity, surface charge, porosity, and topology) can be controlled and can be optimized for various types of vascular cells. Traditionally, ECs are seeded on 2D bioactive hydrogel substrates to assess for their vasculogenic potential by quantifying cell spread, proliferation, tubule formation, and expression of prominent vasculogenic markers (CD31, VE-Cadherin, VEGFA, etc.). In some cases, 2.5D cultures are established where ECs seeded on the top surface of a hydrogel scaffold can transmigrate through the underlying porous network and infiltrate deeper regions of the scaffold. However, to accurately mimic native physiological vascular morphogenesis, it is necessary to establish 3D cultures of hydrogel-encapsulated cells. Single ECs when encapsulated within permissive hydrogel matrices at specific cell densities can interconnect with each other through filopodial protrusions and elongated cell bodies to form rudimentary vascular networks. These rudimentary networks can be stabilized by surrounding mural cells (e.g., pericytes, fibroblasts, MSCs) until they anastomose into integrated vascular lumens (Peters et al. 2016).

Hydrogels employed for vascular applications can be broadly grouped into natural, synthetic and hybrid materials. Natural ones (e.g., Matrigel, collagen, fibrin, gelatin, alginate and agarose) are obtained from animal or plant sources and have long been used for both in vitro modeling and in vivo vascular regeneration studies. Synthetic hydrogels (e.g., poly(ethylene glycol) (PEG), poly(lactic-*co*-glycolic acid), poly(caprolactone)) are obtained through chemical synthesis. Hybrid hydrogels (e.g., gelatin methacryloyl, methacrylated hyaluronic acid, methacrylated dextran, PEGylated proteins) are obtained by synthetically modifying natural components to improve their suitability for vascular applications.

Compared to natural hydrogels, synthetic polymers allow better control over their chemistry (by functionalization with polysaccharides, proteins, or peptides) and structure to fabricate highly tailored 3D scaffolds (in terms of mechanical properties, biofunctions and degradability) for vascular regeneration and tumor angiogenesis. Moreover, since these polymers are chemically synthesized, the problem of

availability and batch-to-batch variability is almost negligible, thus promoting highly reproducible scaffold properties. Inherent inertness of most synthetic polymers guarantees unintentional immune responses when implanted as vascular grafts even for long-term regeneration. Some of natural (Table 8.1), synthetic, and hybrid (Table 8.2) scaffolds are described in detail below.

8.4.1 Matrigel

Matrigel, isolated from Engelbreth-Holm-Swarm (EHS) mouse sarcoma and rich in laminin, collagen IV and other growth factors, is a popular choice amongst biologists as a reconstituted basement membrane for 3D cellular studies. Owing to its tumorigenic source, Matrigel contains a wide range of pro-angiogenic growth factors and proteomic components which makes it ideal for angiogenesis assays (i.e., assess the potential of vascular cells to form tubulogenic networks, vascular sprouts). Moreover, these assays are increasingly being integrated with other technologies to build more complex angiogenesis evaluation methods, particularly for high-throughput, reliable, and effective readouts (Akhtar et al. 2002; Kleinman and Martin 2005; Khoo et al. 2011) (Fig. 8.2a). Matrigel has also been shown to enhance the rate of epithelialization and wound healing and promotes the retention of keratinocytes in deep wounds, although its clinical translation is questionable. Matrigel owing to its ability to support tumor growth can be used for co-culture studies of cancer cells with ECs. It is routinely used for in vivo implantation of tumors and in invasion assays. However, it is inherently limited in its material properties due to its animal origin. The heterogeneity in its chemical composition and the inability to control rigidity of gels across different batches makes it difficult to reproduce results across large study sets. This has led to the gradual development of synthetic materials as an alternative to Matrigel (Aisenbrey and Murphy 2020).

Similar to Matrigel, various decellularized ECM (dECM) have also been prepared from various animal (porcine, bovine, etc.) and human sources that have been implemented for vascular tissue engineering (Hackethal et al. 2021). dECM contains a multitude of native growth factors, hormones, and other bound signaling factors necessary for optimal vascular morphogenesis and have been successfully implemented in various regenerative studies. However, the heterogeneity in composition coupled with unreliability of source material makes it challenging to implement it extensively at a clinical scale.

8.4.2 Collagen and Gelatin

Collagen (primarily type I) and its hydrolytic product, gelatin, have been widely used for vascular tissue engineering applications and for in vitro cancer studies, owing to its ease of isolation and abundance in animal tissues (Fig. 8.2a). When thermally/physically crosslinked, collagen and gelatin assume a fibrillar architecture, whose microstructural properties (including fiber alignment, thickness, stiffness, and

Table 8.1 Representative examples of natural biomaterials/hydrogels used for vascular studies and angiogenesis

Biomaterial	Application/findings	Reference(s)
Natural biomaterials:		
Pros: Availability of wide range of biochemical signaling cues, compatible with a wide range of vascular cell types, easy moldability and processability with other natural biomaterials, improved biocompatibility and hemocompatibility *Cons: Poor mechanical strength, source heterogeneity and unreliability, difficult to independently tune matrix physical, biochemical, and mechanical properties*		
Matrigel and dECM	Human placenta-based extract tested for 2D and 3D vasculogenic potential using HUVECs	Hackethal et al. (2021)
	SMCs grown on Matrigel demonstrated reduced proliferative index and higher contractility	Li et al. (1994)
	Formation of vascularized adipose tissue via optimized differentiation from vascular stromal fraction cells in Matrigel	Muller et al. (2019)
	Quantification of angiogenic processes of ECs in Matrigel in the presence of various pro- and anti-angiogenic agents	Akhtar et al. (2002); Khoo et al. (2011)
Collagen and gelatin	ECs self-assembled into perfusable, patent, and scale-spanning networks in collagen gels of varying densities	Morgan et al. (2019)
	Denser collagen microspheres within softer collagen bulk scaffolds promoted HUVEC migration and invasion	Celie et al. (2019)
	hESC-ECs formed sprouting tubules within a microfluidic collagen-gel based device under dynamic perfusion	Redd et al. (2019)
	Modular collagen/gelatin hydrogel constructs packed into a cylindrical conduit, seeded with ECs and perfused to form vascularized tissue	McGuigan and Sefton (2006, 2007)
Fibrin	ECs seeded on gelatin-coated microspheres embedded within fibrin gel evaluated for sprouting angiogenesis under different pro-angiogenic stimulators and in co-culture with SMCs, pericytes and fibroblasts	Nehls et al. (1994); Nehls and Drenckhahn (1995)
	Fibrin matrices with encoded recombinant VEGF enabled stable, long-lasting capillary network formation	Sacchi et al. (2014, p. 164)
	Mechanisms of sprouting angiogenesis and lumen formation by HUVECs within fibrin gel under different regulatory factors	Nakatsu et al. (2003)
	Local stiffening and dynamic remodeling of fibrin matrix under sprouting capillary formation	Juliar et al. (2018)
Alginate and agarose	RGD- and VEGF-mimetic peptide conjugated with alginate gel for vasculogenesis of pericyte-EC co-culture	Barrs et al. (2021)
	RGD-functionalized alginate used for co-culture of ECs, fibroblasts and breast epithelial cells for studying heterotypic cell-cell interactions	Teixeira et al. (2021)

(continued)

Table 8.1 (continued)

Biomaterial	Application/findings	Reference(s)
	Alginate hydrogels loaded with ECs and alginate lyase for controlled degradation and delivery of cells for revascularization	Campbell et al. (2018)
Silk	Heparinized silk scaffold used for culturing ECs and SMCs with high cytocompatibility, low hemolysis and thrombogenicity	Zamani et al. (2017)
	Desferrioxamine-loaded silk nanofiber scaffolds underwent controlled release and improved tissue vascularization, wound repair and tissue growth	Ding et al. (2019)
	Silk fibroin gels of varying concentrations used for vascularization of adipose derived stem cells into functional adipose tissue both in vitro and in vivo	Kayabolen et al. (2017)
	Peptide-modified silk fibroin scaffolds used for co-culture of MSCs and ECs for enhanced vascularization	Sun et al. (2016)
Chitosan	Growth factor loaded photocrosslinkable chitosan hydrogels enabled enhanced vascularization upon degradation in vivo	Ishihara et al. (2003)
	Chitosan-collagen hydrogels with loaded growth factors demonstrated improved endothelial growth, vascular signaling, and functional tissue repair	Deng et al. (2010); Chiu et al. (2012)
Hyaluronic acid (HA)	Thiolated heparin loaded HA hydrogel enabled stable, perfusable capillary network formation by hiPSC-ECs	Natividad-Diaz et al. (2019)
	HA modified with VEGF-mimetic peptide demonstrated enhanced endothelial spread, and proliferation leading to improved tissue repair in traumatic brain injury in vivo	Lu et al. (2019)
	Acrylated HA hydrogels with varying stiffness and oxygen concentrations were used for culturing fibrosarcoma cells with corresponding effect on endothelial sprouting and invasion	Shen et al. (2014)

density) can be controlled by modulating concentration, temperature, and pH, thereby resulting in gels with widely varying bulk properties (McGuigan and Sefton 2006; Celie et al. 2019; Morgan et al. 2019; Redd et al. 2019). Alignment of collagen/gelatin fibers is of particular interest as they promote contact-guidance dependent assembly and migration of cells encapsulated in 3D gels or topographical guidance on 2D substrates. ECs seeded in scaffolds of aligned, isotropic collagen fibers can form rapid and robust vascular networks compared to those in random, anisotropic collagen scaffolds. Collagen and gelatin hydrogels have also been used extensively for spheroid cultures to study 3D vascular sprouting and vascular morphogenesis assays.

In addition to physical crosslinking, collagen and gelatin can also be chemically or enzymatically crosslinked using various functional side groups on the macromolecular backbone to form more robust hydrogels. Although stiffness of these

Table 8.2 Representative examples of synthetic and hybrid biomaterials/hydrogels used for vascular studies and angiogenesis

Biomaterial	Application/findings	Reference(s)
Synthetic biomaterials:		
Pros: Matrix properties can be independently tuned for mechanistic investigations with, high degree of control, improved mechanical properties, lower rate of degradation, longer life and highly stable *Cons: Lack the full range of biochemical cues necessary for cellular attachment, vascular structure, and function, need to be optimized for each individual cell type, may not be conducive for organ-specific vascular cell types*		
Poly(ethylene glycol)	Star-PEG hydrogels conjugated to heparin, growth factors, RGD and MMP-sensitive peptides used for multiculture of cancer cells, HUVECs and MSCs and for angiogenesis assays	Zieris et al. (2010); Bray et al. (2015); Chwalek et al. (2015); Taubenberger et al. (2016)
	PEG hydrogels conjugated to RGD and MMP sensitive peptide sequences used for bi-layered culture of lung adenocarcinoma cells and ECs to study biochemical signaling and cellular crosstalk	Roudsari et al. (2016)
	PEG-collagen scaffolds of varying composition encapsulating ECs and fibroblasts showed capillary network formation in vitro	Singh et al. (2013)
Poly(lactic-*co*-glycolic acid) (PLGA)	RGD and graphene oxide functionalized PLGA nanofibrous scaffold promoted SMC spreading and growth	Shin et al. (2017)
	Pre-vascularization of PLGA scaffolds led to improved functional integration with host vasculature	Laschke et al. (2008)
	PLGA scaffolds functionalized with VEGF and loaded with MSCs led to improved growth of functional microvasculature	Kampmann et al. (2013)
Poly (ε-caprolactone) (PCL)	PCL scaffolds loaded with heparin and VEGF with endothelial progenitor cells promoted improved vascularization and anastomoses with host vasculature	Singh et al. (2011)
Hybrid biomaterials:		
Pros: Improved mechanical properties, wider range of biochemical functionality than purely synthetic biomaterials *Cons: Needs careful optimization of matrix composition and properties to suit multiple vascular cell types*		
Gelatin methacrylate (GelMA)	GelMA and PEG dimethacrylate dual crosslinked hydrogels was fabricated	Kim et al. (2020)

(continued)

Table 8.2 (continued)

Biomaterial	Application/findings	Reference(s)
	for endothelialization of perfusable channels and sprouting angiogenesis	
	Odontoblasts and ECFCs cultured within microstructured GelMA hydrogels formed vascularized dental pulp tissue via angiogenic sprouting	Athirasala et al. (2017)
	GelMA cryogel microspheres promoted osteogenic differentiation and vascularization in human bone marrow stromal cell (hBMSC) and HUVECs	Yuan et al. (2021)
Methacrylated hyaluronic acid (MeHA)	GelMA and MeHA hydrogels promoted vascularization and creation of functional adipose tissue with ADSCs	Eke et al. (2017)
	Macroporous MeHA hydrogels promoted enhanced proliferation, spreading and vascularization	Lu et al. (2022)

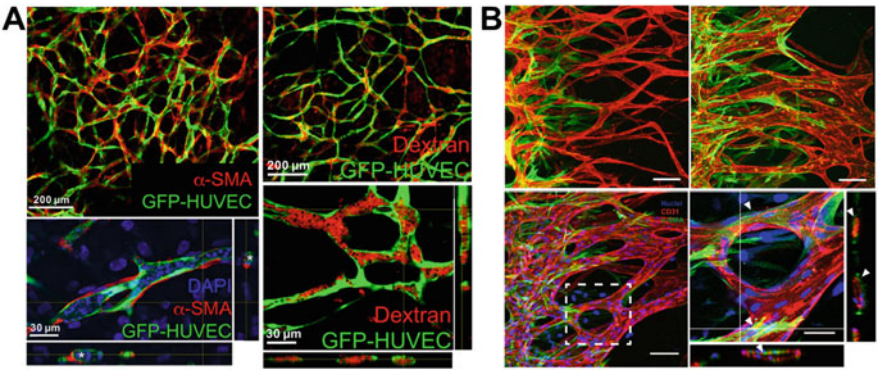


Fig. 8.2 Representative examples of natural hydrogels enabling vascularization and angiogenesis. (**a**) GFP-labelled HUVECs co-cultured with ADSCs in Matrigel/rat tail collagen I hydrogels after 14 days of culture in serum-free media demonstrate stable, lumenized, and patent microvascular networks as evidenced by fluorescent dextran perfusion. Adapted from (Andrée et al. 2019). (**b**) HUVECs (red) and pericytes (green) co-cultured within fibrin gels undergo angiogenic sprouting towards a biochemical gradient within a microfluidic chip as imaged on day 3 (left) and day 6 (right). Pericytes gradually wrap around endothelial lumens to stabilize the neovessels and form patent lumens. Scale bar: 100 μm. Adapted from (Kim et al. 2015)

hydrogels can be increased with additional crosslinks, it is still lower than that observed in native tumor tissues, and they are susceptible to rapid degradation post implantation in vivo. Further, various matrix properties including stiffness, porosity, degradability, and adhesivity of collagen/gelatin are intimately coupled with each other, making it difficult to independently study the role of these

parameters in vascular regeneration and tumor angiogenesis. In recent approaches, collagen and gelatin have also been modified with functional methacrylate groups that can be chemically crosslinked to form stiffer hydrogels (Yuan et al. 2021). The degree of methacrylation and polymer concentration determines the degree of crosslinking and the resulting macromolecular architecture of the hydrogels. Owing to the cheap and facile nature of handling collagen and gelatin, these are popular choices for both in vitro and in vivo vascular applications.

8.4.3 Fibrin

Fibrin hydrogels, obtained by thrombin-mediated cleavage and polymerization of blood-isolated fibrinogen protein, have often been used for various vascular regeneration as well as for in vitro mechanistic studies of vasculogenesis and angiogenesis (Fig. 8.2b). Owing to its good hemocompatibility, ability to bind to large number of blood proteins, and rapid crosslinking properties, fibrin is an attractive material of choice, particularly for wound healing and tissue sealing applications. In addition to endothelial cells, fibrin is conducive to other supportive cell types, including MSCs, SMCs, pericytes, adipose-derived stromal cells (ADSCs), that help form integrated and stable vascular networks in three-dimensional constructs. Fibrin hydrogels are also commonly used for vascular sprouting assays to assess the vasculogenic potential of isolated cell types (Nehls et al. 1994; Nehls and Drenckhahn 1995; Nakatsu et al. 2003).

The fibrillar microarchitecture of fibrin hydrogels allows directional migration and rapid interlinking of encapsulated vascular cells and rudimentary tubules. However, fibrin hydrogels, owing to their high in vivo degradation rates, result in rapid generation of new capillaries that are unstable, tortuous, and highly branched. High fibril density is also detrimental to the vascular sprouting process. Implantation and long-term studies using fibrin can be conducted by modulating the fibrin microarchitecture or by mixing with other materials (collagen, gelatin, alginate, etc.) to form integrated and multi-crosslinked constructs. Depending upon the polymerization mechanism and dynamics (pH, temperature, additional crosslinkers like transglutaminase, etc.), the mechanical and architectural properties of the fibrillar network can be modified in various ways (Juliar et al. 2018). This enables users to adjust the bulk stiffness, stability, and biodegradability of fibrin gels at the macro-scale as well as modulate cellular-level responses and morphology at the micro-scale. These abilities make fibrin an attractive material of choice for vasculogenic and angiogenic applications.

8.4.4 Alginate and Agarose

Alginate and agarose, linear polysaccharide-based co-polymers obtained from seaweed, have been extensively used as a cheap source of vascular-compatible biomaterials for several in vitro and in vivo applications. Alginate is composed of

β-(1–4)-linked D-mannuronic acid (M) and β-(1–4)-linked L-guluronic acid (G) units, while agarose is composed of α-(1-3) linked D-galactose and β-(1-4) linked 3,6-anhydro-L-galactopyranose. Sodium alginate, when treated with calcium ions, gets chelated rapidly to form calcium alginate hydrogels via ionic crosslinking. Agarose when dissolved in water and heated cools back to form hydrogels via thermal gelation. Alginate and agarose do not have any inherent cell-adhesive ligands; hence vasculo-mimetic ligands need to be incorporated separately when preparing scaffolds of these materials. Some of these ligands include the ubiquitous RGD peptide motif and the protease-sensitive VEGF receptor binding peptide GPQGIAGKLTWQELYQLKYKGI amongst others (Barrs et al. 2021; Teixeira et al. 2021). Additionally, alginate and agarose can also be combined with other materials like gelatin, fibrin, and others to form hybrid hydrogels with improved mechanical and biochemical properties (Kinoshita et al. 2016).

Some major advantages of alginate hydrogels include the ability to modulate its degree of crosslinking and stiffness by regulating the concentration of Ca^{2+} ions, high degree of stability and relatively lower rate of biodegradation, 2D and 3D printability and injectability (due to it viscoelastic nature), amongst others. In certain applications, faster degradation of alginate matrices is desirable which can be attained by loading alginate lyase (in a dose-dependent manner) along with encapsulated cells (Campbell et al. 2018; Antunes et al. 2021). The ability to closely modulate the mechanical properties and 3D architecture of alginate also enables it to be used for creating modular scaffolds and create hierarchically structured patterns for more complex 3D vascular designs. Calcium alginate can also be degraded using ethylenediaminetetraacetic acid (EDTA), and this strategy can be exploited to form sacrificial scaffolds and templated 3D structures for various vascular applications.

8.4.5 Other Natural Materials

A few other emerging natural materials implemented for vascular tissue engineering and modeling of tumor angiogenesis include silk, chitosan, hyaluronic acid, and other GAG-based hydrogels. Although they are biocompatible and have low immunogenicity, the vasculo-mimetic potential is variable and often depends on the source of the material, molecular characteristics, and biochemical composition. Silk is primarily obtained from the cocoons of silkworms (mulberry or non-mulberry in origin). Silk fibroin is a promising protein-based biomaterial that has high hemocompatibility, excellent mechanical strength, and the ability to be molded into various shapes and sizes from nano-scale to centimeter-scale constructs (Blanco-Fernandez et al. 2021; Gupta and Mandal 2021). Silk fibroin has both amorphous and crystalline regions which transition from α-helix and random coil structure to β-sheets during isolation and processing. Additionally, sericin protein found in silk also acts as a glue to bind fibroin protein fibrils. Although sericin has been used to a lesser extent, it is primarily fibroin protein which is predominant for vascular applications (e.g., tissue-engineered vascular grafts, TEVGs) (Zamani et al. 2017; van Uden et al. 2019). Silk fibroin scaffolds or nanoparticles loaded with

VEGF or heparin have been used extensively to enhance angiogenic repair in combination with various vascular cell types (ECs, SMC, ADSCs, and others) (Zamani et al. 2017; Zhang et al. 2018). The stiffness of silk-based hydrogels can be controlled by tuning initial polymer concentration, by combining with other natural or synthetic materials, or by inducing additional crosslinking of the tyrosine groups using horseradish peroxidase (HRP)/H_2O_2. In general, silk biomaterial is still in its nascent stages in terms of being applied for vascular tissue engineering (Gupta and Mandal 2021).

Chitosan, a linear polysaccharide, composed of repeating units of D-glucosamine and *N*-acetyl-D-glucosamine linked by β-(1,4) glycosidic bonds has often been used with other biomaterials to enhance vascularization and angiogenesis in the context of wound healing, fabrication of small diameter vascular grafts, and tissue engineered blood vessels. Chitosan has hydrophilic groups and is biocompatible and biodegradable with minimal immunogenicity. Additionally, it can be blended with a wide variety of natural and synthetic materials to improve mechanical properties, surface properties, in vivo stability, and vasculogenic potential of engineered constructs (Islam et al. 2020). Further, the processability of chitosan into various scale-spanning structures like nano/microparticles, flat layers, scaffolds and matrices, tubes and fibers helps in creating a wide range of desired vascular constructs (Deng et al. 2010; Badhe et al. 2017; Wang et al. 2020). Interestingly, chitosan has also been used to inhibit tumor angiogenesis through blocking of the VEGF signaling pathway as well as a drug carrier for several compounds that inhibit tumor angiogenesis and thereby tumor growth (Li et al. 2019b; Yadav et al. 2020).

Hyaluronic acid (HA), a linear glycosaminoglycan (GAG) polysaccharide, composed of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by β-(1,4) glycosidic bonds is another popular choice for vascular tissue engineering. Although HA does not have any integrin binding sites, it has receptors for CD44 and receptor for HA-mediated motility (RHAMM). However, for encapsulation or culture of ECs, it is often necessary to incorporate vasculo-mimetic factors that engage directly for improved vascular signaling and morphogenesis. These factors include the RGD peptide sequence for improved cell adhesion, VEGF-mimetic peptide sequence, and conjugation with heparin/thiolated heparin for improved vasculogenesis. In addition, inclusion of MMP-sensitive peptide sequences and dynamic induction of hypoxia within hydrogels also helps improve vascularization (Shen et al. 2014; Lu et al. 2019; Natividad-Diaz et al. 2019). In previous studies, HA has also been chemically modified with other materials including collagen, chitosan, and poly(ε-caprolactone) (PCL) to create improved scaffolds for vascularization (Vignesh et al. 2018; Kang et al. 2019; Li et al. 2019a). While using HA, it is necessary to optimize the molecular weight of the polymer chains and viscoelasticity of the resulting scaffold, as these play important roles in determining spreading, adhesion, and vascular morphogenesis of ECs.

8.4.6 Poly(ethylene Glycol)

Poly(ethylene glycol) (PEG) is a synthetic linear or multi-arm polymer that serves as a "blank slate" on which various biochemical modifications can be made to make it suitable for vascular applications. Although PEG by itself does not have any bioactive sites required for cell adhesion or degradation, it can be modified by chemical conjugation with various cell-binding and proteolytically degradable peptide sequences, ECM proteins, growth factors, and hormones to produce bioactive and bioresponsive scaffolds (Moore and West 2019). Multi-arm PEG chains provide the added advantage of covalently conjugating multiple bioactive moieties in the same macromolecular network. PEG-chains end-modified with diacrylate (DA) or dimethacrylate (DMA) groups can be covalently crosslinked via UV- or visible light-based photoinitiators or via click-based chemistries to form scaffolds and hydrogels (Zieris et al. 2010; Chwalek et al. 2015; Taubenberger et al. 2016). Further, by controlling the molecular weight, initial polymer concentration, and crosslinker concentration, the overall stiffness, porosity, and other microarchitectural features of PEG-based scaffolds can be tuned for the required cell type used. PEGDA hydrogels with RGD- and MMP-degradable peptide sequences have been used for vascular regeneration, in vitro, in vivo, and in PDMS-based microfluidic models to form patent lumen-like vessels that provide diffusive and convective mass transport to adjacent tissue space (Suresh and West 2020) (Fig. 8.3a). PEGDA hydrogels can also be modified by conjugation with

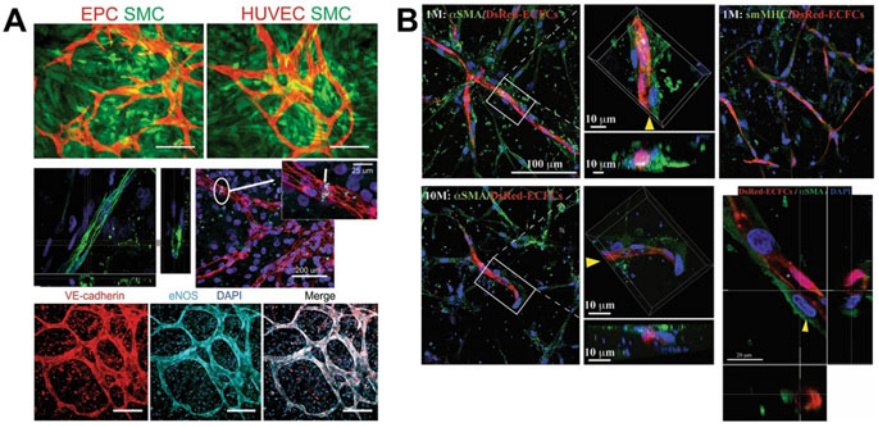


Fig. 8.3 Representative examples of synthetic/hybrid hydrogels used for vascular tissue engineering. (**a**) EPCs and HUVECs (red) co-cultured with SMCs (green) within PEG-based hydrogels with adhesive RGDS and MMP-sensitive peptide sequences after 14 days of culture form stable microvascular networks. Scale bar: 200 μm. Cells are stained for VE-Cadherin (green/red), connexin-32 gap junction (cyan), and nuclei (blue) (central panel) and endothelial nitric oxide synthase (eNOS, cyan) (bottom panel). Scale bar: 100 μm. Adapted from (Peters et al. 2016). (**b**) ECFCs (red) co-encapsulated with MSCs within GelMA hydrogels at varying cell densities as imaged on day 7 of culture forming rudimentary microvascular networks with hollow patent lumens. Scale bar: 100 μm (left), 20 μm (bottom right). Adapted from (Chen et al. 2012)

photo-degradable peptide sequences that can be selectively degraded using light of specific wavelengths (or a laser source) to create patterned and user-guided vascular channels (Kloxin et al. 2009). Co-culture of human umbilical vein endothelial cells (HUVEC) and human vascular pericytes (HVP) with lung adenocarcinoma cells within a two-layered PEG-peptide based hydrogels demonstrated that proximity to vascular cells helped promote higher tumor growth in large, disorganized clusters (Roudsari et al. 2016). Overall, PEG-based scaffolds provide immense potential and opportunities for researchers to elucidate specific microenvironmental factors regulating vascular regeneration and tumor angiogenesis (Bray et al. 2015; Taubenberger et al. 2016).

8.4.7 Poly(lactic-*co*-Glycolic Acid) and Poly(caprolactone)

Poly(lactic-*co*-glycolic acid) (PLGA), a co-polymer of lactic acid and glycolic acid, has been used a cheap alternative for various vascular tissue engineering applications due to its biocompatibility and biodegradability (Pan and Ding 2012). The rate of PLGA biodegradation (via hydrolytic cleavage of the ester bonds) can be controlled by tuning the relative ratios of the monomers used to form the PLGA scaffold. Although hydrophilic, PLGA by itself does not have any cell-adhesive ligands necessary for vascular cell attachment. Hence, it is often combined with other natural polymers (e.g., collagen, fibrin, silk) or specific peptide sequences (e.g., RGD) to promote cell adhesion and spreading (Shin et al. 2017). PLGA is often molded into microspheres and nanoparticles for controlled released of vascular growth factors (e.g., VEGF, BMP-2) that promote vascular regeneration and wound healing (Golub et al. 2010). PLGA can also be molded into electrospun scaffolds with tunable microarchitectural properties (e.g., fiber thickness, alignment, porosity), and in conjunction with other cell-adhesive materials, be used to make small-diameter vascular grafts and porous scaffolds (Han et al. 2011). Incorporation of growth factor loaded-PLGA microspheres within larger scaffolds also helps in recruitment of ECs seeded on the scaffold surface, thereby mimicking angiogenic growth in 3D microenvironments.

Similar to PLGA, poly(ε-caprolactone) (PCL) is another biodegradable polyester that is employed as small diameter vascular grafts and bioactive scaffolds for vascular regeneration. By combining with other materials, including fibrin, collagen, silk, chitosan, PLA, PVA, and others, controlled release of vascular growth factors, and by fabricating into molded structures, including electrospun fibers, multi-layered scaffolds, PCL can be used for a wide variety of applications involving vasculogenesis and angiogenesis (Fuchs et al. 2009; Singh et al. 2011; Gniesmer et al. 2019).

8.4.8 Hybrid Hydrogels

In order to overcome the limitations of natural and synthetic matrices, hybrid hydrogels have been developed comprising multiple natural and synthetic components that provide a wider range of bioactive cues to encapsulated ECs, thereby promoting vasculogenic and angiogenic processes. Since natural materials are mechanically weak and susceptible to rapid degradation in vivo, and synthetic materials lack the wide range of biochemical cues necessary for vascular functionalization, it is beneficial to develop hybrid materials that incorporate the "best of both worlds" and thereby provide optimized and tunable microenvironments necessary for vascular growth and development.

Some of these include protein-based materials (gelatin, collagen, silk, Matrigel, and others) that are functionalized with synthetic side/end-groups to provide additional cross-link sites that increase the overall mechanical strength of the fabricated scaffolds (Fig. 8.3b). Proteins can also be blended with synthetic polymers to improve mechanical properties of hybrid hydrogels provided there is no molecular level phase separation between different macromolecules. Protein-mimetic or protein-derived peptide sequences (e.g., collagen-mimetic peptide, VEGF-mimetic peptide, and self-assembling peptides) can be incorporated into polymer-based hydrogels to provide additional biochemical or biophysical signaling cues required for ECs (Jia and Kiick 2009; Singh et al. 2013; Klotz et al. 2019; Kim et al. 2020).

In addition to proteins, carbohydrate polymers including dextran, alginate, and hyaluronic acid can also be functionalized with synthetic groups to improve structural and functional properties of scaffolds. Methacrylate groups covalently coupled to hyaluronic acid and dextran have been used to create methacrylated HA (MeHA) and methacrylated dextran (DexMA), respectively, (Möller et al. 2007; Jin et al. 2010; Liu and Chan-Park 2010; Eke et al. 2017; Lu et al. 2022). In these polymers, choosing the optimum molecular weight and degree of crosslink is critical to ensuring success in vascularization strategies. Too high molecular weight polymer chains can increase the matrix viscoelasticity through chain entanglements that may hinder vascular migration, network formation, and vessel branching. Too low molecular weight polymer chains may cause inflammatory responses and dysfunctional behavior in encapsulated or seeded cells. Similarly, degree of crosslink determines the matrix porosity, stiffness, and overall permissivity towards vascular growth and spreading. MeHa and DexMA can be used in ratiometric combinations with gelatin to form interpenetrating polymer networks (IPNs) that promote vascularization.

Overall, a wide range of polymeric biomaterials are available for tissue revascularization and for modeling tumor angiogenesis. Although various materials-chemistry based methods have been developed to create improved scaffolds, novel innovations are under way to improve hemocompatibility of scaffolds and matrices, create stable and long-lasting vasculature, incorporate tissue-specific vascular and stromal cells, and to fabricate functionally consistent vascularized constructs. In addition, hydrogel-based scaffolds for modeling of tumor angiogenesis have also

been developed in an effort to understand cancer-vascular crosstalk, disrupt tumor angiogenic signaling and thereby prevent vascular growth.

8.5 Biofabrication Strategies for Vasculogenesis and Angiogenesis

Developing in vitro vascular models requires not only the selection of the appropriate biomaterial but also the optimization of various fabrication and processing techniques that integrate the cells and the biomaterial to create structurally and functionally stable vascularized constructs. In this regard, various chemical, mechanical, and optical processing techniques have been developed including self-assembly, bioprinting, micromolding, photolithography, and laser-based techniques, amongst others (Bajaj et al. 2014; Moroni et al. 2018; Pradhan et al. 2020). These techniques are either additive or subtractive in nature. Additive techniques (3D bioprinting, stereolithography, electrospinning, etc.) include step-by-step controlled addition of cell/biomaterial mixture to the growing mass of the final construct that is regulated by photo/thermal/chemical crosslinking. Additive techniques are useful for creating well-designed scaffolds in a high-throughput fashion with good degree of repeatability. Subtractive techniques (laser-based degradation, sacrificial micromolding, etc.) include subjecting a bulk scaffold to optical/chemical treatment that removes controlled volume of material from the scaffold leaving behind a network of porous structures that can be readily occupied by vascular cells to form the vasculature. This approach helps attain finer resolution vascular networks compared to additive bulk processing techniques, but its throughput is much less.

Depending on the end-application, the user must optimize between speed/fabrication throughput, resolution, and complexity of the vascular structure, and reproducibility of the vascularized structures. In some tissue engineering applications (e.g., tissue vascularization and wound healing), fabrication speed and time are more critical than resolution. However, in other applications involving disease modeling and mechanistic studies (e.g., tumor angiogenesis, vascular dysfunction), resolution, architectural complexity, and repeatability are more important. Some of these techniques are discussed in more detail below (Table 8.3).

8.5.1 Self-Assembly

The technique of self-assembly primarily involves 3D encapsulation of vascular cells within hydrogel-based scaffolds and reliance on cellular migration and morphogenesis to form 3D interconnected vascular networks (Blinder et al. 2016) (Fig. 8.4a). Alternatively, cells seeded on top of a 3D scaffold can also migrate inwards into the material to form vascular protrusions and neo-vessel like formations, reminiscent of angiogenic growth. In this approach, both the material micro- and macro-scale properties as well as the cellular features (cell density, migration potential, etc.) are important considerations to achieve optimum

Table 8.3 Comparison of various biofabrication strategies for construction of vascularized networks for vascular tissue engineering and angiogenesis

Technique	Advantages	Limitations	Reference(s)
Self-assembly	• Facile • Easy to adopt for a wide variety of biomaterial/hydrogel scaffolds • Amenable to wide variety of vascular cell types including organ-specific ECs • Best suited for sprouting angiogenesis studies and quantification of vasculogenic potential	• Poor control over network morphology • Capillary network formed may not be homogenous • Difficult to form hierarchical vascular structures • Network formed may not be stable, patent or long-lasting	Levenberg et al. (2005); Moon et al. (2010); Chen et al. (2012); Singh et al. (2013); Blinder et al. (2016)
Bioprinting	• High degree of engineering precision during construction of vascular network • High spatiotemporal control of dispensed cell-gel mixture • Suited for both thermally and light-mediated crosslinkable gels • Wide range of bioinks available for different vascular cell types	• Bioinks need to be optimized for individual applications and cell types • Higher capital cost and operational knowledge of process parameters required • Rheological and mechanical properties need to be tuned to reduce cell damage and toxicity during printing process	Bhattacharjee et al. (2015); Highley et al. (2015); Hinton et al. (2015); Ouyang et al. (2017); Millik et al. (2019)
Electrospinning	• Highly porous scaffolds with controlled degree of fiber alignment and micro- to macro-scale stiffness • Suited for studies of cell-fiber interactions, contact guidance, and topography effects on vascular cell behavior • Electro-responsive polymers can be coated with natural biomaterials to enhance bioactivity	• Applicable with specific electro-responsive polymers only • Viscosity and concentration need to be optimized for every formulation • Cells can be seeded in 2D only for further migration into porous 3D scaffold	Ahn et al. (2015); Guo et al. (2017); Bertlein et al. (2018); Li et al. (2019a)
Micromolding	• Facile, cheap, and scalable	• Medium degree of control over resolution	Chrobak et al. (2006); Zheng et al. (2012);

(continued)

Table 8.3 (continued)

Technique	Advantages	Limitations	Reference(s)
	• Reproducible and consistent networks obtained • Can be adopted for a large number of soft hydrogels	• Limited in complexity of vascular networks • Large number of handling steps	Jiménez-Torres et al. (2016); DiVito et al. (2017)
Photolithography	• Layer-by-layer or point-by-point fabrication makes it possible to obtain finer structures with high resolution • Complex network structures with defined geometries achievable • High degree of repeatability	• Relatively lower fabrication speed and limited throughput • Scale-up is limited by fabrication speed and ability of cells to survive during the fabrication process	Gaebel et al. (2011); Slater et al. (2011); Kazemzadeh-Narbat et al. (2017); Zhu et al. (2017); Grigoryan et al. (2019)
Laser-based degradation	• Highest degree of spatial control over network geometry and complex features • Amenable to wide range of hydrogels	• Relatively low speed and throughput • Limited scale-up • High capital cost and operational cost	Hribar et al. (2015); Brandenberg and Lutolf (2016); Heintz et al. (2016); Arakawa et al. (2017)

vascularization. Higher cell density leads to faster interconnections between encapsulated cells and better anastomoses between the neovessels. As the cells tunnel through the matrix to connect with their neighboring cells, the matrix compliance and permissiveness determine the efficiency of the process. If the matrix is nanoporous or poorly degradable, it is challenging for encapsulated vascular cells to form lumenized networks, and they eventually regress and die. Since this technique is highly dependent on the migration potential of the cells and is stochastic in nature, several strategies have been developed to improve and accelerate the self-assembly process.

The most facile strategy to alter matrix microarchitecture is to control the degree of crosslinking either by controlling polymer concentration, crosslinker concentration, or changing pH/temperature during crosslinking process to tune the scaffold porosity, fiber density, fiber thickness, pore size, etc. (Cross et al. 2010; McCoy et al. 2018). Matrix porosity can also be altered by employing techniques like gas foaming, salt leaching, lyophilization or sacrificial molding (Harris et al. 1998; Ford et al. 2006). Gas foaming involves fabrication of scaffolds under high pressure with non-reactive gases (e.g., carbon dioxide), which is released at the end to leave behind macroporous hydrogel scaffolds that permit cell spreading and migration. Salt leaching involves soluble porogens (e.g., sodium chloride) that are added along with the polymer precursor at specific concentration. After completion of crosslinking, the porogens leach out into the surrounding buffer leaving behind

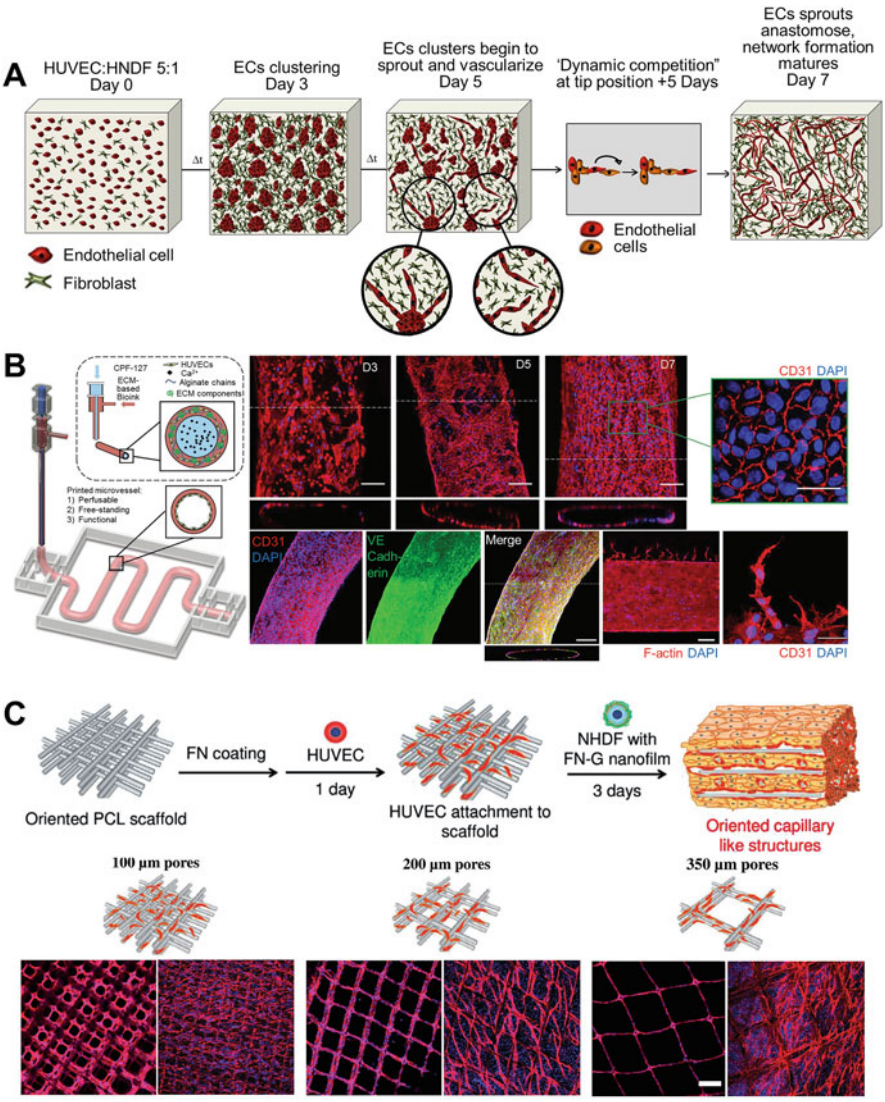


Fig. 8.4 Representative biofabrication techniques for construction of vascularized tissues. (**a**) Schematic of the dynamic vascular self-assembly process of HUVECs and HNDFs in a fibrin gel loaded in macroporous PLLA/PLGA scaffold over 7 days in culture. Adapted from (Blinder et al. 2016). (**b**) Schematic of the coaxial extrusion bioprinting of perfusable patent 3D vascular structures and HUVECs stained for characteristic markers (CD31: red; VE-Cadherin: green; nuclei: blue) demonstrating patent lumen and sprouting microvessels after 7 days. Scale bar: 100 µm. Adapted with permission from (Gao et al. 2018). (**c**) Electrospun PCL scaffolds of controlled spacing coated with fibronectin and seeded with HUVECs and fibroblasts and stacked with cell-accumulation technology to create thick vascularized constructs with contact-guidance patterned oriented vascular networks. Scale bar: 200 µm. Adapted with permission from (Bertlein et al. 2018)

voids and pores in the scaffold. Freeze-drying/lyophilization of polymer scaffolds is also used to create highly porous constructs. Alternately, thermo-sensitive polymers (e.g., gelatin, dextran) can also be incorporated within scaffolds in the form of microspheres or other intricate structures, which can later melt and dissolve away leaving behind templated pores and channels within the crosslinked polymer scaffolds. In recent developments, macroporous scaffolds have been achieved through annealing polymeric microspheres of various sizes to form larger scaffolds so that seeded cells can migrate through the void spaces between the microspheres and connect with neighboring cells (Griffin et al. 2015). The size of the microspheres determines the void fraction and porosity of the overall scaffold, while the stiffness and surface adhesivity of the microspheres determine the level of cell-matrix engagement through the void volumes.

Co-encapsulation of supporting vascular cell types like fibroblasts, MSCs, and pericytes along with ECs also helps accelerate and stabilize the vascular network formation, as these mural cells provide the initial tunneled paths within the matrix which is followed by the ECs to form lumenized networks (Peters et al. 2016). In case of modeling tumor angiogenesis, the most common strategy is to provide a gradient of biochemical or biophysical cues that enables ECs to form sprouting vasculature through chemotaxis or durotaxis, respectively (Kim et al. 2015). The biochemical gradient can be induced through various growth or angiogenic factors that are either encoded within the matrix or through simple diffusion. Biophysical cues can be incorporated through strain-assisted stretching and alignment of fibrillar components of the matrix/scaffold which enables encapsulated cells to directionally migrate along the aligned fibers and form aligned vascular networks (McCoy et al. 2018). Overall, multiple biochemical, biophysical, and meso-scale materials processing techniques have greatly improved the vascularization potential and performance of scaffolds towards guided self-assembly of vascular networks.

8.5.2 Bioprinting

Three-dimensional (3D) bioprinting has emerged as one of the most popular choices for high-throughput, repeatable, and reliable techniques for the fabrication of vascularized constructs. Although various types of 3D bioprinting (e.g., ink-jet/droplet, valve-jet) have been developed, the most prominent technique is extrusion-based bioprinting/bioplotting which involves controlled dispensing of cell-polymer mixture in a 3D volume which is then crosslinked to form an integrated scaffold (Fig. 8.4b). Most major vascularization applications have adopted extrusion-printing owing to its ability to fabricate at high speed, high resolution, low cell damage and toxicity, ability to print intricate and complex patterns, and suitability with a wide range of bioinks. Considering that tissue engineered vascularized scaffolds are usually millimeter- to centimeter-scale thick, this approach ensures that highest efficiency in terms of speed and resolution achievable.

The bioink used for extrusion-printing needs to be carefully selected and optimized for the cell type as well as for the printing process itself (Ouyang et al.

2017). The viscosity of the hydrogel precursor to be used as the bioink, its temperature-sensitivity, and the shear forces experienced by the cells during extrusion through the nozzle need to be carefully monitored to optimize the process. If the bioink is thermally-crosslinked, the dispensing rate needs to be controlled to allow sufficient time for the extruded material to crosslink before further material can be added on top of it to create hierarchical structures. This approach can slow down the overall throughput; hence, researchers have explored other avenues, including shear-thinning, self-healing hydrogels, or rapidly photocrosslinkable hydrogels as potential bioinks. MeHA hydrogels with adamantane and β-cyclodextrin groups which employ the guest-host bonding chemistry and which can be further photocrosslinked using UV light have been used as a potential bioink (Ouyang et al. 2016, 2017). The bioinks used can either be pre-photocrosslinked and dispensed to maintain patency of the structure, or photocrosslinked during the dispensing process itself to ensure structural integrity with the fabricated scaffold. The dispensing mechanism can itself be fluidically controlled to obtain various morphologies of the extruded material including linearly uniform fibers, core-shell fibers, and hollow core fibers.

In recent developments, the bioprinting process can be achieved by extruding the bioink within a gel-like support medium composed of granular particles (e.g., gelatin, Carbopol) (Bhattacharjee et al. 2015; Hinton et al. 2015). As the nozzle-head moves through the support bath and extrudes the bioink, the local region is temporarily fluidized and holds the extruded material in place after the nozzle moves away. After the entire structure is printed in 3D, the support bath is dissolved or melted to extract the 3D printed material. This process enables creation of relatively high resolution and hierarchically complex vascular patterns in 3D volumes. In an inverse approach, sacrificial bioinks (composed of water-soluble dextran, etc.) can be printed into 3D vascular-mimetic templates, which can then be encapsulated within polymeric hydrogel scaffolds and the dextran template is dissolved away to leave behind a vascular channel-based network (Miller et al. 2012). Thus, multiple approaches to 3D bioprinting have been developed to create more defined, patterned, and user-guided large-scale vascularized constructs for various tissue engineering applications.

8.5.3 Electrospinning

Similar to extrusion-bioprinting, electrospinning is another technique that is used to produce nano- to micron-scale polymer fibers that are integrated to form larger porous scaffolds. However, these fibers are produced via application of an electric field which can be tuned to vary the dimensions of the fibers formed. The polymer biomaterial chosen for this technique needs to be electrically conductive (e.g., PCL, PVA amongst others), have low surface tension and sufficiently high viscosity to be spun into fibers. The polymer melt or polymer-solvent solution is subjected to an electrical field within a nozzle, which when it overcomes the surface tension of the liquid, generates a stream of liquid jet. The dried liquid jet is collected in the form of fibers on a rotating mandrel to obtain electrospun mats. Both the polymer solution

properties as well as the operating parameters can be tuned to obtain fibers of various thickness and alignment degree.

Highly aligned electrospun scaffolds help in directional migration of seeded vascular cells and improve network connectivity (Ahn et al. 2015; Bertlein et al. 2018) (Fig. 8.4c). The inter-fiber spacing should be tuned in a way to allow optimized matrix porosity, cellular migration and motility, and overall matrix stiffness to ensure accelerated vascularization. The overall stiffness of electrospun scaffolds arises from the stiffness of individual fibers (determined by initial polymer concentration and molecular weight) and the relative density, thickness, and alignment of fibers in the scaffold. Thicker fibrils enable higher matrix stiffness but reduced porosity, while thinner fibrils allow higher porosity and contact-guidance based cellular migration. Consequently, this technique is particularly suited to study the role of contact-guidance based endothelial migration and spreading. In studies of tumor angiogenesis, where surrounding ECs exploit the biophysical matrix cues to preferentially grow and migrate towards the central tumor mass, electrospun scaffolds of varying fiber thickness, spacing, stiffness and alignment can help elucidate the mechanisms of such contact guidance-based migration. Overall, electrospinning is one of the key techniques that can exploit the cell-matrix biophysical engagement and thereby enable studies of vascular migration and broader tissue vascularization.

8.5.4 Micromolding

Traditional soft lithography approaches including micromolding have been used extensively as a facile technique for making large- to small-scale vascular channels within large biomaterial scaffolds. The most common material used for this purpose is poly(dimethyl siloxane) (PDMS) which can be poured and molded into a wide variety of shapes and sizes on negative masters and thermally cured to obtain positive molds. These PDMS molds can then be used as templates by encapsulating them within biomaterial/hydrogel scaffold of choice and physical removal of the mold to obtain vessel-like structures of the desired template (Jiménez-Torres et al. 2016). PDMS is widely preferred due to its moldability, flexibility, facile handling and low cytotoxicity post removal from the hydrogel scaffold. However, other materials, including metal wires, viscoelastic fluids, ice, and others, can also be used as templating agents to obtain vascular networks of choice (Chrobak et al. 2006; de Graaf et al. 2019; Wang et al. 2019). This technique is limited by some inherent challenges like resolution (inability to obtain micron-scale small features), large number of handling steps involved, and inability to obtain hierarchically complex structures, although some advances have been made in these respects for obtaining vascularized constructs. However, once the workflow has been optimized based on process parameters, this technique can be scaled up easily and enable highly reproducible scaffolds with in-laden vascular channels. Hence, it is also a popular choice for making organ-on-chip or microfluidics-based devices which are used extensively for a wide range of in vitro vascular studies.

8.5.5 Photolithography and Laser-Based Techniques

Photolithography-based techniques depend on the manipulation of light as it is projected on a 3D volume of biomaterial/hydrogel precursor to obtain desired features in the final fabricated scaffold. Photolithography enables fabrication of highly complex patterns with high resolutions and relatively high speeds by controlling the light path as it traverses the polymer mixture to photocrosslink into solid scaffolds. Depending on the mode of operation, it can be classified into mask-based photolithography, stereolithography, and laser-based lithography. Mask-based photolithography involves placing photomasks of defined features in the light path and selectively allowing light to expose certain areas/volumes of the polymer precursor to crosslink it (Aubin et al. 2010; Kazemzadeh-Narbat et al. 2017). Although facile, it is limited in terms of resolution and complexity of the vascular patterns achievable at increasing depths as the projected light gets diffused while traversing through the 3D volume. The use of photoabsorbers in the polymer precursor can mitigate the optical dispersal to a certain extent. Inversely, using an optical diffuser helps achieve feature height gradation that improves the resolution of the complex vascular patterns.

Stereolithography, also called maskless photolithography, involves manipulation of projected light using an array of mirrors (digital mirror device, DMD) that can be guided and controlled through a computer-aided design (CAD) software. This approach allows digital light projection in a dynamic fashion in a layer-by-layer fashion where each thin section of the polymer precursor volume is crosslinked by bringing it into focus with the projected light. Once crosslinking is complete, the next layer is brought into focus with an altered projected light path and the process continues until the entire scaffold is fabricated to its last layer. This approach provides the advantages of building higher resolution features one layer at a time to create intricate, complex, and tortuous vascular patterns with a high degree of spatiotemporal control. By optimizing the type and chemistry of the photocrosslinkers used, relatively high speeds can be achieved to obtain centimeter-scale vascularized constructs (Zhang and Larsen 2017; Zhu et al. 2017; Grigoryan et al. 2019).

Laser-based photolithography depends on the ability of coherent light beam of specific wavelength and power to penetrate a given 3D volume of a polymer precursor and initiate photocrosslinking to form fabricated scaffolds with highly defined and localized patterns (Aizawa et al. 2010). The spatial coherence of the laser beam, obtained through nano- to femto-scale pulses, combined with a two-photon (2P) microscopy platform enables delivery of light energy to specific points in a 3D volume with high degree of lateral and axial resolution and this enables creation of image-guided patterned hydrogels for highly specific and repeatable applications. The polymer precursor chosen for the application can be made to be responsive at specific wavelengths either through chemical modification of the backbone or via appropriate photocrosslinkers. Vascular patterns obtained from 2D/3D scans of in vivo/patient samples can be digitally processed and recreated to obtain in vitro laser-guided patterns in 3D hydrogel volumes with high fidelity

(Culver et al. 2012). Although highly precise structures can be achieved through the technique, it is limited in terms of speed and throughput owing to the 3D scanning rates of the laser and microscopy system. Hence, scale-up of this technique to large areas/volumes is currently limited and the fabricated scaffolds are primarily used for mechanistic studies of vasculogenesis and tumor angiogenesis.

Alternate to additive methods, laser-based techniques can be used for subtractive fabrication of hydrogels scaffolds as well. A large-volume, prefabricated hydrogel scaffold can be degraded in a user-defined, image-guided manner to form interconnected and highly precise vascular network with high degree of fidelity to the original vascular pattern. This can be achieved by optimizing the fluence, power, and pulse duration of the laser source combined with 2P-microscopy to generate microchannels through laser-induced photocavitation of water molecules present within the polymer scaffold (Brandenberg and Lutolf 2016; Heintz et al. 2016). Another approach is to use polymers with encoded photocleavable moieties that can undergo chemical scission under exposure to lasers of specific wavelength (Arakawa et al. 2017). This approach vastly improves the speed of the fabrication process as the scission and photocavitation occur at much faster rates compared to the scanning speed of the laser and microscopy stage itself. Thermally crosslinked hydrogels can also be laser-degraded by incorporation of heat-absorbing gold nanorods/nanoparticles which can locally melt the polymer macromolecules upon laser excitation and heating (Hribar et al. 2015).

Overall, several novel biofabrication technologies, coupled with biochemical and biophysical modifications of biomaterials/hydrogels, have been developed to construct vascularized scaffolds for a wide range of applications. Based on the end need, various operational parameters including speed, resolution, scalability of the fabrication processes need to be optimized. Although some techniques like self-assembly and 3D bioprinting produce scaffolds with high degree of scalability and throughput which may be suitable for in vivo implantation, the process is stochastic and may vary between different materials and different cell types. On the other hand, laser-based techniques produce highly precise vascularized scaffolds that may not be scalable but can be used for mechanistic investigation of disease progression, including mechanisms of tumor angiogenesis.

8.6 Conclusions

Hydrogels, owing to their versatility, moldability, ease of handling and processing, and relatively low cost, have become a popular choice of biomaterial for a wide range of vascular tissue engineering applications. Their biocompatibility with multiple vascular cell types and hemocompatibility with existing vasculature enables their translation towards regenerative medicine. In recent times, there has been an increasing need to create vascularized thick tissues with high degree of patency, structural and functional stability as well as architectural complexity. Although various biofabrication techniques have been developed to address this need, further improvements are necessary to match the required resolution while maintaining

efficient fabrication speed at scale. In addition to cardiovascular system, the synthetic generation and modeling of the lymphatic system has gained attention due to critical role of lymphatics in interstitial fluid clearance and dynamic transport. It is hoped that with the continued discovery of new biomaterials, as well as novel advances in biophysical and biochemical modifications to existing ones, a wider repertoire of biomaterials will be available for individualized and specific applications.

The domain of tumor angiogenesis has also received impetus with the implementation of hydrogel-based vascularized scaffolds. Particularly, with the development of lab-on-a-chip/organ-on-a-chip devices, it has become possible to integrate hydrogel-based scaffolds with microfluidic systems to study mechanisms of cancer cell-endothelial cell crosstalk and investigate angiogenic growth of tumors. By tuning hydrogel properties, the cellular functional and morphological responses that mediate tumor angiogenesis can be studied, and the results gained thereof can be even extended towards other angiogenic processes in the body. These integrated systems can pave the way for testing pre-clinical efficiency of candidate anti-angiogenic drugs and provide valuable data that would be too expensive to obtain from animal models. In the future, these hydrogel-based tissue mimics are expected to be widely implemented for various biomedical applications.

Acknowledgement This work was supported by funding from DBT Ramalingaswami Fellowship (BT/RLF/Re-entry/16/2019) (SP), DST-SERB Startup Research Grant (SRG/2020/000343) (SP), IITM Exploratory Research Project (BT1920893RFER008955) (SP), IITM Seed Grant (BT1920587NFIG008955 and BT2021084NFSC008955) (SP), Prime Minister's Research Fellowship (PMRF) (AB), and UGC Junior Research Fellowship (IPP).

Conflict of Interest Shantanu Pradhan is the co-founder, director, and holds equity in ISMO Biophotonics Pvt. Ltd. registered in Chennai, India.

References

Ahn H et al (2015) Engineered small diameter vascular grafts by combining cell sheet engineering and electrospinning technology. Acta Biomater 16:14–22. https://doi.org/10.1016/j.actbio.2015.01.030

Aisenbrey EA, Murphy WL (2020) Synthetic alternatives to Matrigel. Nat Rev Mater 5(7):539–551. https://doi.org/10.1038/s41578-020-0199-8

Aizawa Y, Wylie R, Shoichet M (2010) Endothelial cell guidance in 3D patterned scaffolds. Adv Mater 22(43):4831–4835. https://doi.org/10.1002/adma.201001855

Akhtar N, Dickerson EB, Auerbach R (2002) The sponge/Matrigel angiogenesis assay. Angiogenesis 5(1–2):75–80. https://doi.org/10.1023/a:1021507031486

Andrée B et al (2019) Formation of three-dimensional tubular endothelial cell networks under defined serum-free cell culture conditions in human collagen hydrogels. Sci Rep 9(1):5437. https://doi.org/10.1038/s41598-019-41985-6

Antunes M et al (2021) Development of alginate-based hydrogels for blood vessel engineering. Mater Sci Eng C 134:112588. https://doi.org/10.1016/j.msec.2021.112588

Arakawa CK et al (2017) Multicellular vascularized engineered tissues through user-programmable biomaterial photodegradation. Adv Mater 29(37):1703156. https://doi.org/10.1002/adma.201703156

Athirasala A et al (2017) A novel strategy to engineer pre-vascularized full-length dental pulp-like tissue constructs. Sci Rep 7(1):3323. https://doi.org/10.1038/s41598-017-02532-3
Aubin H et al (2010) Directed 3D cell alignment and elongation in microengineered hydrogels. Biomaterials 31(27):6941–6951. https://doi.org/10.1016/j.biomaterials.2010.05.056
Auger FA, Gibot L, Lacroix D (2013) The pivotal role of vascularization in tissue engineering. Annu Rev Biomed Eng 15(1):177–200. https://doi.org/10.1146/annurev-bioeng-071812-152428
Badhe RV et al (2017) A composite chitosan-gelatin bi-layered, biomimetic macroporous scaffold for blood vessel tissue engineering. Carbohydr Polym 157:1215–1225. https://doi.org/10.1016/j.carbpol.2016.09.095
Bajaj P et al (2014) 3D biofabrication strategies for tissue engineering and regenerative medicine. Annu Rev Biomed Eng 16(1):247–276. https://doi.org/10.1146/annurev-bioeng-071813-105155
Barrs RW et al (2021) Engineering a chemically defined hydrogel bioink for direct bioprinting of microvasculature. Biomacromolecules 22(2):275–288. https://doi.org/10.1021/acs.biomac.0c00947
Bertlein S et al (2018) Development of endothelial cell networks in 3D tissues by combination of melt electrospinning writing with cell-accumulation technology. Small 14(2):1701521. https://doi.org/10.1002/smll.201701521
Bhattacharjee T et al (2015) Writing in the granular gel medium. Sci Adv 1(8):e1500655. https://doi.org/10.1126/sciadv.1500655
Blanco-Fernandez B et al (2021) Proteinaceous hydrogels for bioengineering advanced 3D tumor models. Adv Sci 8(4):2003129. https://doi.org/10.1002/advs.202003129
Blinder YJ et al (2016) Vasculogenic dynamics in 3D engineered tissue constructs. Sci Rep 5(1):17840. https://doi.org/10.1038/srep17840
Brandenberg N, Lutolf MP (2016) In situ patterning of microfluidic networks in 3D cell-laden hydrogels. Adv Mater 28(34):7450–7456. https://doi.org/10.1002/adma.201601099
Bray LJ et al (2015) Multi-parametric hydrogels support 3D in vitro bioengineered microenvironment models of tumour angiogenesis. Biomaterials 53:609–620. https://doi.org/10.1016/j.biomaterials.2015.02.124
Campbell KT, Stilhano RS, Silva EA (2018) Enzymatically degradable alginate hydrogel systems to deliver endothelial progenitor cells for potential revasculature applications. Biomaterials 179:109–121. https://doi.org/10.1016/j.biomaterials.2018.06.038
Celie K-B et al (2019) Microstructured hydrogel scaffolds containing differential density interfaces promote rapid cellular invasion and vascularization. Acta Biomater 91:144–158. https://doi.org/10.1016/j.actbio.2019.04.027
Chang WG, Niklason LE (2017) A short discourse on vascular tissue engineering. NPJ Regen Med 2(1):1–8. https://doi.org/10.1038/s41536-017-0011-6
Charbonier FW, Zamani M, Huang NF (2019) Endothelial cell mechanotransduction in the dynamic vascular environment. Adv Biosyst 3(2):1800252. https://doi.org/10.1002/adbi.201800252
Chen Y-C et al (2012) Functional human vascular network generated in photocrosslinkable gelatin methacrylate hydrogels. Adv Funct Mater 22(10):2027–2039. https://doi.org/10.1002/adfm.201101662
Chiu LLY et al (2012) Perfusable branching microvessel bed for vascularization of engineered tissues. Proc Natl Acad Sci 109(50):E3414–E3423. https://doi.org/10.1073/pnas.1210580109
Chrobak KM, Potter DR, Tien J (2006) Formation of perfused, functional microvascular tubes in vitro. Microvasc Res 71(3):185–196. https://doi.org/10.1016/j.mvr.2006.02.005
Chwalek K et al (2015) Glycosaminoglycan-based hydrogels to modulate heterocellular communication in in vitro angiogenesis models. Sci Rep 4(1):4414. https://doi.org/10.1038/srep04414
Cross VL et al (2010) Dense type I collagen matrices that support cellular remodeling and microfabrication for studies of tumor angiogenesis and vasculogenesis in vitro. Biomaterials 31(33):8596–8607. https://doi.org/10.1016/j.biomaterials.2010.07.072

Culver JC et al (2012) Three-dimensional biomimetic patterning in hydrogels to guide cellular organization. Adv Mater 24(17):2344–2348. https://doi.org/10.1002/adma.201200395

de Graaf MNS et al (2019) Scalable microphysiological system to model three-dimensional blood vessels. APL Bioeng 3(2):026105. https://doi.org/10.1063/1.5090986

Deng C et al (2010) A collagen–chitosan hydrogel for endothelial differentiation and angiogenesis. Tissue Eng A 16(10):3099–3109. https://doi.org/10.1089/ten.tea.2009.0504

Ding Z et al (2019) Injectable silk nanofiber hydrogels for sustained release of small-molecule drugs and vascularization. ACS Biomater Sci Eng 5(8):4077–4088. https://doi.org/10.1021/acsbiomaterials.9b00621

DiVito KA et al (2017) Microfabricated blood vessels undergo neoangiogenesis. Biomaterials 138: 142–152. https://doi.org/10.1016/j.biomaterials.2017.05.012

Dudley AC (2012) Tumor endothelial cells. Cold Spring Harb Perspect Med 2(3):a006536–a006536. https://doi.org/10.1101/cshperspect.a006536

Eke G et al (2017) Development of a UV crosslinked biodegradable hydrogel containing adipose derived stem cells to promote vascularization for skin wounds and tissue engineering. Biomaterials 129:188–198. https://doi.org/10.1016/j.biomaterials.2017.03.021

Ford MC et al (2006) A macroporous hydrogel for the coculture of neural progenitor and endothelial cells to form functional vascular networks in vivo. Proc Natl Acad Sci 103(8):2512–2517. https://doi.org/10.1073/pnas.0506020102

Fuchs S et al (2009) Contribution of outgrowth endothelial cells from human peripheral blood on in vivo vascularization of bone tissue engineered constructs based on starch polycaprolactone scaffolds. Biomaterials 30(4):526–534. https://doi.org/10.1016/j.biomaterials.2008.09.058

Gaebel R et al (2011) Patterning human stem cells and endothelial cells with laser printing for cardiac regeneration. Biomaterials 32(35):9218–9230. https://doi.org/10.1016/j.biomaterials.2011.08.071

Gao G et al (2018) Coaxial cell printing of freestanding, perfusable, and functional in vitro vascular models for recapitulation of native vascular endothelium pathophysiology. Adv Healthc Mater 7(23):1801102. https://doi.org/10.1002/adhm.201801102

Gniesmer S et al (2019) In vivo analysis of vascularization and biocompatibility of electrospun polycaprolactone fibre mats in the rat femur chamber. J Tissue Eng Regen Med 13(7): 1190–1202. https://doi.org/10.1002/term.2868

Golub JS et al (2010) Sustained VEGF delivery via PLGA nanoparticles promotes vascular growth. Am J Physiol Heart Circ Physiol 298(6):H1959–H1965. https://doi.org/10.1152/ajpheart.00199.2009

Griffin DR et al (2015) Accelerated wound healing by injectable microporous gel scaffolds assembled from annealed building blocks. Nat Mater 14(7):737–744. https://doi.org/10.1038/nmat4294

Grigoryan B et al (2019) Multivascular networks and functional intravascular topologies within biocompatible hydrogels. Science 364(6439):458–464. https://doi.org/10.1126/science.aav9750

Guo H-F et al (2017) A simply prepared small-diameter artificial blood vessel that promotes in situ endothelialization. Acta Biomater 54:107–116. https://doi.org/10.1016/j.actbio.2017.02.038

Gupta P, Mandal BB (2021) Silk biomaterials for vascular tissue engineering applications. Acta Biomater 134:79–106. https://doi.org/10.1016/j.actbio.2021.08.004

Hackethal J et al (2021) Novel human placenta-based extract for vascularization strategies in tissue engineering. Tissue Eng Part C Methods 27(11):616–632. https://doi.org/10.1089/ten.tec.2021.0173

Han J et al (2011) Co-electrospun blends of PLGA, gelatin, and elastin as potential nonthrombogenic scaffolds for vascular tissue engineering. Biomacromolecules 12(2): 399–408. https://doi.org/10.1021/bm101149r

Harris AL (2002) Hypoxia—a key regulatory factor in tumour growth. Nat Rev Cancer 2(1):38–47. https://doi.org/10.1038/nrc704

Harris LD, Kim BS, Mooney DJ (1998) Open pore biodegradable matrices formed with gas foaming. J Biomed Mater Res 42(3):396–402
Heintz KA et al (2016) Fabrication of 3D biomimetic microfluidic networks in hydrogels. Adv Healthc Mater 5(17):2153–2160. https://doi.org/10.1002/adhm.201600351
Highley CB, Rodell CB, Burdick JA (2015) Direct 3D printing of shear-thinning hydrogels into self-healing hydrogels. Adv Mater 27(34):5075–5079. https://doi.org/10.1002/adma.201501234
Hinton TJ et al (2015) Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. Sci Adv 1(9):e1500758. https://doi.org/10.1126/sciadv.1500758
Hribar KC et al (2015) Three-dimensional direct cell patterning in collagen hydrogels with near-infrared femtosecond laser. Sci Rep 5(1):17203. https://doi.org/10.1038/srep17203
Ishihara M et al (2003) Controlled release of fibroblast growth factors and heparin from photocrosslinked chitosan hydrogels and subsequent effect on in vivo vascularization. J Biomed Mater Res A 64A(3):551–559. https://doi.org/10.1002/jbm.a.10427
Islam MM et al (2020) Chitosan based bioactive materials in tissue engineering applications—a review. Bioact Mater 5(1):164–183. https://doi.org/10.1016/j.bioactmat.2020.01.012
Jain RK (2003) Molecular regulation of vessel maturation. Nat Med 9(6):685–693. https://doi.org/10.1038/nm0603-685
James BD, Allen JB (2018) Vascular endothelial cell behavior in complex mechanical microenvironments. ACS Biomater Sci Eng 4(11):3818–3842. https://doi.org/10.1021/acsbiomaterials.8b00628
Jia X, Kiick KL (2009) Hybrid multicomponent hydrogels for tissue engineering: hybrid multicomponent hydrogels for tissue engineering. Macromol Biosci 9(2):140–156. https://doi.org/10.1002/mabi.200800284
Jiménez-Torres JA et al (2016) LumeNEXT: a practical method to pattern luminal structures in ECM gels. Adv Healthc Mater 5(2):198–204. https://doi.org/10.1002/adhm.201500608
Jin R et al (2010) Enzymatically-crosslinked injectable hydrogels based on biomimetic dextran-hyaluronic acid conjugates for cartilage tissue engineering. Biomaterials 31(11):3103–3113. https://doi.org/10.1016/j.biomaterials.2010.01.013
Juliar BA et al (2018) Sprouting angiogenesis induces significant mechanical heterogeneities and ECM stiffening across length scales in fibrin hydrogels. Biomaterials 162:99–108. https://doi.org/10.1016/j.biomaterials.2018.02.012
Kampmann A et al (2013) Additive effect of mesenchymal stem cells and VEGF to vascularization of PLGA scaffolds. Microvasc Res 90:71–79. https://doi.org/10.1016/j.mvr.2013.07.006
Kang L et al (2019) Hyaluronic acid oligosaccharide-modified collagen nanofibers as vascular tissue-engineered scaffold for promoting endothelial cell proliferation. Carbohydr Polym 223:115106. https://doi.org/10.1016/j.carbpol.2019.115106
Kayabolen A et al (2017) Native extracellular matrix/fibroin hydrogels for adipose tissue engineering with enhanced vascularization. Biomed Mater 12(3):035007. https://doi.org/10.1088/1748-605X/aa6a63
Kazemzadeh-Narbat M et al (2017) Engineering photocrosslinkable bicomponent hydrogel constructs for creating 3D vascularized bone. Adv Healthc Mater 6(10):1601122. https://doi.org/10.1002/adhm.201601122
Khoo CP, Micklem K, Watt SM (2011) A comparison of methods for quantifying angiogenesis in the Matrigel assay in vitro, https://home.liebertpub.com/tec. Mary Ann Liebert, Inc. 140 Huguenot Street, 3rd Floor New Rochelle, NY 10801 USA. https://doi.org/10.1089/ten.tec.2011.0150
Kim J et al (2015) Engineering of a biomimetic pericyte-covered 3D microvascular network. PLoS One 10(7):e0133880. https://doi.org/10.1371/journal.pone.0133880
Kim S, Pan C-C, Yang YP (2020) Development of a dual hydrogel model system for vascularization. Macromol Biosci 20(10):2000204. https://doi.org/10.1002/mabi.202000204
Kinoshita K et al (2016) Fabrication of multilayered vascular tissues using microfluidic agarose hydrogel platforms. Biotechnol J 11(11):1415–1423. https://doi.org/10.1002/biot.201600083

Kleinman HK, Martin GR (2005) Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol 15(5):378–386. https://doi.org/10.1016/j.semcancer.2005.05.004

Klotz BJ et al (2019) A versatile biosynthetic hydrogel platform for engineering of tissue analogues. Adv Healthc Mater 8(19):1900979. https://doi.org/10.1002/adhm.201900979

Kloxin AM et al (2009) Photodegradable hydrogels for dynamic tuning of physical and chemical properties. Science 324(5923):59–63. https://doi.org/10.1126/science.1169494

Krock BL, Skuli N, Simon MC (2011) Hypoxia-induced angiogenesis. Genes Cancer 2(12): 1117–1133. https://doi.org/10.1177/1947601911423654

Laschke MW et al (2008) Improvement of vascularization of PLGA scaffolds by inosculation of in situ-preformed functional blood vessels with the host microvasculature. Ann Surg 248(6): 939–948. https://doi.org/10.1097/SLA.0b013e31818fa52f

Levenberg S et al (2005) Engineering vascularized skeletal muscle tissue. Nat Biotechnol 23(7): 879–884. https://doi.org/10.1038/nbt1109

Li X et al (1994) Vascular smooth muscle cells grown on Matrigel. A model of the contractile phenotype with decreased activation of mitogen-activated protein kinase. J Biol Chem 269(30): 19653–19658

Li X et al (2019a) Nanofiber-hydrogel composite–mediated angiogenesis for soft tissue reconstruction. Sci Transl Med 11(490):eaau6210. https://doi.org/10.1126/scitranslmed.aau6210

Li Y et al (2019b) Chitosan sulfate inhibits angiogenesis *via* blocking the VEGF/VEGFR2 pathway and suppresses tumor growth *in vivo*. Biomater Sci 7(4):1584–1597. https://doi.org/10.1039/C8BM01337C

Liu Y, Chan-Park MB (2010) A biomimetic hydrogel based on methacrylated dextran-graft-lysine and gelatin for 3D smooth muscle cell culture. Biomaterials 31(6):1158–1170. https://doi.org/10.1016/j.biomaterials.2009.10.040

Lu J et al (2019) Enhanced angiogenesis by the hyaluronic acid hydrogels immobilized with a VEGF mimetic peptide in a traumatic brain injury model in rats. Regen Biomater 6(6):325–334. https://doi.org/10.1093/rb/rbz027

Lu D et al (2022) Macroporous methacrylated hyaluronic acid hydrogel with different pore sizes for in vitro and in vivo evaluation of vascularization. Biomed Mater 17(2):025006. https://doi.org/10.1088/1748-605X/ac494b

Marcu R et al (2018) Human organ-specific endothelial cell heterogeneity. iScience 4:20–35. https://doi.org/10.1016/j.isci.2018.05.003

McCoy MG et al (2018) Collagen fiber orientation regulates 3D vascular network formation and alignment. ACS Biomater Sci Eng 4(8):2967–2976. https://doi.org/10.1021/acsbiomaterials.8b00384

McGuigan AP, Sefton MV (2006) Vascularized organoid engineered by modular assembly enables blood perfusion. Proc Natl Acad Sci 103(31):11461–11466. https://doi.org/10.1073/pnas.0602740103

McGuigan AP, Sefton MV (2007) Modular tissue engineering: fabrication of a gelatin-based construct. J Tissue Eng Regen Med 1(2):136–145. https://doi.org/10.1002/term.14

Michna R et al (2018) Vascularized microfluidic platforms to mimic the tumor microenvironment. Biotechnol Bioeng 115(11):2793–2806. https://doi.org/10.1002/bit.26778

Miller JS et al (2012) Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. Nat Mater 11(9):768–774. https://doi.org/10.1038/nmat3357

Millik SC et al (2019) 3D printed coaxial nozzles for the extrusion of hydrogel tubes toward modeling vascular endothelium. Biofabrication 11(4):045009. https://doi.org/10.1088/1758-5090/ab2b4d

Möller S et al (2007) Dextran and hyaluronan methacrylate based hydrogels as matrices for soft tissue reconstruction. Biomol Eng 24(5):496–504. https://doi.org/10.1016/j.bioeng.2007.08.014

Moon JJ et al (2010) Biomimetic hydrogels with pro-angiogenic properties. Biomaterials 31(14): 3840–3847. https://doi.org/10.1016/j.biomaterials.2010.01.104

Moore EM, West JL (2019) Bioactive poly(ethylene glycol) acrylate hydrogels for regenerative engineering. Regen Eng Transl Med 5(2):167–179. https://doi.org/10.1007/s40883-018-0074-y

Morgan JT et al (2019) Fabrication of centimeter-scale and geometrically arbitrary vascular networks using in vitro self-assembly. Biomaterials 189:37–47. https://doi.org/10.1016/j.biomaterials.2018.10.021

Moroni L et al (2018) Biofabrication: a guide to technology and terminology. Trends Biotechnol 36(4):384–402. https://doi.org/10.1016/j.tibtech.2017.10.015

Muller S et al (2019) Human adipose stromal-vascular fraction self-organizes to form vascularized adipose tissue in 3D cultures. Sci Rep 9(1):7250. https://doi.org/10.1038/s41598-019-43624-6

Nakatsu MN et al (2003) Angiogenic sprouting and capillary lumen formation modeled by human umbilical vein endothelial cells (HUVEC) in fibrin gels: the role of fibroblasts and Angiopoietin-1☆. Microvasc Res 66(2):102–112. https://doi.org/10.1016/S0026-2862(03)00045-1

Natividad-Diaz SL et al (2019) A combined hiPSC-derived endothelial cell and in vitro microfluidic platform for assessing biomaterial-based angiogenesis. Biomaterials 194:73–83. https://doi.org/10.1016/j.biomaterials.2018.11.032

Nehls V, Drenckhahn D (1995) A novel, microcarrier-based in vitro assay for rapid and reliable quantification of three-dimensional cell migration and angiogenesis. Microvasc Res 50(3):311–322. https://doi.org/10.1006/mvre.1995.1061

Nehls V, Schuchardt E, Drenckhahn D (1994) The effect of fibroblasts, vascular smooth muscle cells, and pericytes on sprout formation of endothelial cells in a fibrin gel angiogenesis system. Microvasc Res 48(3):349–363. https://doi.org/10.1006/mvre.1994.1061

Nerem RM, Seliktar D (2001) Vascular tissue engineering. Annu Rev Biomed Eng 3(1):225–243. https://doi.org/10.1146/annurev.bioeng.3.1.225

Novosel EC, Kleinhans C, Kluger PJ (2011) Vascularization is the key challenge in tissue engineering. Adv Drug Deliv Rev 63(4–5):300–311. https://doi.org/10.1016/j.addr.2011.03.004

Ouyang L et al (2016) 3D printing of shear-thinning hyaluronic acid hydrogels with secondary cross-linking. ACS Biomater Sci Eng 2(10):1743–1751. https://doi.org/10.1021/acsbiomaterials.6b00158

Ouyang L et al (2017) A generalizable strategy for the 3D bioprinting of hydrogels from nonviscous photo-crosslinkable inks. Adv Mater 29(8):1604983. https://doi.org/10.1002/adma.201604983

Pan Z, Ding J (2012) Poly(lactide-co-glycolide) porous scaffolds for tissue engineering and regenerative medicine. Interface Focus 2(3):366–377. https://doi.org/10.1098/rsfs.2011.0123

Peters EB et al (2016) Poly(ethylene glycol) hydrogel scaffolds containing cell-adhesive and protease-sensitive peptides support microvessel formation by endothelial progenitor cells. Cell Mol Bioeng 9(1):38–54. https://doi.org/10.1007/s12195-015-0423-6

Pradhan S et al (2016) Polymeric biomaterials for in vitro cancer tissue engineering and drug testing applications. Tissue Eng Part B Rev 22(6):470–484. https://doi.org/10.1089/ten.teb.2015.0567

Pradhan S et al (2020) Biofabrication strategies and engineered in vitro systems for vascular mechanobiology. Adv Healthc Mater 9(8):1901255. https://doi.org/10.1002/adhm.201901255

Redd MA et al (2019) Patterned human microvascular grafts enable rapid vascularization and increase perfusion in infarcted rat hearts. Nat Commun 10(1):584. https://doi.org/10.1038/s41467-019-08388-7

Roudsari LC et al (2016) A 3D poly(ethylene glycol)-based tumor angiogenesis model to study the influence of vascular cells on lung tumor cell behavior. Sci Rep 6(1):32726. https://doi.org/10.1038/srep32726

Rouwkema J, Khademhosseini A (2016) Vascularization and angiogenesis in tissue engineering: beyond creating static networks. Trends Biotechnol 34(9):733–745. https://doi.org/10.1016/j.tibtech.2016.03.002

Sacchi V et al (2014) Long-lasting fibrin matrices ensure stable and functional angiogenesis by highly tunable, sustained delivery of recombinant VEGF164. Proc Natl Acad Sci 111(19):6952–6957. https://doi.org/10.1073/pnas.1404605111

Shen Y-I et al (2014) Hyaluronic acid hydrogel stiffness and oxygen tension affect cancer cell fate and endothelial sprouting. Biomater Sci 2(5):655. https://doi.org/10.1039/c3bm60274e

Shin YC et al (2017) RGD peptide and graphene oxide co-functionalized PLGA nanofiber scaffolds for vascular tissue engineering. Regen Biomater 4(3):159–166. https://doi.org/10.1093/rb/rbx001

Singh S, Wu BM, Dunn JCY (2011) Accelerating vascularization in polycaprolactone scaffolds by endothelial progenitor cells. Tissue Eng A 17(13–14):1819–1830. https://doi.org/10.1089/ten.tea.2010.0708

Singh RK, Seliktar D, Putnam AJ (2013) Capillary morphogenesis in PEG-collagen hydrogels. Biomaterials 34(37):9331–9340. https://doi.org/10.1016/j.biomaterials.2013.08.016

Slater JH et al (2011) Fabrication of multifaceted micropatterned surfaces with laser scanning lithography. Adv Funct Mater 21(15):2876–2888. https://doi.org/10.1002/adfm.201100297

Stegemann JP, Kaszuba SN, Rowe SL (2007) Review: advances in vascular tissue engineering using protein-based biomaterials. Tissue Eng 13(11):2601–2613. https://doi.org/10.1089/ten.2007.0196

Sun W et al (2016) Co-culture of outgrowth endothelial cells with human mesenchymal stem cells in silk fibroin hydrogels promotes angiogenesis. Biomed Mater 11(3):035009. https://doi.org/10.1088/1748-6041/11/3/035009

Suresh V, West JL (2020) 3D culture facilitates VEGF-stimulated endothelial differentiation of adipose-derived stem cells. Ann Biomed Eng 48(3):1034–1044. https://doi.org/10.1007/s10439-019-02297-y

Taubenberger AV et al (2016) 3D extracellular matrix interactions modulate tumour cell growth, invasion and angiogenesis in engineered tumour microenvironments. Acta Biomater 36:73–85. https://doi.org/10.1016/j.actbio.2016.03.017

Teixeira FC et al (2021) Engineering a vascularized 3D hybrid system to model tumor-stroma interactions in breast cancer. Front Bioeng Biotechnol 9:647031. https://doi.org/10.3389/fbioe.2021.647031

van Uden S et al (2019) A novel hybrid silk-fibroin/polyurethane three-layered vascular graft: towards *in situ* tissue-engineered vascular accesses for haemodialysis. Biomed Mater 14(2):025007. https://doi.org/10.1088/1748-605X/aafc96

Vignesh S et al (2018) Injectable deferoxamine nanoparticles loaded chitosan-hyaluronic acid coacervate hydrogel for therapeutic angiogenesis. Colloids Surf B: Biointerfaces 161:129–138. https://doi.org/10.1016/j.colsurfb.2017.10.033

Wang R et al (2019) Freestanding hierarchical vascular structures engineered from ice. Biomaterials 192:334–345. https://doi.org/10.1016/j.biomaterials.2018.11.011

Wang Y et al (2020) A chitosan modified asymmetric small-diameter vascular graft with anti-thrombotic and anti-bacterial functions for vascular tissue engineering. J Mater Chem B 8(3):568–577. https://doi.org/10.1039/C9TB01755K

Yadav AS et al (2020) RGD functionalized chitosan nanoparticle mediated targeted delivery of raloxifene selectively suppresses angiogenesis and tumor growth in breast cancer. Nanoscale 12(19):10664–10684. https://doi.org/10.1039/C9NR10673A

Yuan Z et al (2021) Injectable GelMA cryogel microspheres for modularized cell delivery and potential vascularized bone regeneration. Small 17(11):2006596. https://doi.org/10.1002/smll.202006596

Zamani M et al (2017) A biomimetic heparinized composite silk-based vascular scaffold with sustained antithrombogenicity. Sci Rep 7(1):4455. https://doi.org/10.1038/s41598-017-04510-1

Zhang R, Larsen NB (2017) Stereolithographic hydrogel printing of 3D culture chips with biofunctionalized complex 3D perfusion networks. Lab Chip 17(24):4273–4282. https://doi.org/10.1039/C7LC00926G

Zhang D et al (2018) The enhanced angiogenesis effect of VEGF-silk fibroin nanospheres-BAMG scaffold composited with adipose derived stem cells in a rabbit model. RSC Adv 8(27): 15158–15165. https://doi.org/10.1039/C7RA11610A

Zheng Y et al (2012) In vitro microvessels for the study of angiogenesis and thrombosis. Proc Natl Acad Sci 109(24):9342–9347. https://doi.org/10.1073/pnas.1201240109

Zhu W et al (2017) Direct 3D bioprinting of prevascularized tissue constructs with complex microarchitecture. Biomaterials 124:106–115. https://doi.org/10.1016/j.biomaterials.2017.01.042

Zieris A et al (2010) FGF-2 and VEGF functionalization of starPEG–heparin hydrogels to modulate biomolecular and physical cues of angiogenesis. Biomaterials 31(31):7985–7994. https://doi.org/10.1016/j.biomaterials.2010.07.021

Advances in 3D Printing Technology for Tissue Engineering

9

Prabhash Dadhich, Parveen Kumar, Anirban Roy, and Khalil N. Bitar

9.1 Introduction

Additive manufacturing is positioning itself as the "State of Art," over the last half a decade, for the reconstruction of grafts in the field of tissue engineering. This technology basket has a lot of variants, such as inkjet printing, bioprinting, laser beam melting, digital laser printing (DLP), fused deposition modeling (FDM), stereolithography, precision extruding deposition (PED), and selective laser sintering (SLS). However, these variants involve the basic principle of successive layer-by-layer material deposition until the formation of the final product. Each technology variant has a unique capability to create complex structures using computer-aided design (CAD) methods (Gibson et al. 2010).

The aforementioned capability and flexibility offered by 3D printing technology to adapt and mimic complex structures have helped garner its popularity in the regenerative medicine community, as complex tissues can be generated with suitable techniques and biomaterials. A plethora of materials like ceramic, metals, and polymers can be 3D printed serving the end-product requirements. The current focus methods are focused on the development of strategies that enable to use of live cells along with supporting hydrogels to bioengineer small organs for the whole human in the future (Ong et al. 2018). Recently, the 3D-cell printing technique is proving to be one of the most promising technologies for precise cell-positioning fabrication methods. The biggest advantage of this technique is that it enables the

P. Dadhich (✉) · K. N. Bitar
Cellfbio LLC, Richmond, VA, USA

P. Kumar
Department of Pharmaceutical Sciences, Shri Ram College of Pharmacy, Karnal, Haryana, India

A. Roy
Department of Chemical Engineering, Birla Institute of Technology and Science, Pilani, K K Birla Goa Campus, Goa, India

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_9

recapitulation of unique features of human tissue. It also can deliver multiple types of cells in controlled distribution.

These advanced strategies are utilized for precise designs of the whole organ or damaged part thereof created using CAD methods based on patient-specific data. Facilitation and control of cell survival, proliferation, tissue porosity, and dynamic tissue growth are the key advantages of 3D printing making it an attractive tool in regenerative medicine and tissue engineering (Egan 2019). The microfluidic approach in 3D cell printing has led to a significant leap in engineering vascularization. Several recent advancements have been reported in the field of genetic engineering and regenerative medicine by involving rapid prototyping.

The development of functional tissues/organs for transplantation is the principal objective of tissue engineering. The analogous orientation of the extracellular matrix along with the cellular components is a preliminary requirement for the success of functional tissues/organs. Investigation of the mechanism of pathophysiology using disease models, toward exploring new therapies is another advantage of these technologies. Recently, several 2D cell cultures or genetically modified transgenic animals were used in the development of different new drugs, and therapeutic targets. These methods were unable to mimic the human physiological conditions and tissue complexity. The generation of 3D printed cellular models is an excellent alternative for mimicking spatial and chemical complexity compared to 2D models. Such biomimicking complex tissue analogs can be used for the transplantation of degenerative tissues.

9.2 Printing Technology

Academic institutions and industries have adopted this technology very quickly and are continuously developing it further to suit their custom requirements by modifying the systems and components. This non-systematic, unorganized proliferation in 3D technology has a flip side, which is incremental innovations by users being filed for trademarks, leading to confusion in breaching and infringement of overlapping technologies. There are several similar terms such as 3D printing, additive manufacturing, solid free-form fabrication, and rapid prototyping/manufacturing which are essentially synonymous. In this chapter, the 3D printing term is used to discuss these technologies in the following three categories (Fig. 9.1). 3D-printing techniques for biomedical applications have been categorized into three

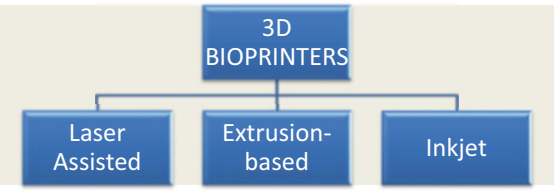


Fig. 9.1 3D cell printing techniques for biomedical applications

major types: (1) Laser-assisted, (2) Extrusion printing system, and (3) Inkjet printing system.

9.2.1 Laser-Assisted

Laser-based technologies are widely used for several biomedical applications for the last four decades (Chua and Leong 2015). Most of the laser-assisted printers used for hydrogel composite fabrications. The laser-assisted printing system can be categorized into two major types: (1) Laser-induced printing (LIB) and (2) laser-guided printing.

9.2.2 Laser-Induced Printing

Laser-induced bioprinting (LIB) was first introduced in 1986 by Bohandy et al., where a high-energy excimer laser was used to print copper film on a silicon layer (Bohandy et al. 1986). A similar principle was proposed by Odde et al. in 1990 for 3D cell patterning and construction of hybrid biological–electronic device, and fabrication of biochip-array for tissue engineering (Odde and Renn 1999). The basic mechanism of LIB involves mainly three parts: a pulsed laser source, targeted on a ribbon coated with liquid biological materials, along with receiving substrate. The ribbon is comprised of three layers, the top donor layer (quartz/glass), followed by an energy-absorbing layer (metal: gold or titanium), and a specialized bioink layer (hydrogel with cells, and bioactive factors) (Fig. 9.2a) (Guillemot et al. 2010). Briefly, the laser beam is focused on the donor layer, energy-absorbing layer pushes the bioink layer by an elevated-pressure bubble, which helps in ejection of required amount of bioink on collecting substrate. The predefined construct is bioengineered in a droplet-by-droplet manner, with a low risk of contamination as the dispenser and bioinks are not in contact (Li et al. 2016). Guillotin et al. demonstrated LIB at microscale resolution using CAD control for the development of tiny tissue-like layouts with de novo high cell density at a speed of 5 kHz (Guillotin et al. 2010).

LIB is mainly suitable for viscous high cell density hydrogels with high resolution. The risk of nozzle clogging is resolved, yet cell viability is a major concern with LIB technology. The cell viability significantly reduced with a longer gelation time after laser fluence in an alginate gelation-based cell printing study (Gudapati et al. 2014). Thermal damage, reduced nutrition, and oxygen transfer due to the thick-viscous hydrogel layer were identified as the main cause of low cell survival (Catros et al. 2011). To overcome these issues, femtosecond lasers were used and it was observed that cell printing in a viscus hydrogel is possible but cell viability was reduced to 85% due to photomechanical influences of the laser pulse (Hopp et al. 2012).

Application of higher thickness of substrate and viscosity was another approach to improve cell viability in LIB. Collagen encapsulated fibroblast and keratinocytes were printed using LIB. Both cells maintained their ability to proliferate without any

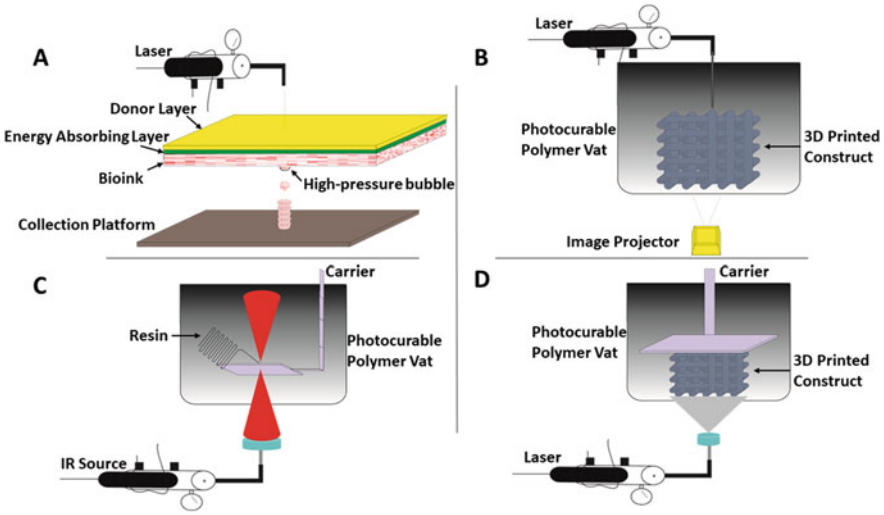


Fig. 9.2 Schematic images of laser-based hydrogel 3D printing systems: (**a**) Laser-induced printing; (**b**) Stereolithography apparatus (SLA); (**c**) Two-photon polymerization (TPP); (**d**) Digital light projection (DLP)

apoptosis or DNA fragmentation after printing, ultimately proven to be resistant to laser-induced cell death (Koch et al. 2010). There are very few printers developed on the LIB principle as this is an expensive process and can be used only for large-scale projects with the risk of laser-induced cell death (Zaszczyńska et al. 2021).

9.2.3 Laser-Guided Printing

The basic principle of laser-assisted 3D printing is the construction of hydrogel composite using the laser as a source of energy. In this method, the laser beam is directed to a suspension of cells and works on the principle of refractive index differences between the medium and cell. It enables the laser beam to capture and push the cells on the receiving substrate.

In this technology, a 3D structure is built in a vat of photocurable hydrogel using a laser beam. The UV laser is exposed to the photocurable liquid which results in hydrogel formation. The UV beam successively moves in a specific pattern which allows development of the layers of hydrogel over the preformed structure (Melchels et al. 2010; Billiet et al. 2012). This basic principle has been used in several applications such as tissue engineering, biomedical implants, devices, and drug delivery. There are several versions of this technique developed based on the type of laser, method of laser exposure, stage movement, and delivering the final product. The main laser-guided 3D printer techniques are discussed as follows.

9.2.4 Stereolithography Apparatus (SLA)

SLA has the ability to write a predefined pattern by solidification of a liquid photopolymer using exposure to infrared (IR), ultraviolet (UV), or visible light. The CAD-controlled predesigned 2D patterns are exposed to the bioink mixture in a polymer reservoir. With the help of a layer-by-layer process, the constructed 2D pattern stacked up to build the 3D structure (Fig. 9.2b) (Billiet et al. 2012). This technique has the advantage of fabrication of delicate cellular patterns in complex geometry with high printing quality and speed. The first SLA developed around four decades ago and is one of the most applicable principles of 3D printing (Zaszczyńska et al. 2021).

There are several controllable parameters such as light energy, light intensity, scanning speed, spot size, wavelength, light exposure time, polymerization, and layer thickness which enable this technology to be high quality, accurate, precise, and consistent (Lovell et al. 2001; Yankov and Nikolova 2017; Watters and Bernhardt 2018). The SLA process is beneficial for small-detailed objects (from 200 to 2000 mm) with high-quality resolution and surface finish.

In recent years, the use of complex optical systems resulted in fabrication at a micron scale. The microfabrication using the micro-SLA system is classified into two types based on precise beam projection and scanning system. (1) Scanning μSLA system: in this system, the UV beam is stationary and focused on the spot position and the workpiece stage move along with the vat, resulting in high resolution and free from focusing-based defects (Beluze et al. 1999; Choi et al. 2009). (2) Mask projection μSLA system: single light beam radiation carried out using dynamic pattern generator mask for each hydrogel layer. The precise pattern can be generated using predesigned 2D patterns of 3D structure (Bertsch et al. 2000; Sun et al. 2005).

A narrow range of photocurable hydrogels, post-curing process, and necessity of support structures are the main limitation of this technology. DNA damage and the risk of tumorigenesis in cells due to UV-induced bioprinting is another challenge with SLA (de Gruijl et al. 2001; Sinha and Häder 2002). In this context, visible light-based SLA was developed using photoinitiators such as GelMA, PEGDA, and other resins (Wang et al. 2015). This system resulted in high cell viability along with low-cost manufacturing. The precision and versatility of SLA examined, and the mechanical properties and pore architecture can be controlled by varying compositions of macromeres. Complex porous scaffolds fabricated using GelMA exhibited successful cell growth in high density after seeding with HUVECs (Gauvin et al. 2012). The 3D construct of polyurethane-based photosensitive materials with hyaluronic acid was printed using blue light SLA displayed human Wharton's jelly mesenchymal stem cells differentiation toward cartilage repair (Shie et al. 2017).

9.2.5 Digital Light Projection (DLP)

DLP comes from the image projection technology of SLA. This basic mechanism is the processing and control of working light sources to photosensitive materials using optical micro-electromechanical technology. A group of micron-sized, controllable mirrors known as digital mirror devices (DMD) rotates and control the light beam and projects to photosensitive resin in a CAD-controlled pattern (Zhang et al. 2020a). DLP is a top-down construction approach compared to the bottom-up principle of other 3D printers. The light beam is positioned beneath the vat and projected on the photocurable hydrogel underneath the vat. On the light exposure, each layer is printed with the help of a building carrier. After polymerization of each layer, the carrier moves in the vertically upward direction and the fresh hydrogel is supplied automatically to the bottom space through capillary action (Fig. 9.2d).

Compared to conventional SLA, planarization was not required and resulted in precision and high speed (Jang et al. 2018). In recent years liquid crystals are also used instead of DMD. The individual on-off beam signal enables DLP to fabricate high-resolution 3D constructs (25 and 150 μm) (Melchels et al. 2010; Billiet et al. 2012). The ambient conditions of printing are mild, at low temperature, pressure, and without any shear stress compared to nozzle-based printing, therefore, cell survival is high (85–95%) and suitable for live-cell printing. The DLP is faster compared to LAB as it is able to print the entire layer compared to spot printing in LAB.

DLP-based 3D printing has been widely used in fabricating various tissues and organs. DLP is used in the creation of a series of nerve conduits, microchannels, and bionic conduits for nerve repair (Zhu et al. 2018). A 3D triculture hepatic model was also constructed using human-induced pluripotent stem cells (hiPSC)-hematopoietic progenitor cells (HPCs), human umbilical vein endothelial cells (HUVECs), and adipose-derived stem cells in a DLP printer (Ma et al. 2016). DLP 3D printer was significant in the bioengineering of a functional, vascularized alveolar model, which exhibited oxygen exchange and blood flow (Grigoryan et al. 2019). The methacrylate polyvinyl alcohol (PVA-MA) and GelMA encapsulated mesenchymal stem cells (MSCs) printed precise micro-scaffolds. The cell survival rate was high (~90%) and MSCs differentiated to osteogenic differentiation and cartilage-specific ECM toward bone regeneration (Lim et al. 2018).

9.2.6 Two-Photon Polymerization (TPP)

TPP is one of the recent stereolithographic techniques to build 3D micro/nanostructures at sub-diffraction-limit resolution without a layer-by-layer process. TPP is a photochemical process, in which a beam of femtosecond infrared laser pulses focused into photosensitive resins and photolytic polymerization is carried out using a high-numerical-aperture (NA) objective (Fig. 9.2c) (Melchels et al. 2010; Billiet et al. 2012; Zhou et al. 2015). The 3D printing is carried out within the photosensitive liquid by polymerization threshold intensity of nonlinear infrared light. Therefore, the TPP is able to fabricate construct at a higher structural resolution

(200 nm), compared to other stereolithography methods. In TPP, an ultra-short laser pulse scans a piezoelectric stage, which is regulated by a computer control system or optical scanning devices, leading in an ultra-small concentrating point by the narrowly focused beam (Nguyen and Narayan 2017). The photoinitiator is excited by this ultra-small, focused beam, consequently, free radicals are produced, which initiate polymerizations and subsequently construction of the 3D object. The laser intensity, beam focus size, scan position, and free radical density are the main control parameters for the polymerization process. Insufficient free radical density, multi-photon ionization, and subsequent dielectric breakdown are the main challenges with TPP (Zhou et al. 2015).

TPP also demonstrated potential for drug delivery, fabrication of implants, and biosensors (Weiß et al. 2009). The nanoscale precision of TPP allows precise control of porosity within the scaffolds and results in elevated cell attachment, migration, and organization within the scaffold. The IR laser beam is safe for cells, therefore, the TPP can be promising for 3D bioprinting using live cells. Yet TPP is not preferred for tissue engineering due to the limitation of fabrication of mesoscale structures and toxicity of feedstock materials (Nguyen and Narayan 2017). Currently, research is going on the exploration of new photopolymers suitable for tissue engineering applications that can provide improved cell viability, biocompatibility, and avoid the risk of toxicity.

9.2.7 Extrusion-Based Printing System

Extrusion and micro-extrusion printing system uses pneumatic pressure or physical forces of mechanical devices to dispense viscous hydrogel, or bio-ink through the nozzle. The computer-controlled coordinated movements of the printing head construct a 3D structure through sequential extrusion of biomaterial layer-by-layer predefined pattern. The interlayer adhesion between layers is critical for successful fabrication (Ning and Chen 2017; Unagolla and Jayasuriya 2020). This method has the capability of producing 3D tissue-mimetic structures. It enables the extrusion of bioinks of a broad variety of viscosities by increasing the ejection power.

The extrusion-based mechanism enables these printers to print high cell density, and dynamic crosslinking between viscous hydrogels. Moreover, this technology can deal with various types of biomaterials, bioinks, and viscous hydrogels (Fig. 9.3a). Extrusion-based printers are capable to print cell-laden bioink into large-scale biomimetic structures at high speed and accuracy (Ning and Chen 2017; Unagolla and Jayasuriya 2020). Inferior resolution and cell loss due to shear caused by printing nozzle during extrusion are the main challenges with this technology (Blaeser et al. 2016; Jang et al. 2016). There are different variants of extrusion-based printing technology that have been developed.

The First 3D plotter was developed around two decades ago targeting soft tissue engineering (Landers and Mülhaupt 2000). This is the basic extrusion-based printing technology, in which viscous hydrogel is injected through a micro-needle from a syringe into a solution of liquid with the density like that of injected hydrogel. The

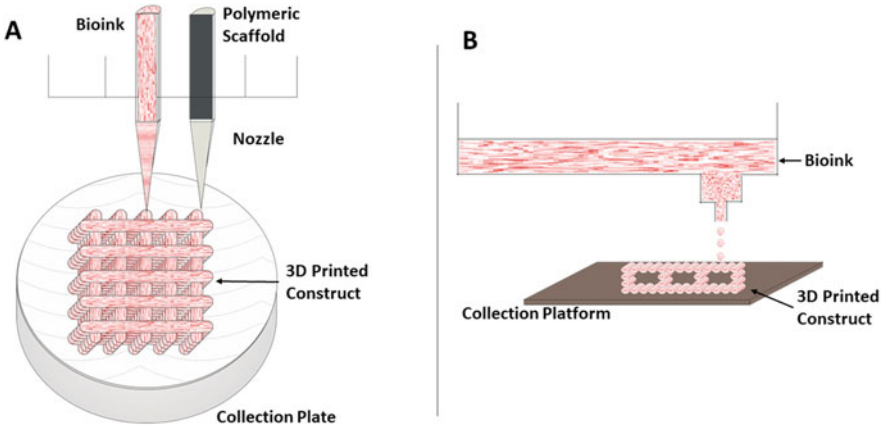


Fig. 9.3 Schematic images of 3D printing systems: (**a**) Extrusion-based printing system; (**b**) Inkjet printing system

ink can be extruded as a micro-dot or continuous strand, which depends on the viscosity of the ink, nozzle diameter, extrusion speed, and pressure.

The coordinated movements of the printing head in different axes enable fabrication of a 3D pattern. There are different types of materials that can be printed using a 3D plotter such as hydrogel, polymer sol, cell, and growth factors (Yilgor et al. 2008; Billiet et al. 2014; Akkineni et al. 2015).

DIW (direct ink writing) or direct write arrangement (DWA) is the fundamental type of extrusion-based printing technology. A hydrogel-filled syringe with nozzle, compressed air, moving platform, and optical microscope for real-time monitoring is the main component of DIW. In DIW, the hydrogel is extruded through a nozzle in the *Z* direction on the collector platform moving in the *X*-*Y* direction. The viscosity of hydrogel and diameter of nozzle control the precision and speed of printing and determine the resolution of the final product ranging from 100 nm to 1000 μm (Jang et al. 2018).

The rheology of hydrogel is a critical parameter and needs to have the self-supporting ability and be set instantly to allow shape retention of desired printed object. The hydrogel needs to be colloidal (high concentration of nanoparticle or colloid) to prevent shrinkage during the drying of the final structure and avoid spreading during extrusion (Ahn et al. 2009).

The pressure-assisted microsyringe (PAM) is another type of extrusion-based printer. In the PAM technology, pneumatic-driven glass capillary microsyringes were used instead of regular nozzles. Temperature-controlled syringe jackets were also employed to regulate the temperature during printing (Vozzi et al. 2002; Tartarisco et al. 2009). Low temperature of deposition as well as manufacturing (LDM) was invented to overcome the heating process. The extruded hydrogel was collected at a platform (moving *X*-*Y* direction) and at subzero temperature. The solvent is removed from the construct via freeze drying. In the recent upgrade of this

technology, multiple nozzles were developed to dispense different hydrogels to form gradient structures of different materials (Liu et al. 2009, 2019).

9.2.8 Inkjet Printing System

The first inkjet printer was developed around two decades ago, in which liquid was deposited on a binder powder (Williams 2003). Inkjet printing is non-contact technology and is promising in dispensing cells or biomaterial as a droplet. The ink (hydrogel and/or cell-suspension bioink) is dispensed through the printer head which moves in the *X*-*Y* direction onto a collection platform that moves on *Z*-axis. The desired 3D object was designed using computer modeling and printed layer-by-layer with deposition of ink. Inkjet printing generates bioink droplets under the influence of thermal or piezoelectric inkjet heads to achieve a specific pattern. A thermal inkjet printer uses a micro-heater to generate a heating pulse which further vaporizes the bioink. The heating element at the printing head causes gasification at elevated temperature and generates bubbles. These bubbles are non-spontaneously printed on the collection platform (Fig. 9.3b). There is a mechanical pulse that also helps in ejecting small bioink droplets from the printing head of the thermal inkjet printer. The bioink droplet expels forcefully from printing head by direct mechanical pulse generated by piezoelectric actuator. Bioink should be low viscous and need to be solidified instantly. The inkjet printing process can be studied in two different categories; drop-on-demand (DOD) printing and continuous inkjet (CIJ) printing (Mei et al. 2005; Saunders et al. 2008).

Both of the categories dispense the ink/liquid binder as the droplet (ranging from 15 to ~100 μm). In DOD the liquid binder ejects as droplets through thermal or piezoelectric effect according to computer-controlled design. Whereas in CIJ, a continuous jet of ink emerges from the nozzles and breaks up into droplets by Rayleigh instability (Jang et al. 2018). There is excellent controllability in dispensing the droplet and fewer chances of contamination in DOD, therefore, a more preferred technique compared to CIJ (Nakamura et al. 2005). The inkjet-based printer can use powder or liquid as starting material as described.

Inkjet-based printer using powder (IPP) is a solid-phase rapid prototyping technique. In this type of printer, a powder is spread over the collection platform, followed by liquid binder/ink dispensed over the powder in a predefined pattern. The liquid ink binds the adjacent powder particles together and forms a 2D pattern. Another layer is formed by lowering the platform and spreading another layer of powder. The process is continuous until the final structure is formed; the unreacted powder acted as support for the structure. This method is suitable for different types of materials such as ceramic, metal, and polymers (Pfister et al. 2004; Leukers et al. 2005; Cui et al. 2010). In an inkjet-based printer using liquid (IPL), the powder bed is replaced by a liquid chamber. The hydrogel was dispensed from nozzles and crosslinks in the collection liquid chamber. In another variant of direct inkjet printing, the photosensitive resin was dispensed from the nozzle head and printed build simultaneously cured with light (Boland et al. 2007; Sun et al. 2009).

Both IPP and IPL are suitable for a wide variety of materials and can be used at ambient temperature. IPL is preferred over IPP due to higher accuracy and uncross-linked material is easy to remove in IPL. Inkjet-based printer exhibits no deleterious effects on the biological component such as laser-mediated in laser-based printers or shear force as in extrusion and is therefore suitable for soft tissue engineering applications. Clogging of the nozzle, uneven distribution of cells, and difficulty in printing highly viscous materials yet are the challenges of inkjet-based printers.

The human primary dermal fibroblast displayed promising as cell viability and proliferation in a PEG-based hydrogel up for ~50 days (Rimann et al. 2016). In a similar study, the fibroblast population was optimized using alginate gel (Xu et al. 2014). Tissue engineering-related printing using thermal inkjet is not relevant for tissue engineering due to the loss of cells and biomolecules at extreme temperatures (Setti et al. 2004). Yet, piezoelectric inkjet printing systems were reported for bioengineering of cancer models. In this study, Ficoll PM400 demonstrated improved accuracy and eliminated nozzle clogging when used with neutrally buoyant suspensions (Chahal et al. 2012).

9.3 Types of Biomaterial Inks Used in 3D Printing

The previous sections discussed various technologies in 3D printing and keeping this in perspective, it may be noted that there is still tremendous scope for improvement due to the shortage of variety in 3D printable biomaterials and inks used in this technology-intensive process. To prevent shear stress injury, cells are surrounded by fluid and physiologically suitable hydrogels during the printing. Bioinks have a significant benefit in that, they can simulate specific tissue microenvironments by a nanofibrous structure resembling the ECM. A biomaterial must be: (1) biocompatible, (2) non-toxic, and (3) have structural properties. A balance must be struck among all the parameters for the manufacture and eventual use of the biomaterial ink for 3D printing.

9.3.1 Polymer-Based Inks

These polymers are commonly used as hydrogels, which are hydrated networks of cross-linked or polymerized. Hydrogels are hydrophilic and can swell in a high-water content solution. Factors used to control the extent of swelling can be the concentration and type of polymer material, viscosity, and shear stress among others. Several hydrogel combinations have been explored using synthetic and natural polymers, such that their properties can be tailored as per need. Depending on the extent of swelling of the polymers, they can be used in appropriate applications, such as hydrogel with higher water retention capacity is used in soft tissue applications and hydrogel with low water retention capacity is used in drug delivery applications.

Polymers are the most widely material as bioinks in printing due to compatibility with cells and processability. The critical properties (chemical, physical, and

biological) of a polymer can be easily tailored by altering the monomers and processing techniques. The natural polymer is considered the most preferred material for TE applications due to its inherent bioactivity, biodegradability, and low immunogenic reactions. Collagen, chitosan, and cellulose are being widely used biopolymers that support viability and cell proliferation (Kim et al. 2015; Wu et al. 2018; Duarte Campos et al. 2019; Arefin et al. 2021; John et al. 2021; Lawlor et al. 2021). Gelatin which is an irreversibly hydrolyzed form of collagen is another preferred biopolymer used alone or in the combination with ceramic in different tissue engineering applications (Kołbuk et al. 2020; Huang et al. 2021).

Polymers in 3D printing are used in both types of two physical phases: liquid and solid. The liquid phase is used for polymerization or crosslinking of monomers and/or oligomers in laser-assisted 3D printing techniques. The solid phase is commonly used in the extrusion of inkjet printing techniques. Another most important class of polymers used in 3D printing is degradable synthetic polymers such as aliphatic polyesters, polycaprolactone (PCL), poly-lactic-*co*-glycolic acid (PLGA), polylactic acid (PLA), which are used in various TE applications (Ma 2004; Russmueller et al. 2015; Garot et al. 2021). These degradable synthetic polymers are considered to cause relatively low toxicity, yet, the degraded oligomers may cause an inflammatory reaction. However, the rate of degradation is found different in different studies and showed that the degradation kinetics can be controlled and customized as per the requirement (Seyednejad et al. 2011; Park et al. 2012; Zaaba and Jaafar 2020; Wu et al. 2021; Zwawi 2021). Co-polymerization is an effective way to control or alter the physico-chemical and degradation properties of polymers such as poly(hydroxybutyrate) (PHB), poly(propylene fumarate) (PPF), and polyglycolic acid (PGA) (Frone et al. 2020; Koons et al. 2021; Suo et al. 2021). The combination of growth factors such as vascular endothelial growth factor (VEGF) and bone morphogenic protein (BMP) and other biomolecules is another method for enhancing the biological activity of polymers (Park et al. 2015). Particle-reinforcement, fiber-reinforcement, and nanocomposite are the recent practice to modify and control the printability and strength of the final product using polymers (Wang et al. 2017; Oladapo et al. 2019; John et al. 2021).

9.3.2 Ceramic-Based Inks

Ceramic-based products are rigid as well as provide a surface that is naturally required for hard tissue growth. These materials can be used in different forms in different types of 3D printers such as powder-based, slurry-based, or solid-based 3D printers (Chen et al. 2019).

The most commonly used ceramic-based ink is calcium phosphate (CaP). CaP including hydroxyapatite (HA) particles can stimulate the expression and secretion of cytokines and proteases related to bone regeneration (Müller et al. 2008). CaP has significant bioactive properties; it dissolves and promotes the formation of biological apatite resulting in direct bonding without the mediation of any fibrous connective tissue interface (El-Ghannam 2005). Calcium phosphate bioceramic has been

extensively used for bone repairs and as coatings for metallic prostheses owing to osteoconductive, osteoinductive, and bioresorption ability (Ducheyne and Qiu 1999). Amorphous calcium phosphate (ACP), hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), calcium-deficient hydroxyapatite (CDHA), β-tricalcium phosphate (β-TCP, $Ca_3(PO_4)_2$), ortho calcium phosphate (OCP), derivatives and their combinations are the most common CaP derivative used for bone substitute (Peña and Vallet-Regı 2003). The CaP-based inks are bioresorbable and induce new bone ingrowth, stimulating cell differentiation toward bone-forming cell lineages. 3D constructs printed using ceramic-based ink are an excellent choice for bone tissue engineering due to their superior strength, and bioactivity (Do et al. 2015; Dadhich et al. 2016, 2021; Roohani-Esfahani et al. 2016). Bioglass, graphene oxide, and zirconium are other important ceramics, which are used as bioink in the combination of CaP or polymers (Özcan et al. 2021).

9.3.3 Composite Inks

3D printing technology initially was aimed at metals and polymers. But with advancements in technology, composite inks started being used. They are primarily used in tissue engineering applications (Roohani-Esfahani et al. 2016; Fox et al. 2020). Improvement of bioactivity and physico-mechanical strength is the main objective to explore composite bioinks. These types of inks range from a combination of polymer, ceramics, and biomolecules. Studies reported accelerated healing and enhanced bioactivity of composite printed grafts for bone tissue engineering (Vallet-Regi and Arcos Navarrete 2008). However, rapid dissolution, inferior mechanical properties, and poor wear resistance limited its application (Yuan and De Groot 2004; Dorozhkin 2011; Bose et al. 2012).

In this context, efforts have been carried out to develop calcium phosphate-based composite grafts. These composites are a combination of two or more different types of biomaterial in a synergistic way that works as complementary to each other (Amini et al. 2012). Moreover, bones are also a composite material of high elastic modulus minerals embedded in an organic matrix of the low elastic modulus (Wegst et al. 2014). Previously, several attempts have been made to fabricate hybrid composite coupled with CaP and polymers with potential physicomechanical properties as bone grafts (Kim et al. 2020; Hodásová et al. 2021). Different studies reported calcium phosphate-based scaffolds with different polymers like chitosan, silk, collagen, alginate, polycaprolactone (PCL), polylactic acid (PLA), poly L-lactide-glycolic acid (PLGA), and polyethylene glycol diacrylate (PEGDA) (Zhang and Zhang 2001; Collins et al. 2009; Alge and Chu 2010; Luo et al. 2013; Inzana et al. 2014) as reinforcement. There are different minerals such as silica, graphene oxide, zirconium, and bioglass were used as a composite to achieve mechanical strength equivalent to bone and enhance vascularization and bioactivity (Özcan et al. 2021). Many studies reported on the feasibility of the fabrication of 3D constructs using polymeric composites through additive manufacturing, which resulted in improvement of cytocompatibility and mechanical properties (Ma et al.

2018). The bioceramic reinforced with polymer-based 3D printed scaffolds demonstrated control degradability and enhanced mechanical strength (Gao et al. 2014).

9.4 Application of 3D Printing in Tissue Engineering

In last two decades, the printing technology is widely explored for applications in different biomedical applications such as fundamental research, drug delivery, drug screening, tissue engineering, and regenerative medicine. Several non-biological implants such as scaffolds, dental implants, prostheses are fabricated using 3D printing and used clinically for patients. With the aid of 3D imaging and reverse engineering, 3D bioprinting enables more precise customized fabrication of constructs tailored for patient. Figure 9.2 describes the chronological development of 3D printing applications. Tissue engineering is capable of alleviating the crisis of organ transplantation. The vascularization of large three-dimensional constructed organs remains a huge task. Recent advancements in 3D printing offer potential solutions to this problem.

Encapsulating molecules in bio-based scaffolds could not focus on ensuring that cell lines have been accurately implanted into the internal scaffolds, as well as growth factors would only influence surface cell growth and divisions. As a result, studies have recognized growth factors direct-printing advanced technologies in order to produce organs and tissues. Tissue formations with biological functions could be created by layering different materials as well as "physiological ink" comprising growth factors, nutritional elements, seed cells, whereupon culturing these printable organs or tissue. Rather than the production process itself, one of the most difficult technical tasks of organ printing, attempting to recreate the complicated inner vascular organs network. As a result, numerous researchers have directed their attention to the printing of blood vessels. Ganovo, a company based in the United States, first used the technology of 3D printing to manufacture vascular prostheses in 2009 (Mabrouk et al. 2020). The Southern California and Michigan Colleges of Health Sciences used a device of 3D printing to co-print a network of blood vascular systems fewer than the size of 3 mm with agarose as support (Mironov et al. 2011). Researchers from Harvard University's Wyss Institute for Biological systems Influenced Engineering noted a three-dimensional bioprinting technique for manufacturing complicated living systems to embedded microvessels utilizing innumerable special ink (Kolesky et al. 2014). A three-dimensional bioprinter with several electrically controlled printheads has been aimed at creating such structures with different materials that must be printed accurately and concurrently, as during the tissue fabrication incorporated with the blood vessels, multiple cell types as well as extracellular matrix. Several researchers have successfully printed tissue or organ cases. Michael et al. applied laser bioprinting to make a proper cellularized substituted skin by arranging fibroblasts as well as keratinocytes in an accurate 3D spatial structure (Michael et al. 2013). The printable skin framework, which consists of fibroblast cells labeled in keratinocytes classified in green on

the upper side of MatriDerm, is positioned into a mouse skin wound. In a contrast, the rest of the skin of mouse skin is still intact. When the skin frameworks were tested in mice using the dorsal epidermis fold chamber the printable cells stayed active, expanded, as well as secreted extracellular matrix. Some blood vessels have been discovered expanding out from wound edges. A technique for printing numerous cell layers is required to create extra extremely complicated tissue. Mannoor et al. (2013) created a bionic ear by 3D printing matrix hydrogel of chondrocyte seeded alginate as well as infusing nanoparticles of silver in the geometry of anatomy of an ear, as well as electrodes of cochlea shaped. The printable bionic ear outperformed the ear in radio frequency auditory sensing (Mannoor et al. 2013). A Cornell University biologist used stem cells as well as biopolymer substances to print a working cardiovascular valve and the stem cells progressively incorporated into the living cells. There are currently some 3D printing technology organs for clinical use. A study by the University of Michigan transplanted a three-dimensional artificial respiratory tract into an infant's windpipe with a birth defect to support breathing and marking the world's first commercial human 3D printed organ transplant (Yan et al. 2018). The recent progress in 3D printers of the TE scaffolds has been discussed further, with a focus on the endless possibilities for reiteration of complicated tissue constructs presented by 3D printing methodologies (Fig. 9.4).

9.4.1 Nervous Tissue

The peripheral and central nervous systems are the key challenges to repairing tissues. An in vitro brain model was created using 3D printing of microchannels of collagen. Mouse nerve cells were cultured in collagen microchannels, resulting in brain microvasculature regeneration. This study demonstrated that the blood-brain barrier model could be used for physiological and pathological assessments, as well as a wide range of applications including tissue regeneration, and pharmaceuticals (Kim et al. 2015). Several studies reported on application of 3D printing with peripheral nerve conduits. The gel of copolymerized methacryloyl gelatin cellularized to adipose-based stem cells has been used to 3D print cellularized channels for peripheral regeneration of nerves. The fabricated conduits exhibited in vivo functional re-innervation and functionality (Hu et al. 2016).

A 3D neural mini tissue could be directly printed using neural stem cell (NSC) in alginate, agarose, and carboxymethyl-chitosan bioink (Gu et al. 2016). Mimicking a natural fibrin clot, aligned fibrin-factor XIII-HA scaffolds were prepared by an extrusion-based printer along with Schwann cells which support neurite regeneration (England et al. 2017). Schwann cell survival and functionality were studied in gelatin–alginate-based bioink; it was observed that the neurotrophic factors released was significantly high in 3D scaffolds compared to 2D culture (Wu et al. 2020). Recently, a bi-layered nerve conduit was reported along with a gelatin methacrylate (GelMA)-based bioink using bone marrow stem cells printed in a 3D tubular structure, where the inner layer provided the base for cell growth and the outer layer offered mechanical strength (Wu et al. 2020).

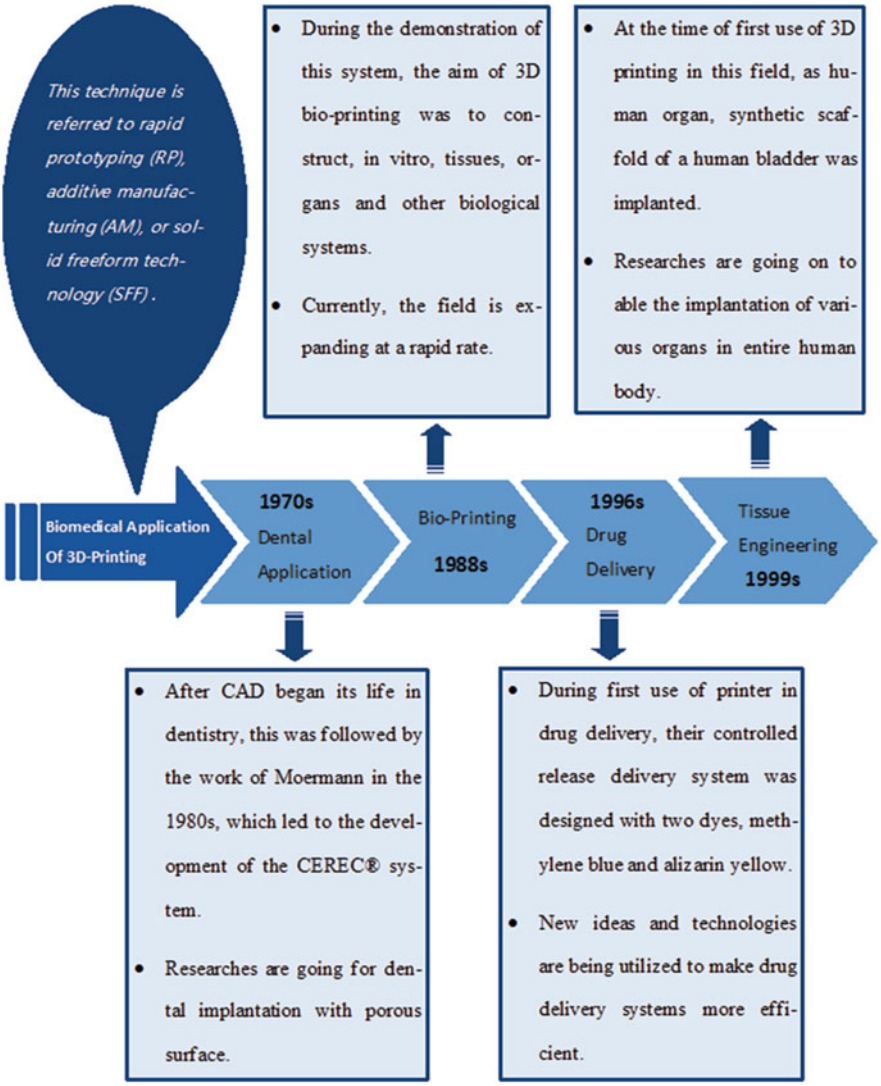


Fig. 9.4 Chronology of development and application of 3D printing technology

9.4.2 Liver

The liver is crucial for the metabolic activities of the body. The stem cells were printed using a liver decellularized extracellular matrix (dECM) bioink, the dECM induces higher differentiation of seeded stem cell and improved functioning of human hepatocellular carcinoma (HepG2) cell compared to the collagen-based hydrogel. PCL was also used to make the hybrid scaffold for mechanical support (Lee et al. 2017). Similarly, dECM was incorporated with GelMA bioink to fabricate

an inner gear-like construct using a DLP bioprinter. The 3D architecture of dECM exhibited improved cell viability, and liver function activities such as albumin and blood urea (Mao et al. 2020). In another study, a liver-mimetic honeycomb 3D structure was effective bioprinted using an extrusion-based bioprinter. The fibroblast, hepatoma cells were mixed with cellulose nanocrystals (CNCs) and alginate-based bioink followed by extruded successfully through the nozzle (100 μm inner diameter) readily without clogging (Wu et al. 2018). Another study of alginate bioink mixed with PF127 and printed into porous structures. Compared to the 2D structure of the same composition, it was observed that metabolic activity and liver functions were improved with 3D structures (Gori et al. 2020). In advanced studies, the hepatorganoids were bioprinted using HepaRG cells and bioink, after initial cell differentiation for 7 days, the hepatorganoids were transplanted into the Fah-deficient mice model of liver injury. It was observed that transplanted 3D hepatorganoids elevated increased synthesis of liver-specific proteins, material transport, and liver functions (Yang et al. 2021).

9.4.3 Kidney

The human embryonic kidney tissue-derived cells were printed into a macroporous tissue-like construct and long-term construct stability and cell viability were studied. The transfected cells displayed promising proliferation and strong cell–cell interactions and functionality. The study confirmed that functional embryonic tissues can be printed in vitro (Ouyang et al. 2015). Recently, extrusion-based 3D bioprinting was used to generate rapid and high-throughput kidney organoids. The 3D bioprinting allows precise control of organoid, size, conformation, cellular population, and biophysical properties. It enables the bioengineering of functional proximal tubular segments with uniformly patterned sheets of kidney cells. These organoid and kidney tissues sheets can be utilized for drug testing models and in vivo applications (Lawlor et al. 2021).

9.4.4 Skin

Fibroblast and keratinocytes were printed in MatriDerm hydrogel-based bioink using laser-assisted printers. The 3D printed bioengineered graft was directly printed on murine skin and resulted in epidermis formation (Michael et al. 2013). There is always a demand for 3D skin equivalents (morphological and functionally comparable to the skin) for drug, cosmetology testing, and disease model. In this context, several studies were carried out using a different combination of epidermal cells (Admane et al. 2019; Albanna et al. 2019; Kim et al. 2019). In another study, human amniotic fluid-derived stem (AFS) cells were encapsulated and combined encapsulated MSCs, where the AFS cells accelerated the wound closure by secreting growth factors and angiogenesis (Skardal et al. 2012). Further, it was evident that 3D printed skin grafts using silk and gelatin were more stable dimensionally compared

to collagen-based skin constructs (Admane et al. 2019). Recently, a method of bioprinting of skin equivalent through extrusion was optimized using fibroblasts and keratinocytes in a gelatin-based hydrogel. The final structure was made up of all three skin layers: basal layer, dermis, and epidermis and could be used to model skin diseases in vitro (Derr et al. 2019).

9.4.5 Bone and Cartilage

Among all the tissue engineering applications, restoration of cartilage and bone defects is one of the most explored regenerative processes. There are numerous studies available using different 3D printing modalities, among few key studies are discussed in this chapter. The majority of the studies proposed a combination of different materials (ceramic, polymers, and/or metals) to satisfy both biological and mechanical characteristics (Dadhich et al. 2016, 2021; Neufurth et al. 2017; Srivas et al. 2017; Kankala et al. 2018; Oladapo et al. 2019). An injectable hydrogel is frequently combined into a porous structure of 3D-printed scaffolds resulting in a hybrid structural framework to enhance cell seeding efficiency (Bahcecioglu et al. 2019).

There are several materials used to engineer 3D-printed bone and cartilage scaffolds, yet biodegradable aliphatic polyesters and hydrogels are considered as most promising, in bone and cartilage tissue engineering (Bahcecioglu et al. 2019). The physical or chemical modification with bioactive components is a common strategy to enhance the biological activity of hydrogel. In this context, the osteogenic and chondrogenic effects of mesenchymal stem cells were evaluated in a hybrid polymerized PEG-GelMA scaffold during layer-by-layer assembly. The results were promising, exhibiting good cell viability and excellent osteogenic and chondrogenic differentiation capacity compared to PEG alone (Gao et al. 2015). The bioactivity of alginate-based bioink was improved by supplementing with methacrylated (Ma)-decellularized extracellular matrix (dECM) derived from bone tissues. The bioink-laden human adipose-derived stem cells demonstrated improvement in cell proliferation and osteogenic differentiation (Lee et al. 2020). The hybrid bioink containing a mixture of alginate and gelatin with human mesenchymal stem cells was optimized for bioprinting a porous bone-like tissue. The study concluded with a successful low-cost cell-laden bioink, optimized for stiffness and cell density toward bone tissue engineering applications (Zhang et al. 2020b).

Another hybrid bioink was developed using chondrocyte-laden GelMA and PCL for bioprinting of 3D constructs toward nasal reconstruction. Different parameters such as the temperature, needle gauge, crosslinking time, and different concentrations of GelMA were optimized to achieve the best possible viability and functionality of chondrocytes in bioprinted constructs (Ruiz-Cantu et al. 2020). Recently, hydroxypropyl methylcellulose was mixed with silk fibrin to print bone marrow mesenchymal stem cells (BMSC) for cartilage tissue repair. Compared to the single network hydrogel, the combination of these two polymers developed double network (DN) hydrogel resulted in improvement in the fracture strength,

breaking elongation, and compressive reproducibility. Further biological assays confirmed that hydrogel could warrant adequate nutrient supply and excellent biological supportability in tissue engineering (Ni et al. 2020).

9.4.6 Ocular Tissues

The use of 3D printing technology is slowly growing for ophthalmology applications. The retinal ganglion cells and glial cells from adult rats were printed using a piezoelectric inkjet printer to bioengineer the 3D retina. It has been demonstrated how these retinal cell types can be effectively printed without losing viability or phenotypic characteristics (Lorber et al. 2016). The fabrication of corneal TE scaffold using collagen bioink comprising corneal keratocytes seems to be another proof of the successful application of 3D printing technology in ocular tissue engineering (Isaacson et al. 2018; Duarte Campos et al. 2019). ARPE-19 and Y79 cells were printed as two subsequent monolayers on each other using a microvalve-based bioprinter. The evenly distributed cells formed a high-quality monolayer in 2 weeks and were efficient and accurate as in vitro model for realistic prediction of pathological responses and mechanisms to human diseases (Shi et al. 2018).

9.4.7 Ears

The initial studies demonstrated bioengineering of outer ear using cell-laden hydrogel on a 3D printed ear of poly-caprolactone (PCL). The adipose derived stem cells were differentiated into chondrocytes and adipocytes and mixed with the hydrogel. This cell laden hydrogel further applied into the cartilage and fat regions of the 3D printed ear constructs. The study concluded that 3D printing technology can regenerate complex shapes using two different types of cells (Lee et al. 2014). In a recent study, a bionic ear was developed by merging cellular and nanoelectronic functionalities via 3D printing. The bionic ear was fabricated in the shape of the human ear with a computer-aided design via printing using bioink comprising cells, conductive polymers, and silver nanoparticles. The printing allowed cartilage to grow on an inductive coil antenna, which facilitates cochlea-shaped electrodes toward retrieval of inductively coupled signals. Ultimately, the developed ear demonstrated improvement in auditory sensing for radio frequency and stereo audio music (Mannoor et al. 2013).

9.5 Conclusion

As the world grapples through the pandemic, one of the lessons learned for the human race is that finding solutions reliably and fast is the need of the "New Normal." From the COVID vaccine to sterilized fabrics for masks, the last 2 years

have provided more innovations than the last two decades, proving the age-old adage "Necessity is the Mother of Invention."

3D printing provides an unmatchable platform for rapid testing and verification of biomedical innovations. The advantage of 3D printing is being interdisciplinary in nature, cross functionalities can be introduced and adopted very quickly for up-gradation and quick innovation. However, this can be baneful too as overlapping or repeated innovations can raise infringement issues, primarily because this sector is still unorganized and in severe need of organized database development as well as technology archiving. Making this technology available at every medical center and working seamlessly with surgeons to test concepts is the urgent need of the hour. Minimum human interventions and easy usage empowering everyone to utilize this technology would improve market penetration and accessibility. In this regard, integrating the Internet of Things (IoT) as well as AI-ML in 3D printing would enable seamless integration with technology as well as develop next-generation "Smart Printers." The "Holy Grail" for 3D printing would be reaching a technological level when "Thinking and Printing" becomes a reality, where the designs conceived in mind could be directly in use in a matter of minutes. After all, we live in a world where we are limited by our imagination, and everything is fiction till it becomes a reality.

References

Admane P et al (2019) Direct 3D bioprinted full-thickness skin constructs recapitulate regulatory signaling pathways and physiology of human skin. Bioprinting 15:e00051. https://doi.org/10.1016/j.bprint.2019.e00051

Ahn BY et al (2009) Omnidirectional printing of flexible, stretchable, and spanning silver microelectrodes. Science 323(5921):1590–1593. https://doi.org/10.1126/science.1168375

Akkineni AR et al (2015) 3D plotting of growth factor loaded calcium phosphate cement scaffolds. Acta Biomater 27:264–274. https://doi.org/10.1016/j.actbio.2015.08.036. England

Albanna M et al (2019) In situ bioprinting of autologous skin cells accelerates wound healing of extensive excisional full-thickness wounds. Sci Rep 9(1):1856. https://doi.org/10.1038/s41598-018-38366-w

Alge DL, Chu T-MG (2010) Calcium phosphate cement reinforcement by polymer infiltration and in situ curing: a method for 3D scaffold reinforcement. J Biomed Mater Res A 94(2):547–555. https://doi.org/10.1002/jbm.a.32742. United States

Amini AR, Laurencin CT, Nukavarapu SP (2012) Bone tissue engineering: recent advances and challenges. Crit Rev Biomed Eng 40(5):363–408. https://doi.org/10.1615/CritRevBiomedEng.v40.i5.10

Arefin AME et al (2021) Polymer 3D printing review: materials, process, and design strategies for medical applications. Polymers 13(9):1499. https://doi.org/10.3390/polym13091499. MDPI

Bahcecioglu G et al (2019) A 3D printed {PCL}/hydrogel construct with zone-specific biochemical composition mimicking that of the meniscus. Biofabrication 11(2):25002. https://doi.org/10.1088/1758-5090/aaf707. {IOP} Publishing

Beluze L, Bertsch A, Renaud P (1999) Microstereolithography: a new process to build complex 3D objects. In: Courtois B et al (eds) {SPIE} Proceedings. SPIE. https://doi.org/10.1117/12.341277

Bertsch A et al (2000) Rapid prototyping of small size objects. Rapid Prototyp J 6(4):259–266. https://doi.org/10.1108/13552540010373362. MCB UP Ltd

Billiet T et al (2012) A review of trends and limitations in hydrogel-rapid prototyping for tissue engineering. Biomaterials 33(26):6020–6041. https://doi.org/10.1016/j.biomaterials.2012.04.050. Netherlands

Billiet T et al (2014) The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. Biomaterials 35(1):49–62. https://doi.org/10.1016/j.biomaterials.2013.09.078. Netherlands

Blaeser A et al (2016) Controlling shear stress in 3D bioprinting is a key factor to balance printing resolution and stem cell integrity. Adv Healthc Mater 5(3):326–333. https://doi.org/10.1002/adhm.201500677

Bohandy J, Kim BF, Adrian FJ (1986) Metal deposition from a supported metal film using an excimer laser. J Appl Phys 60(4):1538–1539. https://doi.org/10.1063/1.337287

Boland T et al (2007) Drop-on-demand printing of cells and materials for designer tissue constructs. Mater Sci Eng C 27(3):372–376. https://doi.org/10.1016/j.msec.2006.05.047

Bose S, Roy M, Bandyopadhyay A (2012) Recent advances in bone tissue engineering scaffolds. Trends Biotechnol 30(10):546–554. https://doi.org/10.1016/j.tibtech.2012.07.005. Elsevier Ltd

Catros S et al (2011) Effect of laser energy, substrate film thickness and bioink viscosity on viability of endothelial cells printed by Laser-Assisted Bioprinting. Appl Surf Sci 257(12):5142–5147. https://doi.org/10.1016/j.apsusc.2010.11.049

Chahal D, Ahmadi A, Cheung KC (2012) Improving piezoelectric cell printing accuracy and reliability through neutral buoyancy of suspensions. Biotechnol Bioeng 109(11):2932–2940. https://doi.org/10.1002/bit.24562

Chen Z et al (2019) 3D printing of ceramics: a review. J Eur Ceram Soc 39(4):661–687. https://doi.org/10.1016/j.jeurceramsoc.2018.11.013

Choi JS et al (2009) Development of micro-stereolithography technology using a UV lamp and optical fiber. Int J Adv Manuf Technol 41(3–4):281–286. https://doi.org/10.1007/s00170-008-1461-1

Chua CK, Leong KF (2015) 3D printing and additive manufacturing: principles and applications, 5th edn. World Scientific Publishing Co. https://doi.org/10.1142/10200

Collins AM et al (2009) Bone-like resorbable silk-based scaffolds for load-bearing osteoregenerative applications. Adv Mater 21(1):75–78. https://doi.org/10.1002/adma.200802239. WILEY-VCH Verlag

Cui X et al (2010) Cell damage evaluation of thermal inkjet printed Chinese hamster ovary cells. Biotechnol Bioeng 106(6):963–969. https://doi.org/10.1002/bit.22762. United States

Dadhich P et al (2016) A simple approach for an eggshell-based 3D-printed osteoinductive multiphasic calcium phosphate scaffold. ACS Appl Mater Interfaces 8(19):11910–11924. https://doi.org/10.1021/acsami.5b11981. American Chemical Society

Dadhich P et al (2021) Direct 3D printing of seashell precursor toward engineering a multiphasic calcium phosphate bone graft. ACS Biomater Sci Eng 7(8):3806–3820. https://doi.org/10.1021/acsbiomaterials.1c00303. American Chemical Society

de Gruijl FR, van Kranen HJ, Mullenders LHF (2001) UV-induced DNA damage, repair, mutations and oncogenic pathways in skin cancer. J Photochem Photobiol B Biol 63(1):19–27. https://doi.org/10.1016/S1011-1344(01)00199-3

Derr K et al (2019) Fully three-dimensional bioprinted skin equivalent constructs with validated morphology and barrier function. Tissue Eng Part C Methods 25(6):334–343. https://doi.org/10.1089/ten.tec.2018.0318

Do A-V et al (2015) 3D printing of scaffolds for tissue regeneration applications. Adv Healthc Mater 4(12):1742–1762. https://doi.org/10.1002/adhm.201500168

Dorozhkin SV (2011) Calcium orthophosphates: occurrence, properties, biomineralization, pathological calcification and biomimetic applications. Biomatter 1:121–164. https://doi.org/10.4161/biom.18790. United States

Duarte Campos DF et al (2019) Corneal bioprinting utilizing collagen-based bioinks and primary human keratocytes. J Biomed Mater Res A 107(9):1945–1953. https://doi.org/10.1002/jbm.a.36702

Ducheyne P, Qiu Q (1999) Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function. Biomaterials 20(23–24):2287–2303. https://doi.org/10.1016/S0142-9612(99)00181-7

Egan PF (2019) Integrated design approaches for 3D printed tissue scaffolds: review and outlook. Materials 12(15):2355. https://doi.org/10.3390/ma12152355

El-Ghannam A (2005) Bone reconstruction: from bioceramics to tissue engineering. Expert Rev Med Devices 2(1):87–101. https://doi.org/10.1586/17434440.2.1.87. England

England S et al (2017) Bioprinted fibrin-factor XIII-hyaluronate hydrogel scaffolds with encapsulated Schwann cells and their in vitro characterization for use in nerve regeneration. Bioprinting 5:1–9. https://doi.org/10.1016/j.bprint.2016.12.001

Fox K et al (2020) High nanodiamond content-PCL composite for tissue engineering scaffolds. Nanomaterials 10(5):948. https://doi.org/10.3390/nano10050948

Frone AN et al (2020) Morpho-structural, thermal and mechanical properties of PLA/PHB/cellulose biodegradable nanocomposites obtained by compression molding, extrusion, and 3D printing. Nanomaterials 10(1):51. https://doi.org/10.3390/nano10010051

Gao C et al (2014) Current progress in bioactive ceramic scaffolds for bone repair and regeneration. Int J Mol Sci 15(3):4714–4732. https://doi.org/10.3390/ijms15034714

Gao G et al (2015) Improved properties of bone and cartilage tissue from 3D inkjet-bioprinted human mesenchymal stem cells by simultaneous deposition and photocrosslinking in PEG-GelMA. Biotechnol Lett 37(11):2349–2355. https://doi.org/10.1007/s10529-015-1921-2

Garot C, Bettega G, Picart C (2021) Additive manufacturing of material scaffolds for bone regeneration: toward application in the clinics. Adv Funct Mater 31(5):2006967. https://doi.org/10.1002/adfm.202006967

Gauvin R et al (2012) Microfabrication of complex porous tissue engineering scaffolds using 3D projection stereolithography. Biomaterials 33(15):3824–3834. https://doi.org/10.1016/j.biomaterials.2012.01.048

Gibson I, Rosen DW, Stucker B (2010) Development of additive manufacturing technology. In: Additive manufacturing technologies: rapid prototyping to direct digital manufacturing. Springer US, Boston, pp 36–58. https://doi.org/10.1007/978-1-4419-1120-9_2

Gori M et al (2020) Biofabrication of hepatic constructs by 3D bioprinting of a cell-laden thermogel: an effective tool to assess drug-induced hepatotoxic response. Adv Healthc Mater 9(21):2001163. https://doi.org/10.1002/adhm.202001163

Grigoryan B et al (2019) Multivascular networks and functional intravascular topologies within biocompatible hydrogels. Science 364(6439):458–464. https://doi.org/10.1126/science.aav9750

Gu Q et al (2016) Functional 3D neural mini-tissues from printed gel-based bioink and human neural stem cells. Adv Healthc Mater 5(12):1429–1438. https://doi.org/10.1002/adhm.201600095

Gudapati H et al (2014) Alginate gelation-induced cell death during laser-assisted cell printing. Biofabrication 6(3):35022. https://doi.org/10.1088/1758-5082/6/3/035022. {IOP} Publishing

Guillemot F et al (2010) High-throughput laser printing of cells and biomaterials for tissue engineering. Acta Biomater 6(7):2494–2500. https://doi.org/10.1016/j.actbio.2009.09.029

Guillotin B et al (2010) Laser assisted bioprinting of engineered tissue with high cell density and microscale organization. Biomaterials 31(28):7250–7256. https://doi.org/10.1016/j.biomaterials.2010.05.055

Hodásová L et al (2021) Polymer infiltrated ceramic networks with biocompatible adhesive and 3D-printed highly porous scaffolds. Addit Manuf 39:101850. https://doi.org/10.1016/j.addma.2021.101850

Hopp B et al (2012) Femtosecond laser printing of living cells using absorbing film-assisted laser-induced forward transfer. Opt Eng 51(1):1–7. https://doi.org/10.1117/1.OE.51.1.014302

Hu Y et al (2016) 3D-engineering of cellularized conduits for peripheral nerve regeneration. Sci Rep 6(1):32184. https://doi.org/10.1038/srep32184

Huang J et al (2021) 3D printed gelatin/hydroxyapatite scaffolds for stem cell chondrogenic differentiation and articular cartilage repair. Biomater Sci 9(7):2620–2630. https://doi.org/10.1039/D0BM02103B. The Royal Society of Chemistry

Inzana JA et al (2014) 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. Biomaterials 35(13):4026–4034. https://doi.org/10.1016/j.biomaterials.2014.01.064. Elsevier Ltd

Isaacson A, Swioklo S, Connon CJ (2018) 3D bioprinting of a corneal stroma equivalent. Exp Eye Res 173:188–193. https://doi.org/10.1016/j.exer.2018.05.010

Jang J, Yi H-G, Cho D-W (2016) 3D printed tissue models: present and future. ACS Biomater Sci Eng 2(10):1722–1731. https://doi.org/10.1021/acsbiomaterials.6b00129. American Chemical Society

Jang T et al (2018) 1. 3D printing of hydrogel composite systems-recent advances in technology for tissue engineering. p 1–28. http://ijb.whioce.com/index.php/int-j-bioprinting/article/view/126/pdf

John MJ et al (2021) Design and development of cellulosic bionanocomposites from forestry waste residues for 3D printing applications. Materials 14(13):3462. https://doi.org/10.3390/ma14133462

Kankala RK et al (2018) 3D-printing of microfibrous porous scaffolds based on hybrid approaches for bone tissue engineering. Polymers 10(7):807. https://doi.org/10.3390/polym10070807

Kim JA et al (2015) Collagen-based brain microvasculature model in vitro using three-dimensional printed template. Biomicrofluidics 9(2):24115. https://doi.org/10.1063/1.4917508

Kim HS et al (2019) Advanced drug delivery systems and artificial skin grafts for skin wound healing. Adv Drug Deliv Rev 146:209–239. https://doi.org/10.1016/j.addr.2018.12.014

Kim J-W et al (2020) Bone regeneration capability of 3D printed ceramic scaffolds. Int J Mol Sci 21(14):4837. https://doi.org/10.3390/ijms21144837

Koch L et al (2010) Laser printing of skin cells and human stem cells. Tissue Eng Part C Methods 16(5):847–854. https://doi.org/10.1089/ten.tec.2009.0397

Kołbuk D et al (2020) Novel 3D hybrid nanofiber scaffolds for bone regeneration. Polymers 12(3):544. https://doi.org/10.3390/polym12030544

Kolesky DB et al (2014) 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. Adv Mater 26(19):3124–3130. https://doi.org/10.1002/adma.201305506

Koons GL et al (2021) Effect of 3D printing temperature on bioactivity of bone morphogenetic protein-2 released from polymeric constructs. Ann Biomed Eng 49(9):2114–2125. https://doi.org/10.1007/s10439-021-02736-9

Landers R, Mülhaupt R (2000) Desktop manufacturing of complex objects, prototypes and biomedical scaffolds by means of computer-assisted design combined with computer-guided 3D plotting of polymers and reactive oligomers. Macromol Mater Eng 282:17–21

Lawlor KT et al (2021) Cellular extrusion bioprinting improves kidney organoid reproducibility and conformation. Nat Mater 20(2):260–271. https://doi.org/10.1038/s41563-020-00853-9

Lee J-S et al (2014) 3D printing of composite tissue with complex shape applied to ear regeneration. Biofabrication 6(2):24103. https://doi.org/10.1088/1758-5082/6/2/024103. {IOP} Publishing

Lee H et al (2017) Development of liver decellularized extracellular matrix bioink for three-dimensional cell printing-based liver tissue engineering. Biomacromolecules 18(4):1229–1237. https://doi.org/10.1021/acs.biomac.6b01908. American Chemical Society

Lee J et al (2020) Bone-derived dECM/alginate bioink for fabricating a 3D cell-laden mesh structure for bone tissue engineering. Carbohydr Polym 250:116914. https://doi.org/10.1016/j.carbpol.2020.116914

Leukers B et al (2005) Hydroxyapatite scaffolds for bone tissue engineering made by 3D printing. J Mater Sci Mater Med 16(12):1121–1124. https://doi.org/10.1007/s10856-005-4716-5. United States

Li J et al (2016) Recent advances in bioprinting techniques: approaches, applications and future prospects. J Transl Med 14(1):271. https://doi.org/10.1186/s12967-016-1028-0

Lim KS et al (2018) Bio-resin for high resolution lithography-based biofabrication of complex cell-laden constructs. Biofabrication 10(3):34101. https://doi.org/10.1088/1758-5090/aac00c. England

Liu L et al (2009) A novel osteochondral scaffold fabricated via multi-nozzle low-temperature deposition manufacturing. J Bioact Compat Polym. https://doi.org/10.1177/0883911509102347

Liu C et al (2019) Low-temperature deposition manufacturing: a versatile material extrusion-based 3D printing technology for fabricating hierarchically porous materials. J Nanomater 2019: 1291067. https://doi.org/10.1155/2019/1291067. Edited by A. Nasibulin. Hindawi

Lorber B, Hsiao W-K, Martin KR (2016) Three-dimensional printing of the retina. Curr Opin Ophthalmol 27(3):262–267. https://journals.lww.com/co-ophthalmology/Fulltext/2016/05000/Three_dimensional_printing_of_the_retina.13.aspx

Lovell LG et al (2001) The effect of cure rate on the mechanical properties of dental resins. Dent Mater 17(6):504–511. https://doi.org/10.1016/S0109-5641(01)00010-0

Luo Y et al (2013) Well-ordered biphasic calcium phosphate–alginate scaffolds fabricated by multi-channel 3D plotting under mild conditions. J Mater Chem B 1(33):4088. https://doi.org/10.1039/c3tb20511h

Ma PX (2004) Scaffolds for tissue fabrication. Mater Today 7(5):30–40. https://doi.org/10.1016/S1369-7021(04)00233-0

Ma X et al (2016) Deterministically patterned biomimetic human iPSC-derived hepatic model via rapid 3D bioprinting. Proc Natl Acad Sci 113(8):2206–2211. https://doi.org/10.1073/PNAS.1524510113. National Academy of Sciences

Ma H et al (2018) 3D-printed bioceramic scaffolds: from bone tissue engineering to tumor therapy. Acta Biomater 79:37–59. https://doi.org/10.1016/j.actbio.2018.08.026

Mabrouk M, Beherei HH, Das DB (2020) Recent progress in the fabrication techniques of 3D scaffolds for tissue engineering. Mater Sci Eng C Mater Biol Appl 110:110716. https://doi.org/10.1016/j.msec.2020.110716. Netherlands

Mannoor MS et al (2013) 3D printed bionic ears. Nano Lett 13(6):2634–2639. https://doi.org/10.1021/nl4007744. American Chemical Society

Mao Q et al (2020) Fabrication of liver microtissue with liver decellularized extracellular matrix (dECM) bioink by digital light processing (DLP) bioprinting. Mater Sci Eng C 109:110625. https://doi.org/10.1016/j.msec.2020.110625

Mei J, Lovell MR, Mickle MH (2005) Formulation and processing of novel conductive solution inks in continuous inkjet printing of 3-D electric circuits. IEEE Trans Electron Packag Manuf 28(3):265–273. https://doi.org/10.1109/TEPM.2005.852542

Melchels FPW, Feijen J, Grijpma DW (2010) A review on stereolithography and its applications in biomedical engineering. Biomaterials 31(24):6121–6130. https://doi.org/10.1016/j.biomaterials.2010.04.050

Michael S et al (2013) Tissue engineered skin substitutes created by laser-assisted bioprinting form skin-like structures in the dorsal skin fold chamber in mice. PLoS One 8(3):1–12. https://doi.org/10.1371/journal.pone.0057741. Public Library of Science

Mironov V, Kasyanov V, Markwald RR (2011) Organ printing: from bioprinter to organ biofabrication line. Curr Opin Biotechnol 22(5):667–673. https://doi.org/10.1016/j.copbio.2011.02.006

Müller P et al (2008) Calcium phosphate surfaces promote osteogenic differentiation of mesenchymal stem cells. J Cell Mol Med 12(1):281–291. https://doi.org/10.1111/j.1582-4934.2007.00103.x

Nakamura M et al (2005) Biocompatible inkjet printing technique for designed seeding of individual living cells. Tissue Eng 11(11–12):1658–1666. https://doi.org/10.1089/ten.2005.11.1658. United States

Neufurth M et al (2017) 3D printing of hybrid biomaterials for bone tissue engineering: calcium-polyphosphate microparticles encapsulated by polycaprolactone. Acta Biomater 64:377–388. https://doi.org/10.1016/j.actbio.2017.09.031

Nguyen AK, Narayan RJ (2017) Two-photon polymerization for biological applications. Mater Today 20(6):314–322. https://doi.org/10.1016/j.mattod.2017.06.004
Ni T et al (2020) 3D bioprinting of bone marrow mesenchymal stem cell-laden silk fibroin double network scaffolds for cartilage tissue repair. Bioconjug Chem 31(8):1938–1947. https://doi.org/10.1021/acs.bioconjchem.0c00298. American Chemical Society
Ning L, Chen X (2017) A brief review of extrusion-based tissue scaffold bio-printing. Biotechnol J 12(8):1600671. https://doi.org/10.1002/biot.201600671
Odde DJ, Renn MJ (1999) Laser-guided direct writing for applications in biotechnology. Trends Biotechnol 17(10):385–389. https://doi.org/10.1016/S0167-7799(99)01355-4
Oladapo BI, Zahedi SA, Adeoye AOM (2019) 3D printing of bone scaffolds with hybrid biomaterials. Compos Part B Eng 158:428–436. https://doi.org/10.1016/j.compositesb.2018.09.065
Ong CS et al (2018) 3D bioprinting using stem cells. Pediatr Res 83(1–2):223–231. https://doi.org/10.1038/pr.2017.252. Nature Publishing Group
Ouyang L et al (2015) 3D printing of HEK 293FT cell-laden hydrogel into macroporous constructs with high cell viability and normal biological functions. Biofabrication 7(1):15010. https://doi.org/10.1088/1758-5090/7/1/015010. England
Özcan M et al (2021) Materials and manufacturing techniques for polymeric and ceramic scaffolds used in implant dentistry. J Compos Sci 5(3):78. https://doi.org/10.3390/jcs5030078
Park SH et al (2012) Scaffolds for bone tissue engineering fabricated from two different materials by the rapid prototyping technique: PCL versus PLGA. J Mater Sci Mater Med 23(11):2671–2678. https://doi.org/10.1007/s10856-012-4738-8
Park JY et al (2015) 3D printing technology to control BMP-2 and VEGF delivery spatially and temporally to promote large-volume bone regeneration. J Mater Chem B 3(27):5415–5425. https://doi.org/10.1039/C5TB00637F. The Royal Society of Chemistry
Peña J, Vallet-Regı M (2003) Hydroxyapatite, tricalcium phosphate and biphasic materials prepared by a liquid mix technique. J Eur Ceram Soc 23(10):1687–1696. https://doi.org/10.1016/S0955-2219(02)00369-2
Pfister A et al (2004) Biofunctional rapid prototyping for tissue-engineering applications: 3D bioplotting versus 3D printing. J Polym Sci A Polym Chem 42(3):624–638. https://doi.org/10.1002/pola.10807
Rimann M et al (2016) Standardized 3D bioprinting of soft tissue models with human primary cells. J Lab Autom 21(4):496–509. https://doi.org/10.1177/2211068214567146
Roohani-Esfahani S-I, Newman P, Zreiqat H (2016) Design and fabrication of 3D printed scaffolds with a mechanical strength comparable to cortical bone to repair large bone defects. Sci Rep 6(1):19468. https://doi.org/10.1038/srep19468
Ruiz-Cantu L et al (2020) Multi-material 3D bioprinting of porous constructs for cartilage regeneration. Mater Sci Eng C 109:110578. https://doi.org/10.1016/j.msec.2019.110578
Russmueller G et al (2015) 3D printable biophotopolymers for in vivo bone regeneration. Materials 8(6):3685–3700. https://doi.org/10.3390/ma8063685
Saunders RE, Gough JE, Derby B (2008) Delivery of human fibroblast cells by piezoelectric drop-on-demand inkjet printing. Biomaterials 29(2):193–203. https://doi.org/10.1016/j.biomaterials.2007.09.032
Setti L et al (2004) Thermal inkjet technology for the microdeposition of biological molecules as a viable route for the realization of biosensors. Anal Lett 37(8):1559–1570. https://doi.org/10.1081/AL-120037587. Taylor & Francis
Seyednejad H et al (2011) Functional aliphatic polyesters for biomedical and pharmaceutical applications. J Control Release 152(1):168–176. https://doi.org/10.1016/j.jconrel.2010.12.016
Shi P et al (2018) A bilayer photoreceptor-retinal tissue model with gradient cell density design: a study of microvalve-based bioprinting. J Tissue Eng Regen Med 12(5):1297–1306. https://doi.org/10.1002/term.2661

Shie M-Y et al (2017) 3D printing of cytocompatible water-based light-cured polyurethane with hyaluronic acid for cartilage tissue engineering applications. Materials 10(2):136. https://doi.org/10.3390/ma10020136

Sinha RP, Häder DP (2002) UV-induced DNA damage and repair: a review. Photochem Photobiol Sci 1(4):225–236. https://doi.org/10.1039/b201230h. England

Skardal A et al (2012) Bioprinted amniotic fluid-derived stem cells accelerate healing of large skin wounds. Stem Cells Transl Med 1(11):792–802. https://doi.org/10.5966/sctm.2012-0088

Srivas PK et al (2017) Osseointegration assessment of extrusion printed Ti6Al4V scaffold towards accelerated skeletal defect healing via tissue in-growth. Bioprinting 6:8–17. https://doi.org/10.1016/j.bprint.2017.04.002. Elsevier B.V.

Sun C et al (2005) Projection micro-stereolithography using digital micro-mirror dynamic mask. Sensors Actuators A Phys 121(1):113–120. https://doi.org/10.1016/j.sna.2004.12.011

Sun J et al (2009) Comparison of micro-dispensing performance between micro-valve and piezo-electric printhead. Microsyst Technol 15(9):1437–1448. https://doi.org/10.1007/s00542-009-0905-3

Suo H et al (2021) Low-temperature 3D printing of collagen and chitosan composite for tissue engineering. Mater Sci Eng C 123:111963. https://doi.org/10.1016/j.msec.2021.111963

Tartarisco G et al (2009) Polyurethane unimorph bender microfabricated with Pressure Assisted Microsyringe (PAM) for biomedical applications. Mater Sci Eng C 29(6):1835–1841. https://doi.org/10.1016/j.msec.2009.02.017

Unagolla JM, Jayasuriya AC (2020) Hydrogel-based 3D bioprinting: a comprehensive review on cell-laden hydrogels, bioink formulations, and future perspectives. Appl Mater Today 18:100479. https://doi.org/10.1016/j.apmt.2019.100479

Vallet-Regi M, Arcos Navarrete DA (2008) Biomimetic nanoceramics in clinical use: from materials to application, 1st edn. RSC Publishing, The Royal Society of Chemistry (RSC Nanoscience & Nanotechnology), Cambridge. https://doi.org/10.1039/9781847558923

Vozzi G et al (2002) Microsyringe-based deposition of two-dimensional and three-dimensional polymer scaffolds with a well-defined geometry for application to tissue engineering. Tissue Eng 8(6):1089–1098. https://doi.org/10.1089/107632702320934182. United States

Wang Z et al (2015) A simple and high-resolution stereolithography-based 3D bioprinting system using visible light crosslinkable bioinks. Biofabrication 7(4):45009. https://doi.org/10.1088/1758-5090/7/4/045009. {IOP} Publishing

Wang X et al (2017) 3D printing of polymer matrix composites: a review and prospective. Compos Part B Eng 110:442–458. https://doi.org/10.1016/j.compositesb.2016.11.034

Watters MP, Bernhardt ML (2018) Curing parameters to improve the mechanical properties of stereolithographic printed specimens. Rapid Prototyp J 24(1):46–51. https://doi.org/10.1108/RPJ-11-2016-0180. Emerald Publishing Limited

Wegst UGK et al (2014) Bioinspired structural materials. Nat Mater 14:23–36. https://doi.org/10.1038/nmat4089. Nature Publishing Group

Weiß T et al (2009) Two-Photon polymerization for microfabrication of three-dimensional scaffolds for tissue engineering application. Eng Life Sci 9(5):384–390. https://doi.org/10.1002/elsc.200900002

Williams EMSSHJCA (2003) United States Patent (19)

Wu Y et al (2018) 3D bioprinting of liver-mimetic construct with alginate/cellulose nanocrystal hybrid bioink. Bioprinting 9:1–6. https://doi.org/10.1016/j.bprint.2017.12.001

Wu Z et al (2020) In vitro and in vivo biocompatibility evaluation of a 3D bioprinted gelatin-sodium alginate/rat Schwann-cell scaffold. Mater Sci Eng C 109:110530. https://doi.org/10.1016/j.msec.2019.110530

Wu Y, Simpson MC, Jin J (2021) Fast hydrolytically degradable 3D printed object based on aliphatic polycarbonate thiol-yne photoresins. Macromol Chem Phys 222(6):2000435. https://doi.org/10.1002/macp.202000435

Xu C et al (2014) Study of droplet formation process during drop-on-demand inkjetting of living cell-laden bioink. Langmuir 30(30):9130–9138. https://doi.org/10.1021/la501430x. American Chemical Society

Yan Q et al (2018) A review of 3D printing technology for medical applications. Engineering 4(5): 729–742. https://doi.org/10.1016/j.eng.2018.07.021. Chinese Academy of Engineering

Yang H et al (2021) Three-dimensional bioprinted hepatorganoids prolong survival of mice with liver failure. Gut 70(3):567–574. https://doi.org/10.1136/gutjnl-2019-319960. BMJ Publishing Group

Yankov E, Nikolova MP (2017) Comparison of the accuracy of 3D printed prototypes using the stereolithography (SLA) method with the digital CAD models. MATEC Web Conf 137:2014. https://doi.org/10.1051/matecconf/201713702014

Yilgor P et al (2008) 3D plotted PCL scaffolds for stem cell based bone tissue engineering. Macromol Symp 269(1):92–99. https://doi.org/10.1002/masy.200850911

Yuan H, De Groot K (2004) Calcium phosphate biomaterial: an overview. In: Reis RL, Weiner S (eds) Learning from nature how to design implantable biomaterials. Kluwer Academic Publishers, Netherlands, pp 37–57

Zaaba NF, Jaafar M (2020) A review on degradation mechanisms of polylactic acid: hydrolytic, photodegradative, microbial, and enzymatic degradation. Polym Eng Sci 60(9):2061–2075. https://doi.org/10.1002/pen.25511

Zaszczyńska A et al (2021) Advances in 3D printing for tissue engineering. Materials 14(12):3149. https://doi.org/10.3390/ma14123149

Zhang Y, Zhang M (2001) Synthesis and characterization of macroporous chitosan/calcium phosphate composite scaffolds for tissue engineering. J Biomed Mater Res 55(3):304–312. http://www.ncbi.nlm.nih.gov/pubmed/11255183

Zhang J et al (2020a) Digital light processing based three-dimensional printing for medical applications. Int J Bioprint 6(1):12–27. https://doi.org/10.18063/ijb.v6i1.242

Zhang J et al (2020b) Optimization of mechanical stiffness and cell density of 3D bioprinted cell-laden scaffolds improves extracellular matrix mineralization and cellular organization for bone tissue engineering. Acta Biomater 114:307–322. https://doi.org/10.1016/j.actbio.2020.07.016

Zhou X, Hou Y, Lin J (2015) A review on the processing accuracy of two-photon polymerization. AIP Adv 5(3). https://doi.org/10.1063/1.4916886

Zhu W et al (2018) Rapid continuous 3D printing of customizable peripheral nerve guidance conduits. Mater Today 21(9):951–959. https://doi.org/10.1016/j.mattod.2018.04.001

Zwawi M (2021) A review on natural fiber bio-composites, surface modifications and applications. Molecules 26(2):404. https://doi.org/10.3390/molecules26020404

Adult Neurogenesis: A Potential Target for Regenerative Medicine

10

Manoj Kumar Eradath

10.1 History of Adult Neurogenesis

> "In adult. . . , the nerve paths are something fixed and immutable: . . . nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."—(Ramón y Cajal, 1913).

For long it was considered as an established fact in neuroscience that adult brain is incapable of creating new neurons (Koelliker 1896; Cajal 1899; Ramon y Cajal 1913; Leblond 1964). Technological limitations to accurately identify the "birthdates" of neurons helped to maintain this "central Dogma of neuroscience" (Gross 2000) for most part of twentieth century. It should be noted that the idea of neural plasticity, that is, the ability of the existing nerve cells to adapt to the changes in the environment by physiological, biochemical, and morphological changes, was a known an approved notion even from the early times of neuroscience. The debate was always on the idea whether an adult brain can generate new neurons as a mechanism of central nervous system (CNS) plasticity. Classical morphological characterizations using hematoxylin and Nissl stain techniques were not efficient in accurately tracking the development of precursor cells over time into distinct populations. However, the advancements in autoradiography techniques in late 1950s allowed selective and permanent tagging DNA of neuroblasts with radioactive Thymidine (Thymidine-H^3) and thus enabling the researchers to serially track the differentiation and migration of labeled cells (Angevine Jr. 1965). The detailed autoradiographic studies of 1960s subsequently started to provide early evidences for the generation of new neurons from precursor neural stem cells in adult brain

M. K. Eradath (✉)
Princeton Neuroscience Institute, Princeton University, Princeton, NJ, USA
e-mail: meradath@princeton.edu

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_10

(Altman and Das 1965; Angevine Jr. 1965). While studying the glial proliferation in retinogeniculate area by infusing radiolabeled thymidine (precursors of DNA), Altman found that many cells that incorporated radiolabeled Thymidine were in fact morphologically similar to neurons, and such newly formed neurons were specific to the granule cell layer of Dentate Gyrus (DG) area of hippocampus. The studies opened the whole exciting field of adult neurogenesis and the discussions on the potential functional importance of generation of new neurons in adult brains and the clinical possibilities of adult formed neurons in regenerative medicine. However, the technological limitations existed with the autoradiographic techniques in convincingly differentiating the labeled neurons from glia. The early autoradiographic studies used morphological features of the labeled cells to differentiate between newly formed neurons from glia. Subjective nature of the morphological classifications induced confusions and caused strong resistance from the classical neuroscientists who believed in the central dogma of non-replicative neurons, subsequently causing the entire topic of adult neurogenesis getting pushed to the outskirts of established neuroscience field (see Gross 2000 review for a detailed historic timeline of adult neurogenesis). Series of detailed autoradiographic studies in early 1980s in primate brains exploring all major brain structures failed to detect newly formed cells with the conclusive morphological characteristics of neurons in adult animals, further suggesting that the entire concept of adult neurogenesis, may in fact, was a possible experimental error (historical review, Rakic 2002). However, things again started to change in late 80s following the emergence of novel immunohistochemistry techniques, especially the synthetic thymidine analogue BrdU (5-bromo-3′-deoxyuridine) based immunohistochemical procedures and the emergence of cell-type specific markers gave a rebirth to the topic of adult neurogenesis by avoiding the need of autoradiography and by providing more accurate methods of identifying neurons in objective, non-morphological feature dependent manner. Immunohistochemistry in adult human hippocampus using newly introduced methods detected presence of neurogenesis in anatomically similar location and in numbers, in line with the early evidences from the rodent literature (Eriksson et al. 1998). Stem cells with neurogenic potential got isolated from the adult human hippocampus and immunohistochemistry studies showed the presence of markers specific to intermediate and immature neurons, providing further experimental evidences for adult neurogenesis (Kempermann et al. 2015, for detailed review). Numerous studies also started to show the functional relevance of adult neurogenesis, and the decreases and increases in the adult neurogenesis in response to environmental factors, reigniting the excitement of potential therapeutic potentials of adult neurogenesis (Gross 2000). However, good amounts of skepticism remain in the field regarding the extent, rate and functional relevance of the newly formed neurons, especially in higher primates and humans (Rakic 2002). The debate on the timeline of adult neurogenesis and its relevance is far from a settled notion. Most notably, a recent study (Sorrells et al. 2018) suggested neurogenesis in human dentate gyrus sharply drops by childhood and rarely continue in adult DG, a finding sharply contrasting the findings of continued neurogenesis in adult DG in other

mammals like rodents, thereby reigniting the fundamental questions around the notion and timing of adult neurogenesis, especially from a human clinical perspective. However, another study which came in the very same year provided a totally opposite finding, that neurogenesis persists in adult human brain (Boldrini et al. 2018), discussing the need of careful considerations on the specific timeline, quantitative analysis techniques, and the functional relevance of the adult neurogenesis. A review which looked at the techniques presented on the contrasting 2018 papers suggested that the negative neurogenesis finding in Sorrells et al. study may have been emerged from the non-optimal technical aspects used in the study like the delay in sample preparation which may have caused the diminished detection of marker proteins (Kempermann et al. 2018). A detailed metanalysis which compared timelines and rates of adult neurogenesis across multiple species, factoring in the age of the samples and techniques used, suggested that the seemingly prolong neurogenesis in rodent samples compared to primate and human samples can be understood if we consider the differences in neurodevelopmental timings, ages of sexual maturity and the overall lifespan of subjects used in different studies (Snyder 2019). The metanalysis showed comparable dentate gyrus neurogenesis during the conception to pre-birth windows and birth to sexual maturity windows across multiple species (Fig. 10.2, Snyder 2019 review), indicating that there is more alignment in terms of timing and extent of adult neurogenesis if the overall lifespan and developmental milestones are taken into consideration. However, the functional roles of newly formed neurons are still an outstanding question, especially if we consider diverse but specific foci of neurogenesis across the species.

10.2 Factors Influencing Adult Neurogenesis

Although the anatomical extent and the rate and the timeline of neurogenesis in adult brain are still ongoing research topics, it is now an accepted fact that new neurons form in subventricular zone (SVZ) of lateral ventricles and in subgranular zone of DG of adult hippocampus, across species (Gould 2007). Olfactory bulb is another important area where neurogenesis has been observed across multiple species. Adult neurogenesis in birds has extensively been studied and is shown to be different from mammalian species. While neurogenesis is limited in time and location for mammals, new neurons are added throughout most of the avian telencephalon, providing some form of plasticity to avian forebrains. Furthermore, the magnitude of neurogenesis in the avian brain is significantly higher than that seen in mammalian brains. In birds, 0.1–0.7% of all high vocal center (HVC)—an area homologous to Broca's area for speech control in human frontal lobe—neurons and 0.15–0.37% of hippocampal neurons are newly recruited per day on average while only about 0.02% of total hippocampal granule cells in mature macaque monkeys are generated per day, thereby providing evidences for species specific differences in the extent and possibly the functional relevance of adult neurogenesis (Brenowitz et al. 2015). Studies have shown conclusively that the process of neurogenesis respond to multiple external or internal factors like environmental enrichment, aging, local or

general inflammatory factors. The neural progenitor cells, first formed in sub-granular zone (SGZ) of dentate gyrus, subsequently migrate to granular cells layer to form the Dentate Granular Cells (DGC) which further project axons and make connections with CA3 region of hippocampus (Hastings and Gould 1999; Ming and Song 2005; Oomen et al. 2009; Snyder 2019). The development and maturation of neurons in DG follow multiple highly regulated steps starting from Type-1 radial glia-like cells (RGLs) population of DG neural stem cells (NSC) to the mature Dentate Granule neurons (DGN) (Gonçalves et al. 2016). RGLs under internal or external influencing factors get activated and undergo symmetric or asymmetric divisions. Under symmetric divisions RGL divides into 2 RGLs while asymmetric division leads to generation of astrocyte + RGL combination or RGL + neural progenitor combination (Ghosh 2019). The neural progenitor cells first receive GABAergic inputs followed by appearance of dendritic spines facilitating the excitatory transmission in 2–3 weeks of time. The experience dependent dendritic modifications occur during this stage and are mediated through glutaminergic inputs (Snyder 2019; Gonçalves et al. 2016). This stage of experience dependent modifications is also a critical survival phase and is susceptible to environmental factors like enrichment. The dendritic spine growth and modifications continue thereafter throughout the life time of adult formed neurons and the neurons follow a similar integration process with the rest of the existing circuitry (Ming and Song 2005; Snyder 2019). Deeper understanding of the intrinsic and extrinsic factors influencing the specific timeline and rate of neurogenesis can be critical in decoding the elusive functional roles of adult neurogenesis (Fig. 10.1).

Cell cycle transcription factors like E2F1, receptor tyrosine kinases like EphB1–3, EphA4, signaling pathways like the sonic hedgehog (SHH), adhesion molecules like neural cell adhesion molecule (NCAM) signaling pathway are among the key factors shown to be involved in regulating the timing and extent of neurogenesis (Cooper-Kuhn 2002; Conover 2000; McMahon 2003; Lledo et al. 2006). Molecular trophic factors are shown to exert mitogenic proliferative effects in adult neurogenesis and these include epidermal growth factor (EGF), bone morphogenetic protein (BMP), and glial fibrillary acidic protein (GFAP) (Abrous et al. 2005). Blockade of NMDA glutamate receptors are shown to increase the adult neurogenesis while Serotonin and nitric oxide upregulate the adult neurogenesis (Abrous et al. 2005; Lledo et al. 2006). Transcription factors like paired box 6 (PAX6) and oligodendrocyte transcription factor 2 (OLIG2) have shown to be playing potentially opposite roles in adult neurogenesis, with PAX6 promoting while the overexpression of OLIG2 resulting in the reduction in the rate of neurogenesis (Lledo et al. 2006). Other transcription factors like Repressor element 1-silencing transcription (REST), basic helix–loop–helix (bHLH) transcription factors, FoxOs, Tbr2, and CREB are also found to be critical for regulating adult neurogenesis. Epigenetic factors like histone modifiers, DNA methylase/demethylase, and microRNA, play significant roles in formation of new neurons in adult brain (Toda and Gage 2018). Adrenal corticosteroids, Gonadal hormones, especially the female gonadal hormones are studied in relation with the regulation of neurogenesis. Neurosteroids like dihydroxy epiandrosterone (DHEA) and

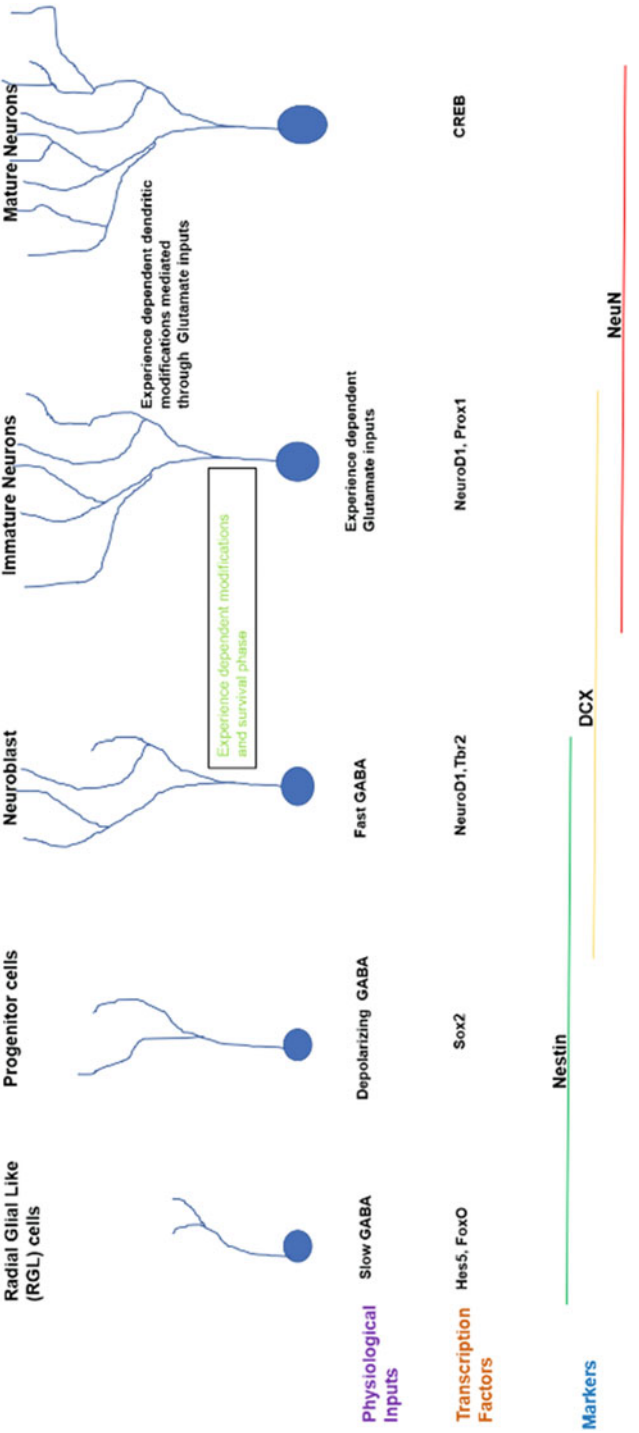


Fig. 10.1 Stages and critical players of adult neurogenesis

pregnenolone sulfate (Preg-S) facilitate neurogenesis by acting as allosteric antagonists of the $GABA_A$ receptors while allopregnanolone (AlloP), by acting as a positive $GABA_A$ receptor modulator, decrease neurogenesis in rodent DG and SVZ (Abrous et al. 2005; Lledo et al. 2006). In addition to the intrinsic factors, a variety of external factors are also known to be influencing and regulating the generation, migration, and maturation of the neurons in adult brain. Most notable external factors are physical activity, environmental enrichment, and contextual learning. These factors appear to enhance the neurogenesis, possibly through upregulation of neurotransmitters and recruitment of trophic factors (Abrous et al. 2005; Lledo et al. 2006; Toda and Gage 2018). Studies have shown increasing levels of brain-derived neurotrophic factor (BDNF) mRNA levels in the dentate gyrus within a few weeks and persistent expression of BDNF protein expression lasting several weeks. The increased BDNF expression has subsequently shown to be associated with enhancement in dentate gyrus neurogenesis and memory improvements. Furthermore, exercise when coupled with TrkB blocking (BDNF binds to tropomyosin receptor kinase B (TrkB) to affect the regulatory control over neurons), reduced the exercise-related dentate gyrus neurogenesis (Liu and Nusslock 2018). Increased neurogenesis has been observed following epilepsy, ischemia and traumatic injuries to brain. Rapid increases in neurogenesis have been observed following experimentally induced Traumatic brain injury (TBI) in mice models. However, following the initial enhancement, neurogenesis subsequently scales back to the preinjury levels and even falls below the preinjury levels indicating the potentially limited supply of neural progenitor cells and rate of adaptability of neurogenesis-mediated repair following acute injuries (Neuberger et al. 2017). Significant reductions in neurogenesis have been documented on neurodegenerative conditions like Huntington's disease, in affective disorders like major depression, and following substance abuse involving alcohol, opiates, nicotine, and cannabinoid (Abrous et al. 2005; Lledo et al. 2006; Toda and Gage 2018). The changes in adult neurogenesis in response to environmental stress factors appear to be the result of corticosteroid-related mechanisms mediated through NMDA receptor-mediated excitatory signaling pathways. However, it should be noted that the relationship between Corticosterone concentrations and the adult neurogenesis follow U-shaped dose–response relationship with complete removal of corticosterone and abnormally elevated concentrations (e.g., as in case of acute stress), both leading to significant cell loss, indicating the requirement of optimal levels of corticosterone (Hanson 2011). The environmental factors influencing neurogenesis have provided significant understandings on the potential functional implications of neurogenesis.

10.3 Anatomical and Physiological Properties of Adult Formed Neurons

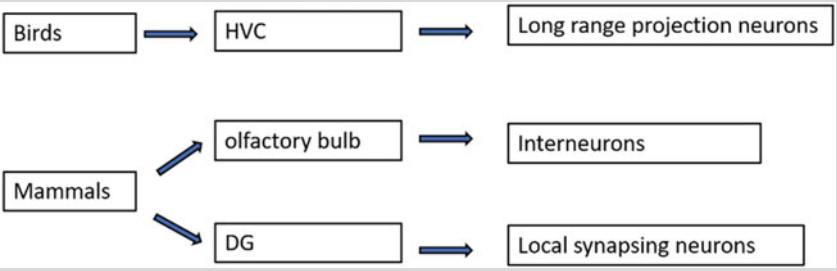
Recent immunohistochemical and electrophysiological studies on the adult formed neurons have provided extensive data on the morphological characteristics and the physiological properties of these newly formed neurons, giving insights on the

potential functional role of adult neurogenesis. Newly formed cells become more complex morphologically and functionally within the first few weeks after their birth. The specific process of maturation depends on the site of neurogenesis and the anatomical and functional inputs of the neurogenic focus.

10.3.1 Distinct Timeline of Evolution of Morphological Features in Adult Formed Neurons

Labeling studies using retroviral vectors showed distinct differences in the timeline of development of morphological features in adult formed neurons when compared with their neonatally born counterparts. The dendritic complexity and initiation of dendritic spine growth appear to be delayed by ~4 days in mice DG in comparison with the adult formed neurons. Given that the dendritic spines serve as the major receptive points for the glutamatergic inputs, the delayed dendritic spine formation subsequently leads to delays in physiological maturations (Zhao et al. 2006). Similarly, axonal growth was also found to be delayed in adult formed neurons. The mossy fiber axons of neonatally formed neurons migrate to positions more distal to the dentate compared adult neurons of similar duration from differentiation indicating a delayed or differential integration of adult formed neurons in the larger network from the site of origin (Zhao et al. 2006). However, it should be noted that, although the developmental trajectory of adult formed neurons is delayed, eventually it catches up to acquire features of developmentally born neurons. Neonatally born cells mature faster than adult-born neurons but then do not undergo additional dendritic growth beyond ~5–6 weeks of cell age. The delayed but extended developmental trajectory of adult formed neurons brings distinct morphological and functional properties providing critical plasticity features for the adult formed neurons. For example, the size of the cell soma and nucleus of adult formed neurons surpass that of neonatally formed neurons over the course development and integration. Over the course of lifespan, the adult-born neurons acquire greater dendritic spine density, larger presynaptic terminals, and more putative efferent filopodial contacts onto inhibitory neurons (Cole et al. 2020). The protracted morphological development timeline in the adult formed neurons may be critical in contributing the physiological basis for the neurogenesis-mediated plasticity within the hippocampal network. Species specificity is also striking while comparing the morphological characteristics of adult formed neurons between mammals and birds. Most of the new neurons added to the adult HVC in birds have long axons projecting 4 mm or more to synapse on target cells in RA nucleus while the newly formed neurons in the olfactory bulb of mammals are interneurons and those added to the DG are granule cells primarily involved in making local synapses (Brenowitz et al. 2015) (Box 10.1).

Box 10.1 Species Specific Differences of Adult Formed Neurons



10.3.2 Physiological Features of Adult Formed Neurons

In general, GABA-mediated synapses are the first formed connections followed by glutaminergic connections. The order of voltage-dependent currents during the maturation process and the specific sequence of synaptic connections formed decide the physiological properties of newly formed neurons from different sites of neurogenesis (Lledo et al. 2006). In periglomerular cells, the maturation of the voltage-dependent sodium current and the ability to generate fully formed action potential appear before the development of synaptic contacts while in newly formed cells of granular zone, the sodium current is generated only after the formation of extensive synaptic connections. The delayed development of action potential generation in adult formed neurons in DG possibly carries a functional importance by not disrupting the pre-formed circuits within the DG (Lledo et al. 2006). The neurogenesis in DG and the physiological properties of adult formed neurons in DG assumes special functional relevance given the causal role of hippocampus in the memory formation, consolidation and retrieval. It is interesting to note that only about 25% of adult formed DG neurons end up integrated in the circuit. This may appear as a waste of resources, but the distinct physiological features of immature neurons during the pathway of adult neurogenesis may be functionally contributing to the normal memory processes of brain even without eventually getting integrated to the circuit (Ghosh 2019). The smaller population of adult formed neurons which eventually get integrated to the circuit also show physiological characteristics that are distinct from the developmentally formed neighboring ones. Most notable features of the newly formed neurons are the enhanced excitability and the lower thresholds for Long-Term Potentiation (LTP) and Long-Term Depression (LTD)—two fundamental neural plasticity mechanisms. These neural plasticity-related features appear to be stemming from the presence of low-threshold T-type Ca^{2+} channels and the low expression of Ca^{2+} binding proteins. These two specific channel characteristics, in combination, effectively lead to the temporal summation of Ca^{2+} signals (Schmidt-Hieber et al. 2004; Ming and Song 2005). However, whether or not the adult formed neurons retain such enhanced plasticity throughout

the lifetime is still an active area of research (Ming and Song 2005; Lledo et al. 2006). While rodent studies have suggested long lasting plasticity in the adult formed neurons (Lemaire et al. 2012), extending such results to primate brain will require more extensive research (La Rosa et al. 2020). Studies using immediate early genes (IEGs) like *Fos*—expression of which is closely associated with the neuronal activity—have further shown distinct physiological properties of adult born neurons. The neuronal activity (in response to physiological events or spontaneous activity) did not induce IEG expression in adult-born neurons in DG up until about 3–4 weeks of age in mice. Interestingly, the IEG expression in adult-born neurons (once it is formed) to learning related and cognitively demanding physiological stimuli was found to be significantly more when compared with the developmentally formed neurons, findings that provide potential links between the protracted morphological development of adult-born neurons and the functional relevance of such neurons (Deng et al. 2010).

10.4 Functional Roles of Adult Neurogenesis

Some of the external factors influencing the extent and the rate of the adult neurogenesis as mentioned in the previous sections also provide evidences toward the potential functional roles of the adult neurogenesis. Evidences suggest that only about 0.004% of neurons are added each day in adult humans (Spalding et al. 2013), which in absolute terms, may appear as a small, functionally insignificant number, but if we consider the evidences suggesting that neurogenesis persists throughout the lifespan, the amount of newly formed neurons can translate to functionally relevant cell mass in hippocampus (Snyder 2019). Studies have shown the functional roles of adult neurogenesis in learning, memory, especially related to the flexibility of such cognitive tasks (Toda and Gage 2018).

10.4.1 Learning and Memory

Replacement of older neurons in the HVC of songbirds with newly formed neurons is shown to be critical in unlearning of older song patterns and acquisition of newer patters, providing a critical role of adult neurogenesis in learning and memory (Thorpe 1958; Nottebohm 1984). Rodent studies in late 1990s further extended the functional roles of adult neurogenesis to learning related to spatial cues and spatial navigation. In rodents, the number of adult formed neurons in DG significantly increased in response to training on tasks which demanded spatial association learnings involving hippocampal circuits (e.g., spatial water-maze training) while the number of adult formed neurons were not significantly different when rodents were trained with tasks like cue-maze training which didn't require hippocampal circuits (Gould et al. 1999). Studies attempted to experimentally manipulate critical components of adult neurogenesis pathways to assess the causal role of neurogenesis at specific anatomical locations and during stages of anatomical development and cognitive learning. Early studies with methyl azoxy methanol acetate (MAM)—an

antimitotic agent, have showed reduction in hippocampal neurogenesis and subsequent impairments with hippocampal-mediated contextual memory learning (Toda and Gage 2018). Selective perturbance of hippocampal neurogenesis by targeted X-ray irradiation or with the usage of transgenic animals further established the causal role of hippocampal neurogenesis with the formation and flexibility of contextual memory. The physiological properties of newly formed DG neurons, specifically the enhanced synaptic plasticity features, point toward its functional role in helping the network to effectively adapt to changes by providing flexibility within the learning networks. Such network level flexibility and adaptability may also be playing important functions when comes to processing and storing new information (Lledo et al. 2006). Computational models incorporating neurogenesis in DG have shown the improvements in recall function by reducing the interactions between previously stored memories and the newly formed ones—a key concept related to the meta-plasticity or the ability of the brain networks to change to effectively adapt to the changes (Lledo et al. 2006; Ming and Song 2011).

10.4.2 Pattern Separation and Pattern Completion

Causal manipulation studies have shown the role of hippocampal neurogenesis with the blockade of hippocampal neurogenesis leading to impairments in the pattern separation functionalities, possibly stemming from the disturbances in population coding dynamics within the medial temporal lobe memory system (Toda and Gage 2018). Computational models have suggested the role of DG neurogenesis in pattern separation and pattern completion. The unique anatomy of DG with highly converging inputs from its primary input area—entorhinal cortex—and the sparse coding property of DG neurons make the DG a potential key area for the neural computational processes related with pattern separation and completion. The extended plasticity features and delayed axonal developments associated with the adult-born neurons provide the specific opportunities for rate modulations or firing specific groups of newly formed neurons, subsequently leading to unique output variations effectively separating the patterns in memory space and thereby providing enough response dissimilarity between the new patterns, resulting in an efficient computational mechanism for pattern separation (Deng et al. 2010).

10.4.3 Higher Order Cognitive Functionalities

Hippocampal neurons modulate hypothalamic–pituitary–adrenal (HPA) axis by a negative feedback loop mechanism in response to circulating stress hormones through the high levels of glucocorticoid and mineralocorticoid receptors expressed. The adult formed neurons in DG have enhanced receptors for stress hormones indicating its critical role in regulating the stress hormone-mediated mechanisms (Schoenfeld and Cameron 2014). Several studies have shown the critical role of hippocampal neurogenesis in the treatment efficiency of antidepressant agents like Fluoxetine—a selective serotonin reuptake inhibitor (SSRI). The antidepressant

effect of fluoxetine depends significantly on its pro-neurogenic action, exerted by promoting proliferation, differentiation, and survival of progenitor cells of the hippocampus (Micheli et al. 2018). The extensive anatomical connections of hippocampus with other brain nodes like hypothalamus Amygdala, and anterior thalamic nuclei further contribute to further possibilities of hippocampal neurogenesis influencing other higher cognitive functions related to emotion, mood, and attention. It has also been suggested that the proposed role of hippocampal neurogenesis in pattern recognition and completion may also be playing a role in higher order cognitive functions like mood and anxiety, by its interactions with memory and by helping to recognize dangerous and stressful signals and to resolve decision conflicts. Impairments in these cognitive pattern separation functionalities may in turn lead to triggering or aggravating anhedonic or depressive behavior (Eisch and Petrik 2012). Studies on the factors influencing the neurogenesis suggest the critical roles of neurogenesis in regulation of higher cognitive functions like emotional control and decision making. The findings of environmental enrichments and physical or social rewarding enhancing the adult neurogenesis and the correlated findings of improvements in depression and anxiety symptoms further provide evidences for the interlinks between adult neurogenesis neuropsychiatric conditions. These findings also suggest the possibility and the need of exploring the functionally relevant neurogenesis in neocortical areas in addition to the hippocampal and SVZ sites (Gould 2007; Ming and Song 2011; Cameron and Glover 2015). For example, the depressive-like state produced in rodents by removal of the olfactory bulbs and the olfactory changes are also observed in human patients with depression and schizophrenia (Schoenfeld and Cameron 2014).

10.5 Adult Neurogenesis: A Target for Regenerative Medicine

From the very early days of the emergence of the topic of adult neurogenesis, it was started being proposed as a potential target for a large variety of neurological conditions. Even with all the (still) ongoing debates on the anatomical extent, timeline, rate, and functional significance of adult formed neurons, there are promising evidences suggesting the clinical possibilities of adult neurogenesis (Fig. 10.2).

10.5.1 Stroke and Injuries

Studies have shown enhanced neurogenesis follows medial temporal lobe epilepsies, ischemia, and traumatic injuries, providing promising directions toward the possibility of natural neural repair mechanisms related to the formation and assimilation of new neurons (Abrous et al. 2005). Experiments on animal models of stroke induced by techniques like transient middle cerebral artery occlusion have shown evidences of neurogenesis following stroke episodes. The neural progenitors proliferate in response to ischemia-induced cell loss. The proliferated progenitor cells differentiate and subsequently migrate to damaged areas, most notably to striatum

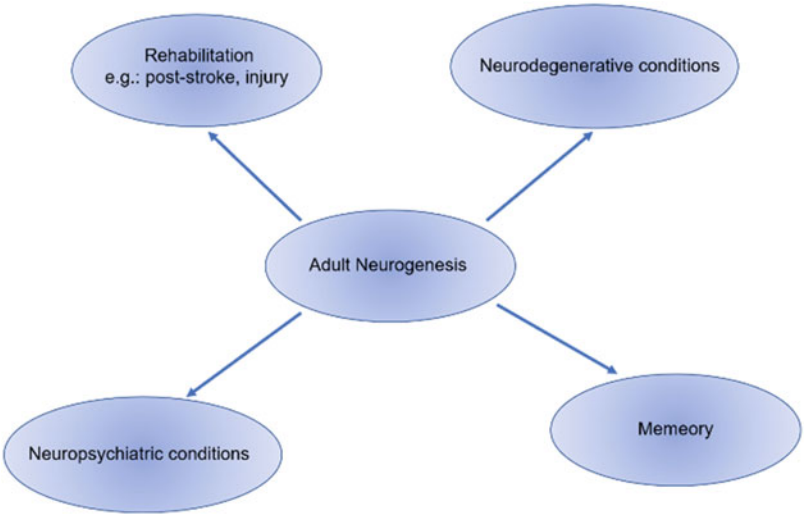


Fig. 10.2 Potential clinical targets of adult neurogenesis

(Lindvall and Kokaia 2015). The migration of neuroblasts from the neurogenesis foci to damaged areas appear to be mediated through the blood vessels. Compared to the post-stroke neurogenesis evidences in striatum, the cortical neurogenesis is less conclusive. Significant regeneration of neocortex out of neurogenesis has not yet been observed in humans. It has been shown that neurogenesis can be induced in stroke damaged cortical areas by introducing agents like growth factors, but it should be noted that majority of the newly differentiated striatal neurons following stroke undergo programmed cell death within the first 2 week of their formation and fails to survive. Ischemia-related inflammatory changes which induced the initial proliferation of progenitor cells also contribute to the poor survival of the newly formed striatal neurons, indicating that limitations of neurogenesis dependent post-stroke self-repair mechanisms (Lindvall and Kokaia 2015; Lu et al. 2017). It has been suggested that reducing ischemia-related inflammatory substrates in critical windows of survival of newly formed neurons and also be stimulating the differential of cells using growth and neurotrophic factors could be potential workarounds for using the potential of neurogenesis as effective post-stroke repair mechanism (Lu et al. 2017). However, it should be noted that inflammatory factors are critical for proliferation of the progenitor cells and the angiogenesis which ultimately control the migration of newly formed neurons to damaged areas. Striking a balance between the interconnected processes is critical for being able to use neurogenesis for post-stroke repair (Rahman et al. 2021). A recent review exploring the role of neurogenesis in post-stroke functional recovery, discuses several factors which needed to be considered and solved before translating the finding of increased post-stroke neurogenesis as a valid treatment pathway for the post-stroke functional recovery (Ceanga et al. 2021). The extent to which the newly formed neurons can

migrate from the currently established foci of neurogenesis to the distant cortices critical for the post-stroke functional recovery being the most important factor to be considered. The timing of neurogenesis is also important given numerous neural network plasticity processes and functional neural compensation mechanisms that occur within a short time window following the stroke events. Efforts to accelerate the neurogenesis, in the hopes for faster and efficient functional and cognitive repair and recovery, need to be approached carefully given our limited understanding on the migration and functional assimilation of newly formed neurons. Careful considerations should be made regarding maladaptive versus beneficial reorganizations, especially when considering motor recoveries (Ceanga et al. 2021).

10.5.2 Neurodegenerative Conditions

Neurodegenerative diseases are another avenue where adult neurogenesis is extensively investigated as a potential therapeutic target. Significant changes in adult neurogenesis have been observed in multiple neurodegenerative diseases like Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD). Although the pathology of these neurodegenerative diseases is linked to different proteins and distinct neuronal populations, the early symptom complexes of these conditions are strikingly similar and include memory and learning-related cognitive impairments, emotional imbalances, and depression, all of which can be linked to hippocampal and olfactory complexes, the primary areas of adult neurogenesis, suggesting a possible causal link between the adult neurogenesis and onset of pathophysiology of the neurodegenerative conditions (Winner and Winkler 2015). Experimental evidences support this hypothesis. Transgenic animal models of PD showed impairments in proliferative activity and survival of newly generated neurons. Similarly, decreased proliferation in correlation with the increase in β-amyloid plaques has been observed in AD models (Winner and Winkler 2015). Impaired hippocampal neurogenesis in Alzheimer's disease and Impaired maturation of striatal neurons in the striatum have been observed (Cheyuo et al. 2019). Postmortem studies on human subjects with neurodegenerative conditions showed abnormal morphological development and changes in differentiation markers in adult hippocampal neurogenesis (Therreros-Roncal et al. 2021). Based on the potential link between the impairments in the adult neurogenesis and the development of pathophysiology of neurodegenerative conditions, strategies aimed at enhancing neurogenesis have been suggested as potential treatment options for neurodegenerative conditions. Milk fat globule-epidermal growth factor-factor VIII (MFG-E8), a secretory glycoprotein that has been shown to be promoting neural stem cell proliferation and migration toward the ischemic brain tissues in post-stroke brains has also been explored as a potential therapeutic agent for repairing neurogenesis impairments in neurodegenerative conditions (Cheyuo et al. 2019). Animal studies on the neurodegenerative models do provide early promising results of functional improvement with enhanced neurogenesis (Winner and Winkler 2015). However, the functional recovery with neural stem cells or artificially

enhancing the in vivo neurogenesis will largely depend on the rate, anatomical extent and the functional integration of newly formed neurons to the existing network, similar to the recovery possibilities discussed with the post-stroke conditions (Ceanga et al. 2021).

10.5.3 Neuropsychiatric Conditions

Decreased neurogenesis in a number of neuropsychiatric conditions including major depression, anxiety, and the reversal of pathologically reduced neurogenesis with existing treatment options point toward yet another therapeutic aspect of adult neurogenesis (Abrous et al. 2005; Ghosh 2019). Pharmacological innervations and other treatment modalities have shown to enhance the neurogenesis and the findings of the dependence of treatment efficacy level of neurogenesis provide direct links between the role of neurogenesis and development of neuropsychiatric conditions (Schoenfeld and Cameron 2014). Decrease in cell proliferation within the dentate gyrus and reduced hippocampal volume have been reported in a number of neuropsychiatric conditions along with impairments in hippocampus-dependent functionalities (Kang et al. 2016). It has been shown that DISC1 gene, a major susceptibility gene for schizophrenia is involved in regulating adult hippocampal neurogenesis, further providing evidences for the neurogenesis related origin of schizophrenia (Eisch et al. 2008). Given the role of anatomical connections between hippocampus and limbic system in reward and motivation, hippocampal neurogenesis was hypothesized to play roles in drug addiction and substance abuse and experimental evidences indeed suggest the possibility of such an association (Eisch et al. 2008). Although these evidences suggest the potential role of neurogenesis in the pathophysiology of a variety of neuropsychiatric conditions, targeting neurogenesis in treatment options is not straightforward as in the case of stroke rehabilitation and neurodegenerative conditions. Many unknown factors remain to be revealed related to the rate of proliferation, survival, and integration of the newly formed neurons within the existing network—the factors which critically determine the potential clinical role of adult neurogenesis in neuropsychiatric conditions.

10.5.4 Potential Roles in Learning and Cognitive Functionalities

It has been shown that environmental factors like enrichment and learning affect adult neurogenesis (Shohayeb et al. 2018). These findings provide a potential avenue of a reverse clinical application, that is, enhancing cognitive abilities related to learning and memory by enhancing hippocampal neurogenesis. Genetically driven expansion of neural stem cells compensated the age-related decline in neurogenesis enhanced navigational learning strategies in mice models (Berdugo-Vega et al. 2020). Interestingly, enhanced hippocampal neurogenesis and related plasticity also leads to a memory reorganization and neurogenesis-induced forgetting as in

the case of contextual fear conditioning experiments in mice models (Evans et al. 2021). Further studies suggest that the neurogenesis-mediated forgetting is not limited to contextual fear memories but extends to broader array of learned memories including spatial, context, or object memories (Scott et al. 2021). These findings while suggesting the potential possibilities for modulating cognitive functionalities using factors influencing hippocampal neurogenesis, also shows the complicated interconnections between the memory and learning nodes within the hippocampal and medial temporal lobe memory system.

While existing research works on the adult neurogenesis do indicate the prospects of adult neurogenesis being emerging as the potential target for many neurological conditions, its limitations, based on what we currently know (and don't know) about the extent and the specific mechanisms, also need to be considered while aiming for therapeutic applications. Manipulating the adult neurogenesis will most possibly bring functional effects related to the local microenvironments at the foci of neurogenesis, but the clinical relevance of such local effects and the causal roles of adult formed neurons in the wider neural networks and in the global brain functionalities will require extensive future research works. Such works will eventually better define the clinical potentials of adult neurogenesis in vascular, neuropsychiatric, or neurodegenerative conditions (Box 10.2).

Box 10.2 Outstanding Questions and Future Directions

- **Search for the other potential loci of adult neurogenesis:** comparative studies with other species showing varying loci of neurogenesis in adult organisms suggest the need of considering developmental milestones and embryological parallelism while exploring the potential neurogenesis loci.
- **Precise mechanisms of migration and integration of adult formed neurons in the existing networks:** A better understanding on these mechanisms are critical for exploring the therapeutic potentials of adult neurogenesis.
- **Physiological and computational mechanisms of adult formed neurons in cognitive domains:** although current evidences show the links between adult neurogenesis and memory functionalities, the network reorganizations with which newly formed neurons involved in new learning while simultaneously affecting the storage of existing memories need to be explored.

References

Abrous DN, Koehl M, Le Moal M (2005) Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85(2):523–569

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124(3):319–335

Angevine JB Jr (1965) Time of neuron origin in the hippocampal region. An autoradiographic study in the mouse. Exp Neurol Suppl 2:1–70. Supplement
Berdugo-Vega G et al (2020) Increasing neurogenesis refines hippocampal activity rejuvenating navigational learning strategies and contextual memory throughout life. Nat Commun 11:135
Brenowitz EA, Larson TA (2015) Neurogenesis in the adult avian song-control system. Cold Spring Harb Perspect Biol 7(6):a019000. https://doi.org/10.1101/cshperspect.a019000
Boldrini M et al (2018) Human hippocampal neurogenesis persists throughout aging. Cell Stem Cell 22:589–599
Cajal S (1899) Texture of the nervous system of man and the vertebrates (trans: Pasik P, Pasik T, from the 1899–1904 Spanish edition). Springer, Vienna (1999)
Cameron HA, Glover LR et al (2015) Annu Rev Psychol 66:53–81. https://doi.org/10.1146/annurev-psych-010814-015006. Epub 2014 Sep 19. PMCID: PMC5612417
Ceanga M, Dahab M, Witte OW, Keiner S (2021) Adult neurogenesis and stroke: a tale of two neurogenic niches. Front Neurosci 15:700297
Cheyuo C et al (2019) Neurogenesis in neurodegenerative diseases: role of MFG-E8. Front Neurosci 13:569. https://doi.org/10.3389/fnins.2019.00569
Cole JD et al (2020) Adult-born hippocampal neurons undergo extended development and are morphologically distinct from neonatally-born neurons. J Neurosci 40(30):5740–5756
Conover JC (2000) Disruption of Eph/ephrin signaling affects migration and proliferation in the adult subventricular zone. Nat Neurosci 3:1091–1097
Cooper-Kuhn CM (2002) Impaired adult neurogenesis in mice lacking the transcription factor E2F1. Mol Cell Neurosci 21:312–323
Deng W et al (2010) New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nat Rev Neurosci 11(5):339–350
Eisch AJ, Petrik D (2012) Depression and hippocampal neurogenesis: a road to remission? Science 338(6103):72–75
Eisch AJ et al (2008) Adult neurogenesis, mental health, and mental illness: hope or hype? J Neurosci 28(46):11785–11791
Eriksson P et al (1998) Neurogenesis in the adult human hippocampus. Nat Med 4:1313–1317
Evans A et al (2021) Neurogenesis-induced forgetting is associated with reduced neuronal activity in CA1 during context fear memory retrieval. Biorxiv. https://doi.org/10.1101/2021.03.24.436893
Ghosh HS (2019) Adult neurogenesis and the promise of adult neural stem cells. J Exp Neurosci 13:1179069519856876
Gonçalves JT, Schafer ST, Gage FH (2016) Adult neurogenesis in the hippocampus: from stem cells to behavior. Cell 167(4):897–914
Gould E (2007) How widespread is adult neurogenesis in mammals? Nat Rev Neurosci 8(6):481–488
Gould E, Beylin A, Tanapat P et al (1999) Learning enhances adult neurogenesis in the hippocampal formation. Nat Neurosci 2:260–265. https://doi.org/10.1038/6365
Gross CG (2000) Neurogenesis in the adult brain: death of a dogma. Nat Rev Neurosci 1(1):67–73
Hanson ND (2011) Depression, antidepressants, and neurogenesis: a critical reappraisal. Neuropsychopharmacology 36(13):2589–2602
Hastings NB, Gould E (1999) Rapid extension of axons into the CA3 region by adult-generated granule cells. J Comp Neurol 413:146–154
Kang E et al (2016) Adult neurogenesis and psychiatric disorders. Cold Spring Harb Perspect Biol 8(9):a019026. https://doi.org/10.1101/cshperspect.a019026
Kempermann G, Song H, Gage FH (2015) Neurogenesis in the adult hippocampus. Cold Spring Harb Perspect Biol 7(9):a018812
Kempermann G et al (2018) Human adult neurogenesis: evidence and remaining questions. Cell Stem Cell 23(1):25–30. https://doi.org/10.1016/j.stem.2018.04.004
Koelliker A (1896) Handbuch der Gewebelehre des Menschen. Engelmann, Leipzig

La Rosa C, Parolisi R, Bonfanti L (2020) Brain structural plasticity: from adult neurogenesis to immature neurons. Front Neurosci 14:75
Leblond CP (1964) Classification of cell populations on the basis of their proliferative behavior. Natl Cancer Inst Monogr 14:119–150
Lemaire V, Tronel S, Montaron M-F, Fabre A, Dugast E, Abrous DN (2012) Long-lasting plasticity of hippocampal adult-born neurons. J Neurosci 32(9):3101–3108
Lindvall O, Kokaia Z (2015) Neurogenesis following stroke affecting the adult brain. Cold Spring Harb Perspect Biol 7(11):a019034
Liu PZ, Nusslock R (2018) Exercise-mediated neurogenesis in the hippocampus via BDNF. Front Neurosci 12:52
Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7(3):179–193
Lu J et al (2017) Targeting adult neurogenesis for poststroke therapy. Stem Cells Int 2017: 5868632
McMahon AP (2003) Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 53:1–114
Micheli L et al (2018) Depression and adult neurogenesis: positive effects of the antidepressant fluoxetine and of physical exercise. Brain Res Bull 143:181–193
Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223–250
Ming GL, Song H (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70(4):687–702
Neuberger EJ et al (2017) Enhanced dentate neurogenesis after brain injury undermines long-term neurogenic potential and promotes seizure susceptibility. Stem Cell Rep 9(3):972–984
Nottebohm F (1984) Birdsong as a model in which to study brain processes related to learning. Condor 86(3):227–236. https://doi.org/10.2307/1366988. JSTOR. Accessed 23 Sep 2022
Oomen CA et al (2009) Opposite effects of early maternal deprivation on neurogenesis in male versus female rats. PLoS One 4(1):e3675
Rahman AA et al (2021) Neurogenesis after stroke: a therapeutic perspective. Transl Stroke Res 12: 1–14
Rakic P (2002) Neurogenesis in adult primates. Prog Brain Res 138:3–14
Ramon y Cajal S (1913–14) Estudios sobre la degeneración y regeneración del sistema nervioso. Madrid, Moya
Schmidt-Hieber C, Jonas P, Bischofberger J (2004) Enhanced synaptic plasticity in newly generated granule cells of the adult hippocampus. Nature 429(6988):184–187
Schoenfeld TJ, Cameron HA (2014) Adult neurogenesis and mental illness. Neuropsychopharmacology 40:113–128
Scott GA et al (2021) Adult neurogenesis mediates forgetting of multiple types of memory in the rat. Biorxiv. https://doi.org/10.1101/2021.03.22.436536
Shohayeb B et al (2018) Factors that influence adult neurogenesis as potential therapy. Transl Neurodegener 7:4
Snyder JS (2019) Recalibrating the relevance of adult neurogenesis. Trends Neurosci 42(3): 164–178
Sorrells SF et al (2018) Human hippocampal neurogenesis drops sharply in children to undetectable levels in adults. Nature 555:377–381
Spalding KL et al (2013) Dynamics of hippocampal neurogenesis in adult humans. Cell 153(6): 1219–1227
Therreros-Roncal J et al (2021) Impact of neurodegenerative diseases on human adult hippocampal neurogenesis. Science 374(6571):1106–1113

Thorpe WH (1958) The learning of song patterns by birds, with especial reference to the song of the chaffinch Fringilla coelebs. Ibis 100(4):535–570. https://doi.org/10.1111/j.1474-919X.1958.tb07960.x

Toda T, Gage FH (2018) Review: adult neurogenesis contributes to hippocampal plasticity. Cell Tissue Res 373(3):693–709

Winner B, Winkler J (2015) Adult neurogenesis in neurodegenerative diseases. Cold Spring Harb Perspect Biol 7(4):a021287

Zhao C et al (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26(1):3–11

Regenerative Approaches in the Nervous System

11

Ronak Reshamwala and Megha Shah

Abbreviations

AD-MSC	Adipose derived mesenchymal stem cells
AEC	Amniotic epithelial cells
AF-MSC	Amniotic foetal mesenchymal stem cells
ANS	Autonomic nervous system
ASIA	American spinal cord injury Association
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BM-MSC	Bone marrow derived mesenchymal stem cells
BSCB	Blood spinal cord barrier
CNI	Central nervous system injuries
CNS	Central nervous system
CSPG	Chondroitin sulphate proteoglycans
CTGF	Connective tissue growth factor
DRG	Dorsal root ganglia
EEG	Electroencephalogram
EGF	Epidermal growth factor
EPC	Endothelial progenitor cells
ESC	Embryonic stem cells
FGF	Fibroblast growth factor

R. Reshamwala (✉) · M. Shah
Menzies Health Institute Queensland, Griffith University, Southport, QLD, Australia

Clem Jones Centre for Neurobiology and Stem Cell Research, Griffith University, Southport, QLD, Australia

School of Pharmacy and Medical Sciences, Griffith University, Southport, QLD, Australia
e-mail: r.reshamwala@griffith.edu.au

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_11

FGF-2	Fibroblast growth factor 2
FIM	Functional independence measure
GCS	Glasgow coma scale
G-CSF	Granulocyte colony stimulating factor
GDNF	Glial cell-line derived neurotrophic factor
GGF	Glial growth factor
hFSC	Human foetal stem cells
HGF	Hepatocyte growth factor
IGF-1	Insulin like growth factor 1
iPSC	Induced pluripotent stem cells
MAP	Myelin-associated proteins
MSC	Mesenchymal stem cells
NGF	Nerve growth factor
NPC	Neural progenitor cells
NSC	Neural stem cells
NT-3	Neurotrophin-3
OEC	Olfactory ensheathing cells
OPC	Oligodendrocyte precursor cells
PCL	Poly caprolactone
PDGF	Platelet derived growth factor
PEG	Polyethylene glycol
pHEMA	Poly hydroxyethyl methacrylate
PLGA	Poly lactic-*co*-glycolic acid
PLL	Poly-L-lysine
PNI	Peripheral nerve injuries
PNS	Peripheral nervous system
PU	Polyurethane
SCI	Spinal cord injuries
SCIM	Spinal cord independence measure
SCs	Schwann cells
TBI	Traumatic brain injuries
TGF-β1	Transforming growth factor B1
TIA	Transient ischemic attacks
UBC-MSC	Umbilical cord blood mesenchymal stem cells
VEGF	Vascular endothelial growth factor

11.1 Background

11.1.1 Anatomy and Organisation of the Nervous System

The nervous system is primarily made up of specialised excitable and conductive cells known neurons (the nerve cells), and their supportive cells known as glia. Neurons are unique in their cellular structure and function as well as their arrangement. Together, they make a control, command and communications network, capable of transmitting impulses at very high speeds. The neurons have large cell bodies and cell processes. The processes or neurites are of two types: (1) axons and (2) dendrites. Each neuron has only one axon and the axons carry outgoing signals from the neuron and relay it to distal organs or other neurons. The dendrites are shorter, smaller, branched processes and neurons can have one or several dendrites. They carry the incoming signals from other cells into the neuron's cell body. Thus, neurons use axons and dendrites to communicate with each other as well as other organs. The cellular connection between axons and dendrites is known as 'synapse'.

The cell bodies of the neurons are arranged together in clusters, known as ganglia or nuclei (not to be confused with nucleus of a cell), that monitor and regulate specific bodily functions or organ systems. These clusters are generally located in the brain or spinal cord, and together, they make the central nervous system (CNS). From here, they send out bundles of long, slender cellular process, known as axons, to communicate with their target organs throughout the body. These bundles of axons are known as nerves, which make the peripheral nervous system (PNS). The axons can also connect and facilitate intercommunications between the nuclei within the brain and spinal cord. Here, in the CNS, they are known as tracts.

Thus, structurally, the nervous system has two parts: (1) CNS and (2) PNS. As mentioned, the CNS is comprised of the brain and spinal cord, which are wrapped inside three protective layers known as the meninges, which are further encased with the skull (or cranial vault) and the vertebral column respectively. The CNS is further organised in the grey matter and the white matter. The grey matter contains the cell bodies of the neurons, and the glial cells, whereas the axonal tracts make up the white matter.

The peripheral nerves emerge from the CNS and supply the rest of the body. There are 12 pairs of nerves that emerge directly from the brain. They are called cranial nerves. Similarly, the spinal cord gives rise to 31 pairs of nerves, the spinal nerves. The spinal nerves exit the spinal column from small spaces between the vertebrae, known as the intervertebral foramina, on either side of the spinal column. There are 8 pairs of cervical spinal nerves, 12 pairs of thoracic, 5 pairs of lumbar, 5 pairs of sacral and 1 pair of coccygeal spinal nerves. These nerves are so called because they exit from the intervertebral foramina of the cervical, thoracic, lumbar, sacral and coccygeal vertebrae respectively. Together, the cranial and spinal nerves make the PNS.

Thus, the CNS and PNS are remarkably different from each other. The CNS contains the neuronal cell bodies and axons, and PNS predominantly contains the axons, with the exception of cell bodies of the sensory neurons located in dorsal root

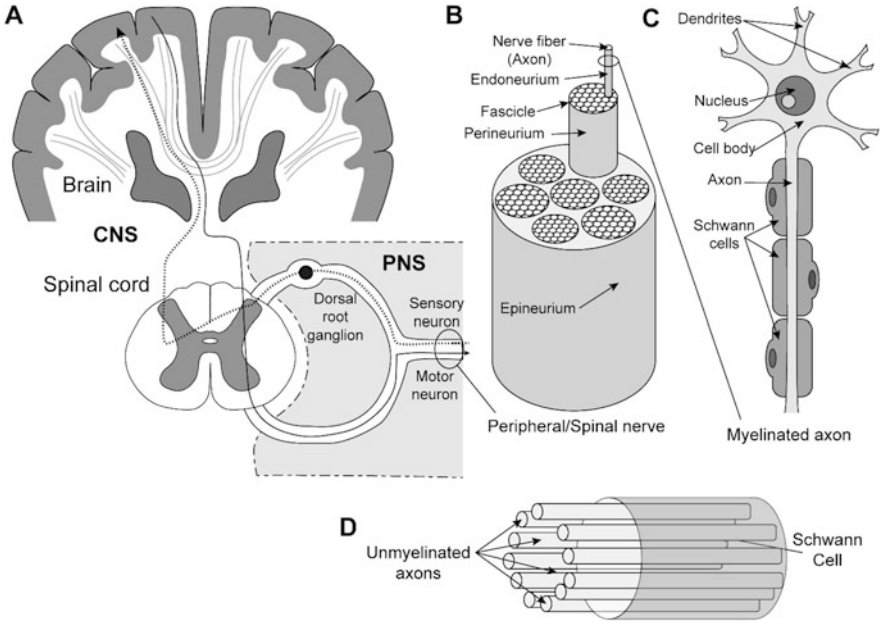


Fig. 11.1 (**a**) The arrangement of CNS and PNS, (**b**) Enlarged view and detailed structure of a peripheral nerve, (**c**) Enlarged view and detailed structure of myelinated axon and neuron. (**d**) Unmyelinated axons where a single Schwann cell envelopes a bundle of axons

ganglia, just outside the dorsal root of spinal nerves. The axons can repair themselves after a small injury in a limited capacity; however, an injury to the cell body is largely irreversible and results in permanent loss of the neuron, and by extension, its function. The supporting cells, or glia, are also a critical part of both CNS and PNS, and play key roles in their normal functions, maintenance of homeostasis and response to injury. The CNS contains several different types of glial cells: astrocytes, oligodendrocytes, microglia, ependymal cells, radial glia and all of these cell types have different functions. The PNS has only one type of glial cells known as Schwann cells (SCs); they support and augment the axons and facilitate the nerve conduction and they wrap the axons in an insulating layer in one of the two manners: (1) myelinating and (2) non-myelinating. The myelinating SCs cover a small segment of individual (myelinated) axons, whereas the non-myelinating SCs wrap around a large bundle of (unmyelinated) axons. Figure 11.1 shows arrangement of CNS and PNS, the neuron and its processes, as well as the two phenotypes—myelinated and unmyelinated nerves.

11.1.2 Function of the Nervous System

The nervous system monitors and regulates all the bodily functions. For better understanding, the function of the nervous system can be classified in two main aspects: (1) motor and (2) sensory. Motor functions broadly include functions related to movements and locomotion, which involve the CNS's control centres that determine, generate and regulate the motor signals and PNS's motor nerves that carry the motor commands to the muscles or glands to execute the commands. Similarly, sensory functions include the sensory nerves of the PNS that gather and transmit the sensations experienced by the body to CNS and the sensory centres of the CNS that interpret and monitor all the incoming sensations such as touch, pain, pressure, temperature, vibrations etc. somatic sensations and smell, sight, sound, taste, hearing etc. special sensations. The nerves that carry motor commands from the CNS to the periphery are called motor or efferent nerves, and the nerves carrying sensory information from the periphery to the CNS are called sensory or afferent nerves. Some nerves may contain both afferent and efferent nerve fibres, which are therefore called mixed nerves.

The CNS also controls and regulates involuntary movements via the autonomic nervous system (ANS), which is a subsection of the motor system. Internal organs such as the intestines and heart, smooth muscles and glands etc. are controlled by the ANS. The ANS is further subdivided in sympathetic and parasympathetic nervous system. The sympathetic system stimulates the body to prepare for the 'fight or flight' response and parasympathetic system primarily regulates the secretomotor functions such as the digestive functions. Overall, the two parts of the ANS work on the same organ systems, seemingly antagonistically, to help maintain the homeostasis.

Thus, the overall function of the CNS is to monitor, control and regulate the body's organ systems and the primary function of the PNS is to connect the CNS to the rest of the body and to carry the signals to and from the CNS.

11.1.3 Barriers Protecting the CNS

The nervous system evidently is the most critical system in the human body, and as such, is protected by several different mechanisms. As discussed before, the CNS is first protected by three layers of meninges wrapped around it. Next, the bony cranial vault protects the brain, and the vertebral column encases the spinal cord. In addition to these solid physical barriers, the CNS is protected by a functional barrier that shields the CNS at a cellular and molecular level from the exterior influences. This barrier is called blood-brain-barrier (BBB) which continues to protect the spinal cord as the blood-spinal cord-barrier (BSCB) (Bartanusz et al. 2011). This barrier is made of primarily endothelial cells of the CNS; however, pericytes and astrocytes also contribute to the formation and function of the BBB and BSCB. Together, these cells form a cellular barrier that acts as a selectively permeable membrane, a secretory

body as well as a metabolic barrier that strictly regulates the microenvironment within the CNS (Rhea and Banks 2019).

While these barriers protect the CNS from physical trauma, microbial attacks and metabolic disruptions, they are likely to be injured themselves in case of a serious trauma or a severe disease. The broken or compromised barriers then stand to compound the injury and interfere with its clinical management.

11.1.4 Pathophysiology of Nervous System Injuries

The nervous system can be injured by trauma, infection, metabolic disease, or malignancies. Injuries to the nervous system are often compounded by neuroinflammation, excitotoxicity, free radicles (oxidative stress) and metabolic disruptions (Cashman and Hoke 2015; Silva et al. 2014).

In CNS, the commonly encountered injuries come from trauma and cerebrovascular incidents. Traumatic brain injuries (TBI) and spinal cord injuries (SCI) frequently result from road-traffic accidents, falls, firearms or sports-related injuries (Valenzuela et al. 2016). Cerebrovascular accidents usually involve transient ischaemic attacks (TIA), ischaemic stroke or haemorrhagic stroke. The peripheral nerve injuries (PNI) commonly occur from trauma, or disease-induced neuropathies (Valenzuela et al. 2016).

The traumatic CNS injuries (CNIs) have a complex pathophysiology. This can be explained in two distinct phases: (1) primary injury and (2) secondary injury (Reshamwala 2020; Stiefel et al. 2005; Werner and Engelhard 2007). The primary injury is the result of the trauma itself. The traumatic impact causes the initial damage to the neural and vascular tissues, and leads to cell membrane disruption, cellular swelling, hypoperfusion, changes in the membrane permeability and eventually neurotoxicity (Stiefel et al. 2005; Werner and Engelhard 2007; Bouma et al. 1991, 1992; Marion et al. 1991; Reilly 2001).

The secondary injury is essentially the result of neuroinflammatory processes that ensue over the hours to days following the primary injury. Parenchymal oedema, increase in the intracranial pressure and hypoperfusion all eventually lead to ischaemia, reperfusion injury and cell death (Hutchinson et al. 2006; Moppett 2007). Thus, the secondary injury leads to an apparent expansion of the primary injury and the neurological deficits.

The effects of the CNIs can vary greatly depending on the severity of injury. There may only be subtle molecular/cellular changes in the signal conduction in mild cases. In more severe cases, however, there may be haemorrhage, contusion, diffuse axonal injury, BBB/BSCB disruption and severe neuroinflammation, which may all lead to dysregulation of the microenvironment at the molecular level (Shlosberg et al. 2010; Shetty et al. 2014). The breach in the BBB/BSCB along with haemorrhage exacerbates the secondary injury (Pearn et al. 2017) and leads to further deterioration in the neurochemical environment causing metabolic and cellular dysregulation (Kumar and Loane 2012).

The PNI can happen as a result of traumas, infections or neuropathies. The different types of traumas can result in different types of PNIs. Injuries from crushing trauma can simply lead to transient stunning of the nerve, whereas penetrating injures can lead to severing of the nerves. Twisting, traction and torsion types of injuries likely lead to impingement of the nerves. Similarly, PNIs associated with bone fractures can have widely varying presentations and may need to be investigated in depth to determine their severity (Isaacs 2010; Shah et al. 2010). After the injury, the severed distal portion of the injured axon slowly degenerates distally from the point of injury. This process is known as the 'Wallerian degeneration'. The debris created by this degenerative process is cleared by the SCs and other macrophages. The proximal stump of the injured axons can then regrow back into the gap left by clearing out the debris, and reinnervate their original targets. This process usually results in functional restoration in varying degrees depending on the defect size and injury severity (Huebner and Strittmatter 2009).

11.2 Issues with Natural Regeneration and Repair

Nervous system injuries are different from the injury to the rest of the body in that the body cannot heal the nervous injuries well. Adult body is unable to replace the lost neurons naturally, damaged axons have difficulty regrowing along their original path and finding their original targets without specific guidance. These factors present the primary challenge to the natural repair and regeneration of the nervous system.

11.2.1 CNIs

As mentioned earlier, neurons do not readily regenerate in adults, and CNS neurons in particular have multiple compounding factors that impede their axonal regrowth following an injury. This leads to negligible to no clinical recovery in current practice. In the aftermath of the CNI, there is axonal degeneration which leads to accumulation of myelin-associated proteins (MAPs). These debris inhibit regrowth of axons by creating physical hurdles. Additionally, the astrocytes, the most abundant glial cells in CNS, become reactive following an injury. The reactive astrocytes undergo hypertrophy and cordon off the injury site from the surrounding healthy parenchyma by forming an astroglial scar tissue. This reactive astrocytic scar physically and chemically prevents axons from regrowing into the injury area. Eventually, the injury site is also occupied by thick fibrotic scar tissue with several inhibitory molecules further inhibiting axonal regrowth (Mackay-Sim and St John 2011; Reshamwala 2020). These molecules include myelin-associated inhibitors and chondroitin sulphate proteoglycans (CSPGs). It has also been found that the upregulation of growth-inducing genes is considerably lacking, which is a significant challenge for axonal regrowth in itself (Huebner and Strittmatter 2009).

Thus, regeneration and functional restoration in CNS are severely compromised and the body's response to CNI further limits what limited reparative efforts the injured CNS may make (Valls-Sole et al. 2011; Valenzuela et al. 2016).

11.2.2 PNI

As explained earlier, the PNS is essentially comprised of the axons and SCs, which means that PNIs are basically axonal injuries. These axons may readily regenerate, as explained before. However, there may be other secondary processes which may hinder successful reinnervation despite a good regenerative effort in the PNS (Valls-Sole et al. 2011; Valenzuela et al. 2016). After the PNI, the SCs start secreting components of the extra-cellular matrix, such as laminin, collagen IV and heparin sulphate proteoglycans. This enables them to rearrange themselves in tunnel like structure to guide axonal regrowth. However, when the SCs are unable to perform this task sufficiently, the tunnels are not formed and the axonal regrowth does not take place (Brosius Lutz and Barres 2014).

11.2.3 Potential Strategies for Regeneration and Repair

In summary, the CNS axons lack a substantial regrowth potential, unlike the PNS axons. The extracellular matrix and microenvironment are conducive to axonal regrowth, whereas the CNS contains more growth-inhibiting factors. Efficient debris clearance is very important for axonal regrowth following an injury. This information can serve as points of interest to devise regenerative strategies and interventions. For example, using peripheral nerve graft to induce CNS regeneration, factors inhibiting accumulation of CSPGs and MAPs or enhancing their clearance, infusing growth inducing factors or upregulating expression of the same, inhibiting the growth inhibiting factors of CNS neurons (such as the NOGO-Rho pathway) etc. are clearly the logical approaches to explore for CNS regeneration. These approaches are discussed in detail later in this chapter.

11.3 Current Approaches for Clinical Management

As of now, majority of current clinical approaches aim to stabilise the patients and focus primarily on the damage control aspect of the management rather than regeneration of the nervous system or restoration of lost function. In essence, the current practice is to secure airway and breathing, achieve haemostasis and provide anti-inflammatory support (Capizzi et al. 2020; Davanzo et al. 2017). The surgical interventions may be performed to achieve this, or to repair the musculoskeletal damage and prevent further complications as necessary. Here, in this section we will address the clinical assessment and diagnostic approaches, which also serve as tools

for functional measurement, and management approaches that are currently in practice.

11.3.1 Brain Injury

While there are several different types of brain injuries, we will primarily focus here on traumatic injuries and cerebrovascular events. There are no regenerative or reparative approaches currently available for neuro-degenerative disease in clinical practice.

11.3.1.1 Diagnostics

The brain injuries are clinically diagnosed by clinical examination, electrophysiological studies and imaging. The Glasgow Coma Scale (GCS) is currently the gold standard for objective clinical assessment of the patient's neurological status. The scale takes into account the ocular, verbal and motor responses to give a score between 3 and 15. The scoring system is easy to implement, globally accept and provide valuable prognostic information, all without the use of any specialised equipment (Marmarou et al. 2007). Pupillary examination is also critical in clinical neurological assessment because it is useful for the patients who are unconscious, sedated or paralysed (Adoni and McNett 2007).

In addition to the clinical assessment, electroencephalogram (EEG) is an important prognostic and monitoring tool, especially for the comatose patients with TBI. EEG monitoring is particularly useful for diagnosis and managing seizure activities to prevent secondary brain injury (Davanzo et al. 2017; Vespa 2005; Vespa et al. 1999), and for monitoring post-concussive patients (Ianof and Anghinah 2017).

Head computed tomography (CT) and magnetic resonance imaging (MRI) remain the preferred imaging modalities for accurate visualisation of the brain, skull, and surrounding tissues for evaluation of the extent of the injury, particularly for determining if a surgical intervention is required (Jinadasa and Boone 2016). CT scans are most useful to detect skull fractures, acute haematomas and intraventricular haemorrhages (Capizzi et al. 2020).

As discussed above, due to the absence of curative or regenerative measures, the mainstay of management of brain injuries is to treat the primary source of injury, mitigate the progression of the damage and attempt to stave off the secondary injury. Injuries such as TIA or small-scale strokes can be reverted almost completely if timely intervention can be provided. However, for more severe injuries such as larger scale strokes and TBI, the primary goal of treatment remains to control the cerebral oedema, reduce the intracranial pressure and to manage cerebral perfusion, and oxygenation (Hutchinson et al. 2006). One week of prophylactic anti-seizure drugs to prevent seizures and hyperosmolar drugs to enhance cerebral perfusion are indicated under the current guidelines (Capizzi et al. 2020). Craniotomy or burr-hole drilling may be indicated in some cases to drain a rather large epidural/subdural haematoma or a contusion to decompression of brain parenchyma (Bullock et al. 2006). Nevertheless, the evidence for the importance of surgical intervention for TBI

management is largely inconclusive, and hence, therapeutic guidelines for the same still remain unavailable (Jinadasa and Boone 2016). Thus, current practice in managing brain injuries rests on regular clinical examinations, thorough monitoring and in some extreme cases, decompressive surgeries (Capizzi et al. 2020).

11.3.2 Spinal Cord Injury

Like the brain injuries, SCIs are also most commonly traumatic in origin.

11.3.2.1 Diagnostics

The first step to make the diagnosis is clinical assessment. Current practice is to assess the neurological function, to determine the deficits and establish the 'neurological level' of injury in reference to the spinal nerves. Additionally, imaging modalities are used to visualise the spinal cord and surround tissues to assess the extent of injury. Modalities such as X-ray and CT scans are best suitable for the visualisation of the bony spinal column and acute haemorrhages, whereas MRI is used for imaging of the soft tissues of spinal cord, nerves and musculature. The severity of the injury is then assessed by using several different scales or scoring systems, of which American Spinal cord Injury Association (ASIA) scoring system is the most widely used (American Spinal Injury Association n.d.). This system grades a SCI from A to E, where grade A is given for a complete loss of function below the level of injury and E signifies no loss of function below the level of injury. For monitoring the progress of the injury and to objectively evaluate the outcome measurements, scores such as Functional Independence Measure (FIM) and Spinal Cord Independence Measure (SCIM) are used.

11.3.2.2 Management

For the management of SCIs, there are no globally agreed upon approaches available in the clinical practice. Although surgical decompression and steroidal anti-inflammatory treatments are very commonly used, they still remain the focus of much debate and controversy (Silva et al. 2014). Depending on the severity of musculoskeletal injuries, spinal stabilisation/decompression and high-dose methylprednisolone are widely employed as the treatment of choice for damage control and stabilisation of the patient for over two decades (Bracken and Holford 2002).

Although surgical decompression is commonly performed after a SCI, its efficacy in neuroprotection remains yet to be proven. There are numerous studies reporting its neuroprotective effects; however, several studies have also shown it to have a negative impact on outcomes (Fehlings and Perrin 2005). More than anything, this disparity is an indication of the highly variable and complex nature of the SCI (Silva et al. 2014). Nevertheless, the removal of bone fragments from the cord parenchyma and haemostasis is the essential first step in most cases of SCI and surgical decompression may be indicated to achieve that.

As explained in the previous section, the secondary injury following the initial injury is a concern for further neurological deterioration in SCI, and

neuroinflammation plays a pivotal role in its pathophysiology. This is why, many decades ago, methylprednisolone was identified to be beneficial in SCI due to its anti-inflammatory actions, free radical scavenging properties and BSCB preservation (Hall and Braughler 1981). In spite of this, later studies have elucidated the increased risks of reduced immunity, wound infections, sepsis, mucosal haemorrhage, pulmonary embolism and pneumonia with the use of methylprednisolone (Hadley et al. 2002). Thus, the role of methylprednisolone is also highly controversial in SCI management, indicating a dire need of newer approaches for initial management. As discussed before, these approaches do not address the repair or regeneration, and as such this is a field that can richly benefit with the progress regenerative medicine.

11.3.3 Peripheral Nerve Injury

Contrary to CNIs, PNIs have much more streamlined diagnostic and management approaches available, with better chances of recovery and regeneration of the injured nerves.

Clinically, the PNIs can be diagnosed and classified using one of the two well stablished systems: (1) Seddon and (2) Sunderland. The Seddon system (Seddon 1943) classifies injuries into neurapraxia (transient stunning), axonotmesis (severed nerve fibres with intact peri-/epineurium) and neurotmesis (complete severing of the nerve) in increasing order of severity (Bhandari 2019). The Sunderland system classifies injuries into five more detailed 'degrees' based on their histological presentation (Sunderland 1951). Here, first-, third- and fifth-degree of injury are equivalent to neurapraxia, axonotmesis and neurotmesis respectively. The second-degree injury has an intact endoneurium and fourth-degree injury has only an intact epineurium (Griffin et al. 2013). As the injury classification progresses to higher degrees, the natural regenerative potential seemingly decreases.

11.3.3.1 Diagnostics

The current gold standard for diagnosis and monitoring prognosis of a PNI is serial electrophysiological studies over the course of weeks to months along with clinical physical examinations (Griffin et al. 2013). This is done to allow the natural regeneration to take place before any surgical intervention is deemed necessary (Bishop and Ring 2009). Initially, nerve conduction tests and electromyography are used to establish the extent of the injury and functional status of the injured nerves. Further periodic examinations indicate the rate of progressive recovery and also help determine the best time for a surgical intervention (Strandberg et al. 2007).

Imaging studies such as ultrasound and MRI are very commonly used for visualisation of the nerve and locating the injury site. Ultrasound is useful as a cheaper, quicker imaging modality that can give information about the nerve injury, perfusion status and injury to the surrounding tissues. MRI, on the other hand, can give high-resolution information regarding the degrees of the injury and can even

differentiate between neurotmesis and high-grade axonotmesis (McDonald et al. 2000).

11.3.3.2 Management

There are clinical guidelines available for surgical PNI repairs based on the size of the defect. The defects are classified into three categories: (1) <1 cm, (2) 1–5 cm, (3) >5 cm (Bassilios Habre et al. 2018).

1. For nerve gaps under 1 cm in size, end-to-end neurorrhaphy (anastomosis) or direct nerve repair with sutures is indicated. It is important to align the fascicles correctly and avoid tension in the repaired nerve to allow best possible recovery and avoid further injuries (Griffin et al. 2013; Al-Qattan 2002).
2. For the defect sizes between 1 and 5 cm, nerve grafting is most suitable. Here, several choices are available for grafting such as nerve autografts, cellular grafts, acellular grafts, or nerve conduits. For larger nerves with a diameter of 7 mm or more, 'group fascicular repair' with cable nerve grafts is indicated (Bassilios Habre et al. 2018).
3. For the defect sizes over 5 cm, nerve transfers and vascularised nerve grafts are suitable. Occasionally, decellularised allografts, harvested from cadavers, may also be used to bridge the gap; however, such large size defects usually do not respond as well as the other smaller defects. Nevertheless, nerve transfer and grafting procedures have seen some promising outcomes in the clinical setting. Nerve transfers essentially sacrifices an intact nerve with redundant or less critical function to regain a more critical function (Castanov et al. 2021). Several types of nerve conduits are commercially available in a 'ready-to-use' format such as collagen, polycaprolactone and polyglycolic acid (Griffin et al. 2013; Bassilios Habre et al. 2018).

Thus, there are several robust surgical options available for PNI repairs. However, these approaches mainly depend on the PNS axons' innate ability to regrow, while the interventions merely augment or sustain their natural regrowth. For the large size PNIs, the functional regain must come from sacrificing other healthy nerves, or from harvesting cadaveric allografts with somewhat reduced efficacy. Novel regenerative approaches that either enhance the axonal regrowth or induce true regeneration after the injury are needed to help progress the field of PNI repair.

11.4 Novel Regenerative Approaches

Several innovative approaches are under exploration with the aim of actively repairing the damage to the nervous system and to restore the lost function resulting from the injury.

11.4.1 Brain Injury

There are several new therapeutic approaches being explored for the treatment of TBI, cerebrovascular events and neuro-degenerative diseases, using neuroprotection and/or neuro-regeneration: (1) cell transplantation-based approaches, (2) biomaterial-based approaches and (3) bioactive molecule-based approaches.

11.4.1.1 Cell Transplantation-Based Approaches

Stem cells are promising candidates for brain injury repairs due to their ability of neurogenesis, angiogenesis, immunomodulation and secretion of bioactive molecules (Galgano et al. 2017).

The most commonly explored stem cells include bone marrow-derived mesenchymal stem cells (BM-MSCs) (Hu et al. 2016), umbilical cord blood mesenchymal stem cells (UBC-MSCs) (Wang et al. 2013), adipose-derived mesenchymal stem cells (AD-MSCs) (Mastro-Martínez et al. 2015), human foetal stem cells (hFSCs) (Skardelly et al. 2011), neural stem/progenitor cells (NSCs/NPCs) and embryonic stem cells (ESCs) (Molcanyi et al. 2007). Recently, induced pluripotent stem cells (iPSCs) have found a crucial place in neuro-generative disease treatments (Yasuhara et al. 2017; Fan and Ng 2020).

MSCs have a strong anti-inflammatory effect by reducing pro-inflammatory factors such as IL-1β, IL-6, TNF-α, CCL2, CCL11, and CXCL (Galindo et al. 2011); they offer neuroprotection by inhibiting apoptosis and increasing AKT, and they have induced functional regeneration in rodent TBI models (Kim et al. 2010). Additionally, MSCs secrete growth factors that improve neurogenesis, angiogenesis and synaptogenesis—such as fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF) and brain-derived neurotrophic factor (BDNF) (Qu et al. 2009; Xiong et al. 2009). Interestingly, BM-MSCs can migrate to the TBI lesion after a systemic injection without any external interference (Mahmood et al. 2004). The hFSCs can also lead to functional regeneration and reduce the injury size as seen in animal trials (Skardelly et al. 2011).

Recently, multipotent NSCs/NPCs were identified and isolated from adult human brain, specifically from the subventricular zone, hippocampus and subcortical white matter (Arsenijevic et al. 2001; Brunet et al. 2002, 2003; Kukekov et al. 1999; Nunes et al. 2003; Richardson et al. 2006; Roy et al. 2000; Windrem et al. 2002). Thus, they have become a likely viable candidate for clinical translation. These cells have shown efficacy in TBI repair (Patel and Sun 2016; Jiang et al. 2016; Chang et al. 2016; Dixon et al. 2015), neuroprotective and neurogenerative properties, as well as immunological acceptance of the graft (Kokaia et al. 2012).

ESCs have reportedly been somewhat controversial in their regenerative role thus far, despite the enhanced neural repairs, the early experiments have revealed poor survival and integration (Molcanyi et al. 2007) and tumorigenic potential (Riess et al. 2007). Conversely, recent advances in the iPSC reprogramming technologies have opened new possibilities for neural regeneration, without the risk of rejection (autologous acquisition of cells), without the ethical and logistical dilemma (Rolfe and Sun 2015).

AD-MSCs and NSCs have shown promise for treating Alzheimer's disease by inducing neuro-regeneration and relieving cognitive loss (Yan et al. 2014; Blurton-Jones et al. 2009). Currently, MSCs are in clinical trials as a potential therapy for the Alzheimer's (Cummings et al. 2020). Similarly, Parkinson's disease has also been treated in animal models with MSCs, ESCs, NSCs and iPSCs with very encouraging outcomes (Fan and Ng 2020). While not in a clinical trial yet, these therapies do provide a hope for a curative approach in the future (Jarrin et al. 2021).

11.4.1.2 Biomaterial-Based Approaches

Biomaterials in brain injury are primarily used as carriers or scaffolds for cell treatments or biomolecule delivery, rather than as a therapeutic agent by themselves. The biomaterials are generally used in an injectable hydrogel format, or a scaffold format that needs to be surgically implanted (Wang et al. 2018). Either of these formats can be made with natural compounds such as alginate (Pawar et al. 2015), cellulose (Wang et al. 2012), chitosan (Azadi et al. 2013; Mo et al. 2010), collagen (Gil and del Río 2012), fibrin (Tate et al. 2009), gelatin (Lim et al. 2012; Lozano et al. 2015) and hyaluronic acid (Moshayedi et al. 2016), or from synthetic polymers such as poly caprolactone (PCL) (Nisbet et al. 2009; Fon et al. 2014), poly ethylene glycol (PEG) (Bjugstad et al. 2010; Lampe et al. 2011), poly hydroxyethyl methacrylate (pHEMA) (Jhaveri et al. 2009), Poly lactic-*co*-glycolic acid (PLGA) (Álvarez et al. 2014) and polyurethane (PU) (Hsieh et al. 2015).

From the natural compounds, alginate hydrogel was used by itself to aid the axonal regrowth, but the rest were used with stem cells and/or neurotrophic factors. In addition to acting as the carriers/scaffolds, the biomaterials also offer some additional desirable properties, such as support to the cells to enhance their survival, proliferation, anti-inflammatory support, axonal guidance and controlled release of the biomolecules that they may carry (Wang et al. 2018). While highly useful, as mentioned above, biomaterials have taken more of a supportive role in brain injury repairs. As such none of the biomaterials have reached clinical trials as a therapeutic approach by themselves.

11.4.1.3 Bioactive Molecule-Based Approaches

As discussed above, several neurotrophic factors have important roles to play in neuroprotection and regeneration. These include VEGF, platelet-derived growth factor (PDGF), nerve growth factor (NGF), FGF, epidermal growth factor (EGF) and glial cell-line derived neurotrophic factor (GDNF) (Ng and Lee 2019).

VEGF administration activates Akt and MEK/ERK pathways, enhances angiogenesis and neurogenesis and enhances neurite outgrowth by Rho inhibition after a TBI (Thau-Zuchman et al. 2010; Lu et al. 2010; Jin et al. 2006). NGF and BDNF enhance neuronal survival and regeneration following TBI and cerebral ischaemia in animal models (Namiki et al. 2000). Similarly, FGF and EGF have also shown to reduce neuronal apoptosis and enhance neurogenesis by inducing the progenitor cells (Laskowski et al. 2005). GDNF is recently being tried to treat degenerative disease such as Parkinson's and Huntington's, due to its ability to protect dopaminergic neurons (Cheng et al. 2018).

Additionally, a few innovative genetic approaches are under investigation to enhance neuro-regeneration. A novel technique known as DNA vaccination is used to elicit an immune response in a rat TBI and stroke models against molecules such as NOGO, MAG and OMGP, which inhibit axonal regrowth (Zhu et al. 2007; Zhang et al. 2009).

A recent discovery of exosomes—the small size vesicles of 50–200 nm diameter containing proteins, RNA, miRNAs, carrying out intercellular signalling (Taylor and Gercel-Taylor 2013)—is being explored as a potential approach. Exosomes made from MSCs were systemically injected in rat and mouse TBI models, upon which restoration of somatic and cognitive functions was observed, in addition to neurogenesis, angiogenesis and reduced inflammatory responses (Zhang et al. 2015; Kim et al. 2016). Since then, it has been discovered that exosomes with miR-17-92 can promote neurogenesis, axonal regrowth and oligodendrogenesis after stroke (Xin et al. 2017), and miR-132 can help regulate vascular integrity in brain (Xu et al. 2017). Thus, exosomes represent a very promising, cell free, novel approach that can provide all the benefits of a cell's secretome, without the risks associated with cell transplantation, immuno-compatibility, and graft rejection.

11.4.2 Spinal Cord Injury

Similar to brain injury, the approaches to help repair the SCI can also be classified as: (1) Cell transplantation-based approaches, (2) biomaterial-based approaches and (3) bioactive molecule-based approaches. In addition to the stand-alone approaches, there are also (4) combination-based approaches that combine one or more of the above-mentioned approaches to achieve synergistic effects.

11.4.2.1 Cell Transplantation-Based Approaches

Numerous different stem and non-stem cells are being investigated for SCI repair. The cells in question are expected to possess one or more of the following properties: (1) functionally viable cellular regeneration in CNS, (O'Shea et al. 2017; Ahuja et al. 2017; Lu et al. 2012; Kadoya et al. 2016; Rosenzweig et al. 2018) (2) neurotrophic support to induce and enhance axonal regrowth, (Kanekiyo et al. 2018) (3) revascularisation by angiogenesis, (Li et al. 2014) (4) nerve guidance to bridge the gap and reconnect severed axons (Reshamwala et al. 2019) and (5) immunomodulation to control or mitigate secondary injury or scarring (Han et al. 2016; Torres-Espín et al. 2013). The transplanted cells may achieve cellular regeneration by either activating the dormant progenitor cells in the host via secretome/paracrine effects or differentiating into mature cells themselves (applicable only to stem cells) (Kobolak et al. 2016; Curtis et al. 2018).

The cell transplantation-based therapies, despite their tremendous promise in pre-clinical trials, are facing significant challenges that reduce the reliability and robustness of their scientific evidence of success. Poor survival of cells in vivo (Anderson et al. 2017; Curtis et al. 2018; Reshamwala et al. 2019), poor integration and migration out of the injury site (Tuszynski et al. 2014; Steward et al. 2014;

Reshamwala et al. 2019), uncontrolled differentiation or proliferation and tumorigenic potential (Johnson et al. 2010a; Nguyen et al. 2017) and anomalous axonal regrowth causing hyperalgesia or allodynia (Hofstetter et al. 2005) are some of the most important factors. Thus, enhancing the cell survival post transplantation, controlling the cell integration and migration, regulating the differentiation and fate of the stem cells are main objectives of the ongoing research (Vismara et al. 2017; Lin and Du 2018; Reshamwala et al. 2019; Reshamwala 2020). Another logistic challenge is to determine optimal timing of cell transplantation after the injury. If the cells are transplanted too soon, the acute inflammatory process may render the injury site too hostile for the transplanted cells to survive. On the contrary, too much delay may result in loss of all neuronal plasticity and regenerative potential of the therapy (Chhabra and Sarda 2017). Hence, studies have found that subacute stage transplantations usually show better outcomes than acute or chronic stage transplantations (O'Shea et al. 2017; Reshamwala et al. 2019).

Some of the preferred cell types for SCI repairs are amniotic foetal mesenchymal stem cells (AF-MSCs), AD-MSCs, BM-MSCs, ESCs, UCB-MSCs, iPSCs, olfactory ensheathing cells (OECs) and Schwann cells (SCs) (Vismara et al. 2017; Ashammakhi et al. 2019). There are several pre-clinical trials reported using these cells; however, due to obvious ethical and logistic concerns, clinical translation of approaches using AF-MSCs and iPSCs has not been done. Additionally, AD-MSCs and ESCs have only been trialled clinically in a very limited capacity a few times. Other than these, a few more cell types have also been investigated with varying levels of success such as oligodendrocyte precursor cells (OPCs), neural progenitor cells (NPCs) (Ashammakhi et al. 2019), endothelial progenitor cells (EPCs), amniotic epithelial cells (AECs), keratinocytes, mast cells, mononuclear cells, T cells and also genetically modified cells (Przekora and Juszkiewicz 2020; Ryabov et al. 2020; Rosenzweig et al. 2018; Saremi et al. 2021). Unfortunately, none of these cell-based therapies have progressed beyond phase I/IIa trials, likely due to the challenges explained previously.

These cell types are chosen because of their own unique neuroprotective and/or regenerative properties. ESCs, iPSCs and NPCs help reconstitute and regenerate the lost neurons and white matter in the injury site. Different types of MSCs have anti-apoptotic (neuroprotective), angiogenic and anti-inflammatory properties; they can provide neurotrophic support and mitigate myelin loss after the injury. OECs are known for their ability to facilitate axonal regeneration, provide directional guidance to the growing axons and offer trophic support to the growing axons. SCs, on the other hand, myelinate the axons and sustain their regrowth (Ashammakhi et al. 2019; Reshamwala et al. 2020).

11.4.2.2 Biomaterial-Based Approaches

Biomaterials form a rather sizeable portion of tissue engineering, which, in turn, is a crucial arm of exploratory regenerative medicine. Thus, biomaterials either by themselves, or in combination with cells or bioactive agents, have been extensively explored for nerve repair and neuro regeneration. Biomaterials can serve as carriers or delivery mechanisms for the cells or drugs (discussed later), or they may help

provide mechanical strength to friable injured tissues, or favourably modulate the host microenvironment, replace extra cellular matrix, help guide regrowing axons and fill the defect by physically bridging the gap (Ashammakhi et al. 2019).

Many different formats and structure types of biomaterials have been tried for SCI repairs including scaffolds (Guest et al. 2018), conduits (He et al. 2009), sheets (Kim et al. 2011), fibres (Tysseling-Mattiace et al. 2008) and hydrogels (Macaya and Spector 2012). While conduits, sheets and fibres are used in SCI repairs, they are found to be more favourable for PNI repairs. The biomaterials for CNI repairs are expected to be biodegradable and easily injectable with minimally invasive surgical approaches (Vismara et al. 2017) and should not compress or sheer/stress the surrounding CNS parenchyma. Hydrogels are more commonly preferred for CNIs, at least in the acute phase, as they can easily be modified to accommodate these criteria. However, conduits and fibres etc. may be used in later stages (Ziemba and Gilbert 2017). So far, many natural, synthetic and hybrid biomaterials have been tried. Alginate, hyaluronic acid, collagen, agarose etc. natural hydrogel forming materials have been experimented with over the years; however, fibrin is emerging as a more promising material, since it is native to human body, is easily digested by cellular enzymes and is already approved for clinical use for dura repairs (Reshamwala 2020; Ashammakhi et al. 2019). Synthetic polymeric biomaterials such as PLGA, poly-L-lysine (PLL), PCL, pHEMA, PEG have also shown encouraging results in pre-clinical trials. The benefit of these materials is that they have been extensively tried for in vivo use in other tissues and been found safe, many of them have been approved for clinical use in other tissues as well and their biodegradation rate is programmable by the users.

The field of biomaterials is too vast to summarise briefly, and the available biomaterials and their possible formulations are essentially endless; however, only one biomaterial has successfully reached clinical testing phase for neuroregeneration so far (Theodore et al. 2016).

11.4.2.3 Bioactive Molecule-Based Approaches

As identified in the potential therapeutic strategies earlier, several molecular events may be modulated to achieve neuro-protective and regenerative outcomes. Several novel molecules have been investigated in pre-clinical and clinical settings, showing promising results.

So far, Riluzole, Minocycline, magnesium, hepatocyte growth factor (HGF) and granulocyte colony stimulating factor (G-CSF) have shown promise as potential acute phase neuroprotective agents in preclinical studies (AOSpine North America Research Network 2016; Casha 2013; Takahashi et al. 2012; Acorda Therapeutics 2018; Kringle Pharma Inc. 2019). Many attempts at improving CNS axonal sprouting have also shown encouraging outcomes. These approaches usually target, inhibit and/or neutralise the molecular pathways responsible for axonal regrowth inhibition in the CNS, such as NOGO (by using anti-NOGO antibodies), Rho-ROCK and PTEN (a downstream target of NOGO-Rho-ROCK pathway) (Bregman et al. 1995; Danilov and Steward 2015; Lewandowski and Steward 2014; Ashammakhi et al. 2019). Another such promising candidate Cethrin

(VX-120), a Rho pathway inhibitor, showed great promise in phase I/IIa trials, but failed to show efficacy in phase IIb and the trial was ceased (Vertex Pharmaceuticals Inc. 2018). Connective tissue growth factor (CTGF) has been found to promote neuro-regeneration and Pregabalin (an approved drug for neuropathic pain) was demonstrated to inhibit axonal growth inhibitors in pre-clinical studies (Mokalled et al. 2016; Tedeschi et al. 2016).

Furthermore, immunomodulation has also been thoroughly investigated for SCI treatments. Several approaches including delivery of chemokines such as interleukin-4 (IL-4), IL-10 and inhibition of IL-7 have shown enhanced SCI repairs in rodents (Bao et al. 2018; Park et al. 2018; Margul et al. 2016; Mokarram et al. 2017). Tissue growth factors such as insulin like growth factor 1 (IGF-1), VEGF, transforming growth factor β1 (TGF-β1) are also promising candidates for neuroprotection and axonal regrowth stimulation (Nakano et al. 2010) and are commonly found in the stem cell conditioned media. Similarly, oligodendrocyte-lineage differentiation factors such as sonic-hedgehog, PDGF and noggin have shown encouraging results in mouse model for treatment of acute phase SCI (Smith et al. 2019; Thomas et al. 2014). Although several promising approaches have been tried pre-clinically and clinically over the last few decades, unfortunately no pharmaceutical agents have been approved so far as a treatment for neuro-regeneration by a regulatory legislative body such as the USFDA (Ashammakhi et al. 2019).

11.4.2.4 Combination-Based Approaches

As mentioned before, combining biomaterials with cells, drugs, growth factors, or more than one of these agents are all promising combination-based approaches for neuro-regeneration (Kadoya et al. 2016; Wilems et al. 2015; Johnson et al. 2010a, b; Rosenzweig et al. 2018; Liu et al. 2017; Führmann et al. 2016, 2018). Studies have commonly reported combining stem cells with hydrogel scaffolding (Agbay et al. 2016), growth factors (Nagashima et al. 2017), anti-inflammatory agents (Führmann et al. 2018) or transfection vectors to genetically modify transplanted cells (Liu et al. 2017) in the recent years. These approaches aim to improve cell survival and integration post-transplantation, and enhance axonal outgrowth as well as provide them better guidance. Some recent studies have also incorporated physical therapy or rehabilitation with cell transplantation (Wang et al. 2016) or bioactive molecules (Chen et al. 2017) for enhanced outcomes.

An approach combining stem cells in a favourable biomaterial scaffolding with growth factors to augment their function is likely to show synergetic effects and yield superior outcomes (Rosenzweig et al. 2018; Lu et al. 2012).

11.4.3 Peripheral Nerve Injury

Innovation in PNIs is a very rich and thriving field of tissue engineering. Although less devastating than the CNIs in general, PNIs are much more common and quite debilitating to the patients. Therefore, there is immense interest in improving the

existing approaches as well as developing novel approaches to repair PNIs more efficiently, to recover more of the lost function faster and more consistently. In spite of this, much of the novel and promising work has not been successfully translated into clinical or commercial products (Carvalho et al. 2019).

As with CNIs, the approaches for PNI repairs can also be categorised into the following: (1) cell-based approaches, (2) biomaterial-based approaches, (3) bioactive molecule-based approaches and (4) combination-based and other approaches.

11.4.3.1 Cell-Based Approaches

Several types of cells have been trialled for PNI repairs. Same as CNI, different types of MSCs have been very commonly studied. These include BM-MSCs (Kubiak et al. 2020) as one of the most frequently used cells, AD-MSCs (Zhang et al. 2018), UBC-MSCs (Guo et al. 2015), NSCs etc. In addition to the stem cells, SCs have been the focus significant research as the primary glial cells of the PNS (Pearse et al. 2018), and OECs have also been used in a number of studies (Woodhall et al. 2001). Interestingly, some recent studies have also used genetically modified SCs to enhance their function and yield improved functional outcomes (Marquardt et al. 2015; Huang et al. 2015). Unlike in CNI repair, the stem cells in PNIs are used for their ability to differentiate into tissue specific cell types i.e. SCs (Ching et al. 2018) and secretion of neurotrophic factors (Kubiak et al. 2020). Studies have found that after the stem cell treatments, better axonal regrowth was observed, the nerve fibres were myelinated and there was complimentary improvement in functional regain (Mimura et al. 2004). UBC-MSCs are shown to secrete up to 14 different growth factors that ultimately enhance neuronal survival, vascularisation, integrin upregulation, enhanced anti-inflammatory activity and better survival and proliferation of SCs (Ma et al. 2019). OECs are also used, albeit in a limited capacity, in PNI repairs for their ability to produce neurotrophins such as NGF, BDNF, neurotrophin–3 (NT-3), NT-4/5, BDNF and GDNF (Woodhall et al. 2001). The genetically modified SCs were programmed to overexpress c-Jun, a constituent of the AP-1 transcription factor. This enables the SCs to produce several growth factors such as NGF, GDNF, BDNF, artemin and leukaemia inhibitory factor (Huang et al. 2018). However, the use of autologous SCs remains the gold standard for cell-based approaches for PNI repairs (Galgano et al. 2017) as they are the native resident glia of the PNS, and they play an active role in debris clearance and axonal guidance during nerve regeneration.

Recently, there have been reports of dental pulp stem cells (Lavorato et al. 2021; Luo et al. 2021; Rayner et al. 2020) and skin-derived precursor cells (McKenzie et al. 2006) being used for PNI repairs. These cells are of neural crest origin, similar to the SCs, they are easy to access and autologously harvest, and they have the ability to differentiate into other cells of neural crest origin such as the peripheral neurons and SCs or Schwann-like cells (Lopes et al. 2022).

11.4.3.2 Biomaterial-Based Approaches

Several biomaterials-based nerve conduits are already commercially available for clinical use. However, they still remain predominant in novel approaches under

investigation for PNI repair; newer technologies in tissue engineering and stem cell advances are being integrated with neural scaffold fabrication to achieve better outcomes (Yi et al. 2019). Some of the most commonly used natural materials include chitosan, silk fibroin and collagen, laminin, fibronectin, elastin etc. ECM components. Synthetic materials are generally polymers made of silicon, polyglycolic acid (PGA), PLGA, PCL and other similar clinical grade compounds. In some instances, scaffolds have been fabricated with bioglass, natural ECM, ceramics and metallic compounds (Yi et al. 2019).

Using these polymers, PNI defects of 10–15 mm have been treated successfully in rodent models over the last few decades (Urabe et al. 1996; Kim et al. 2014). Silicon has been proven safe and effective for clinical use in the early 1990s (Lundborg et al. 1991, 1994).

An advantage of scaffolds using natural materials like chitosan is that the outcomes were comparable to autologous nerve grafting (a current clinical gold standard) in recent studies (Meyer et al. 2016; Stenberg et al. 2016, 2017). ECM materials such as collagen are much better tolerated by the recipient (Waitayawinyu et al. 2007). Laminin scaffolds are reportedly better at integration and supporting neurite growth (Neal et al. 2009). Several approaches using combined natural and synthetic materials have also seen highly favourable outcomes (Neal et al. 2012).

Several tissue-engineered nerve grafts have recently been approved for clinical trials by the USFDA, Conformit EU and China FDA, and have shown very encouraging outcomes. These materials include collagen for large diameter, small size defect repairs (Dienstknecht et al. 2013; Klein et al. 2016; Taras et al. 2011), collagen sponge filled inside PGA tube for defect size upto 65 mm (Inada et al. 2004), and hollowed out chitosan/PGA hybrids nerve graft used for 35 mm long PNI (Fan et al. 2008; Yi et al. 2019).

11.4.3.3 Bioactive Molecule-Based Approaches

As explained above, a significant aspect of PNI repair approaches is the secretion of neurotrophic factors. Additionally, several of these neurotrophic factors are naturally released following an injury (Tajdaran et al. 2019). Therefore, direct delivery of the same has also been explored as a potentially regenerative approach. NGF, glial growth factor (GGF/neuregulin), GDNF, NT-3 (Grinsell and Keating 2014), BDNF (Lopes et al. 2017), and VEGF (Hobson et al. 2000) have been frequently tried with promising successes in vitro as well as in vivo.

NGF is one of the most studied bioactive molecules for PNI repair. It stimulates axonal regrowth in the sympathetic and sensory neurons (Shakhbazau et al. 2012). NGF has shown some interesting outcomes in combination with VEGF. Despite its strong angiogenic activity, VEGF is studied for its neuroprotective and/or neurotropic properties (Hobson et al. 2000). BDNF was first recognised for its role in the hippocampal neurogenesis—specifically in learning, memory formation and synaptic plasticity; however, it is also secreted in PNS by SCs, motor neurons and dorsal root ganglia (DRGs) after PNI (Carvalho et al. 2019). NT-3 has shown neurotrophic activity similar to NGF and BDNF with a wider spectrum (Maisonpierre et al. 1990).

GDNF strongly enhances survival of injury neurons and outgrowth of injured nerves in PNS (Xu et al. 2013).

11.4.3.4 Combination-Based and Other Approaches

Combination of cell-based approaches with biomaterials and/or bioactive molecules is commonly employed for PNI repair studies. In a recent study, BM-MSCs were combined with platelet rich plasma and a scaffold which showed promising objective nerve regeneration in sheep model (Shi et al. 2019). Another such popular approach is to combine nerve guiding conduits and growth factors such as GDNF and NGF (Carvalho et al. 2021), where the conduit also acts as a reservoir for slow sustained release of the growth factors—creating and maintaining a favourable microenvironment for nerve regeneration (Meena et al. 2021).

In other novel approaches, a few noteworthy approaches are as follows: (1) photochemical tissue bonding to enhance upon the suture-based coaptation technique (Lanier et al. 2021; Fairbairn et al. 2016). (2) Axonal fusion using PEG in acute phase injuries (Lanier et al. 2021; Paskal et al. 2019). (3) Combination of low frequency electrical stimulation with physical exercise (Vijayavenkataraman 2020; Alvites et al. 2018; Modrak et al. 2020). And (4) using non-invasive stimulation techniques such as magnetic stimulation and low frequency ultrasound stimulation for nerve regeneration (Alvites et al. 2018; Lopes et al. 2022).

11.5 Translational Needs

The translation of novel regenerative approaches from preclinical to clinical setting is the most critical step where an astonishing number of seemingly effective approaches are failing either to show efficacy or for feasibility from a practical standpoint. There are several additional challenges which make the translational research even more difficult.

In a stem cell-based clinical trial, it was seen that there were significant differences between the stem cells used for rat studies and the cells prepared for the clinical trial. The rodent studies showed encouraging SCI repairs, but the subsequent clinical trial failed to show efficacy, and worse, some negative effects were also seen (Anderson et al. 2017), which understandably hindered the clinical translation of the approach. Several other therapies have not yet progressed to clinical experimentation despite their remarkable performance in pre-clinical trials. Some of the commonly faced challenges include high financial costs, lack of translatable delivery mechanisms, cross-species differences between the cells of choice and their identifying marker expression and availability of species appropriate/ethical sources of the cells.

In regard with the biomaterial-based approaches, a completely different set of hurdles prevent efficient clinical translation. Most of the approaches showing encouraging early outcomes still need to meet the minimum safety and efficacy criteria, which require extensive experimentation. Different researchers using similar biomaterials may have used different animal models or biomaterial formulations

leading varying results. This gives an appearance of significant disparity in outcomes and creates further difficulty in securing required funding and approvals for clinical translation.

While the combination-based approaches may have a better potential for regeneration, the added components increase the complexity for clinical translation. The burden of proof of safety and efficacy increases for each added therapeutic component. Different translational regulations and requirements internationally also make it more challenging to validate any new findings at a global scale.

References

Acorda Therapeutics (2018) AC105 in patients with acute traumatic spinal cord injury [Online]. https://ClinicalTrials.gov/show/NCT01750684 [Accessed]

Adoni A, McNett M (2007) The pupillary response in traumatic brain injury: a guide for trauma nurses. J Trauma Nurs 14:191–196; quiz 197–8

Agbay A, Edgar JM, Robinson M, Styan T, Wilson K, Schroll J, Ko J, Mohtaram NK, Jun MB-G, Willerth SM (2016) Biomaterial strategies for delivering stem cells as a treatment for spinal cord injury. Cells Tissues Organs 202:42–51

Ahuja CS, Nori S, Tetreault L, Wilson J, Kwon B, Harrop J, Choi D, Fehlings MG (2017) Traumatic spinal cord injury—repair and regeneration. Neurosurgery 80:S9–S22

Al-Qattan MM (2002) End-to-side nerve repair. J Hand Surg Am 27:739; author reply 739–40

Álvarez Z, Castaño O, Castells AA, Mateos-Timoneda MA, Planell JA, Engel E, Alcántara S (2014) Neurogenesis and vascularization of the damaged brain using a lactate-releasing biomimetic scaffold. Biomaterials 35:4769–4781

Alvites R, Rita Caseiro A, Santos Pedrosa S, Vieira Branquinho M, Ronchi G, Geuna S, Varejão ASP, Colette Maurício A (2018) Peripheral nerve injury and axonotmesis: state of the art and recent advances. Cogent Med 5:1466404

American Spinal Injury Association (n.d.) Standard neurological classification of spinal cord injury. ASIA Impairment Scale Guidelines. ASIA

Anderson AJ, Piltti KM, Hooshmand MJ, Nishi RA, Cummings BJ (2017) Preclinical efficacy failure of human CNS-derived stem cells for use in the pathway study of cervical spinal cord injury. Stem Cell Reports 8:249–263

AOSpine North America Research Network (2016) Riluzole in spinal cord injury study (RISCIS). ClinicalTrials.gov

Arsenijevic Y, Villemure JG, Brunet JF, Bloch JJ, Déglon N, Kostic C, Zurn A, Aebischer P (2001) Isolation of multipotent neural precursors residing in the cortex of the adult human brain. Exp Neurol 170:48–62

Ashammakhi N, Kim HJ, Ehsanipour A, Bierman RD, Kaarela O, Xue C, Khademhosseini A, Seidlits SK (2019) Regenerative therapies for spinal cord injury. Tissue Eng Part B Rev 25:471–491

Azadi A, Hamidi M, Rouini MR (2013) Methotrexate-loaded chitosan nanogels as 'Trojan Horses' for drug delivery to brain: preparation and in vitro/in vivo characterization. Int J Biol Macromol 62:523–530

Bao C, Wang B, Yang F, Chen L (2018) Blockade of interleukin-7 receptor shapes macrophage alternative activation and promotes functional recovery after spinal cord injury. Neuroscience 371:518–527

Bartanusz V, Jezova D, Alajajian B, Digicaylioglu M (2011) The blood-spinal cord barrier: morphology and clinical implications. Ann Neurol 70:194–206

Bassilios Habre S, Bond G, Jing XL, Kostopoulos E, Wallace RD, Konofaos P (2018) The surgical management of nerve gaps: present and future. Ann Plast Surg 80:252–261

Bhandari PS (2019) Management of peripheral nerve injury. J Clin Orthop Trauma 10:862–866
Bishop J, Ring D (2009) Management of radial nerve palsy associated with humeral shaft fracture: a decision analysis model. J Hand Surg Am 34:991–6.e1
Bjugstad K, Lampe K, Kern D, Mahoney M (2010) Biocompatibility of poly (ethylene glycol)-based hydrogels in the brain: an analysis of the glial response across space and time. J Biomed Mater Res A 95:79–91
Blurton-Jones M, Kitazawa M, Martinez-Coria H, Castello NA, Müller F-J, Loring JF, Yamasaki TR, Poon WW, Green KN, Laferla FM (2009) Neural stem cells improve cognition via BDNF in a transgenic model of Alzheimer disease. Proc Natl Acad Sci 106:13594–13599
Bouma GJ, Muizelaar JP, Choi SC, Newlon PG, Young HF (1991) Cerebral circulation and metabolism after severe traumatic brain injury: the elusive role of ischemia. J Neurosurg 75: 685–693
Bouma GJ, Muizelaar JP, Stringer WA, Choi SC, Fatouros P, Young HF (1992) Ultra-early evaluation of regional cerebral blood flow in severely head-injured patients using xenon-enhanced computerized tomography. J Neurosurg 77:360–368
Bracken MB, Holford TR (2002) Neurological and functional status 1 year after acute spinal cord injury: estimates of functional recovery in National Acute Spinal Cord Injury Study II from results modeled in National Acute Spinal Cord Injury Study III. J Neurosurg Spine 96:259–266
Bregman BS, Kunkel-Bagden E, Schnell L, Dai HN, Gao D, Schwab ME (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. Nature 378:498–501
Brosius Lutz A, Barres BA (2014) Contrasting the glial response to axon injury in the central and peripheral nervous systems. Dev Cell 28:7–17
Brunet JF, Pellerin L, Arsenijevic Y, Magistretti P, Villemure JG (2002) A novel method for in vitro production of human glial-like cells from neurosurgical resection tissue. Lab Invest 82:809–812
Brunet JF, Pellerin L, Magistretti P, Villemure JG (2003) Cryopreservation of human brain tissue allowing timely production of viable adult human brain cells for autologous transplantation. Cryobiology 47:179–183
Bullock MR, Chesnut R, Ghajar J, Gordon D, Hartl R, Newell DW, Servadei F, Walters BC, Wilberger JE (2006) Surgical management of acute subdural hematomas. Neurosurgery 58: S16–S24; discussion Si–iv
Capizzi A, Woo J, Verduzco-Gutierrez M (2020) Traumatic brain injury: an overview of epidemiology, pathophysiology, and medical management. Med Clin North Am 104:213–238
Carvalho CR, Oliveira JM, Reis RL (2019) Modern trends for peripheral nerve repair and regeneration: beyond the hollow nerve guidance conduit. Front Bioeng Biotechnol 7:337
Carvalho CR, Chang W, Silva-Correia J, Reis RL, Oliveira JM, Kohn J (2021) Engineering silk fibroin-based nerve conduit with neurotrophic factors for proximal protection after peripheral nerve injury. Adv Healthc Mater 10:2000753
Casha S (2013) Minocycline in acute spinal cord injury (MASC). NCT01828203. ClinicalTrials.gov
Cashman CR, Hoke A (2015) Mechanisms of distal axonal degeneration in peripheral neuropathies. Neurosci Lett 596:33–50
Castanov V, Berger M, Ritsma B, Trier J, Hendry JM (2021) Optimizing the timing of peripheral nerve transfers for functional re-animation in cervical spinal cord injury: a conceptual framework. J Neurotrauma 38:3365–3375
Chang EH, Adorjan I, Mundim MV, Sun B, Dizon ML, Szele FG (2016) Traumatic brain injury activation of the adult subventricular zone neurogenic niche. Front Neurosci 10:332
Chen K, Marsh BC, Cowan M, Al'Joboori YD, Gigout S, Smith CC, Messenger N, Gamper N, Schwab ME, Ichiyama RM (2017) Sequential therapy of anti-Nogo-A antibody treatment and treadmill training leads to cumulative improvements after spinal cord injury in rats. Exp Neurol 292:135–144
Cheng S, Tereshchenko J, Zimmer V, Vachey G, Pythoud C, Rey M, Liefhebber J, Raina A, Streit F, Mazur A, Bähr M, Konstantinova P, Déglon N, Kügler S (2018) Therapeutic efficacy of

regulable GDNF expression for Huntington's and Parkinson's disease by a high-induction, background-free "GeneSwitch" vector. Exp Neurol 309:79–90
Chhabra HS, Sarda K (2017) Clinical translation of stem cell based interventions for spinal cord injury—are we there yet? Adv Drug Deliv Rev 120:41–49
Ching RC, Wiberg M, Kingham PJ (2018) Schwann cell-like differentiated adipose stem cells promote neurite outgrowth via secreted exosomes and RNA transfer. Stem Cell Res Ther 9:1–12
Cummings J, Lee G, Ritter A, Sabbagh M, Zhong K (2020) Alzheimer's disease drug development pipeline: 2020. Alzheimers Dement 6:e12050
Curtis E, Martin JR, Gabel B, Sidhu N, Rzesiewicz TK, Mandeville R, Van Gorp S, Leerink M, Tadokoro T, Marsala S (2018) A first-in-human, phase I study of neural stem cell transplantation for chronic spinal cord injury. Cell Stem Cell 22(941–950):e6
Danilov CA, Steward O (2015) Conditional genetic deletion of PTEN after a spinal cord injury enhances regenerative growth of CST axons and motor function recovery in mice. Exp Neurol 266:147–160
Davanzo JR, Sieg EP, Timmons SD (2017) Management of traumatic brain injury. Surg Clin North Am 97:1237–1253
Dienstknecht T, Klein S, Vykoukal J, Gehmert S, Koller M, Gosau M, Prantl L (2013) Type I collagen nerve conduits for median nerve repairs in the forearm. J Hand Surg Am 38:1119–1124
Dixon KJ, Theus MH, Nelersa CM, Mier J, Travieso LG, Yu T-S, Kernie SG, Liebl DJ (2015) Endogenous neural stem/progenitor cells stabilize the cortical microenvironment after traumatic brain injury. J Neurotrauma 32:753–764
Fairbairn NG, Ng-Glazier J, Meppelink AM, Randolph MA, Winograd JM, Redmond RW (2016) Improving outcomes in immediate and delayed nerve grafting of peripheral nerve gaps using light-activated sealing of neurorrhaphy sites with human amnion wraps. Plast Reconstr Surg 137:887–895
Fan Y, Ng S-Y (2020) Replacing what's lost: a new era of stem cell therapy for Parkinson's disease. Transl Neurodegener 9:1–10
Fan W, Gu J, Hu W, Deng A, Ma Y, Liu J, Ding F, Gu X (2008) Repairing a 35-mm-long median nerve defect with a chitosan/PGA artificial nerve graft in the human: a case study. Microsurgery 28:238–242
Fehlings MG, Perrin RG (2005) The role and timing of early decompression for cervical spinal cord injury: update with a review of recent clinical evidence. Injury 36(Suppl 2):B13–B26
Fon D, Zhou K, Ercole F, Fehr F, Marchesan S, Minter MR, Crack PJ, Finkelstein DI, Forsythe JS (2014) Nanofibrous scaffolds releasing a small molecule BDNF-mimetic for the re-direction of endogenous neuroblast migration in the brain. Biomaterials 35:2692–2712
Führmann T, Tam R, Ballarin B, Coles B, Donaghue IE, van der Kooy D, Nagy A, Tator C, Morshead C, Shoichet M (2016) Injectable hydrogel promotes early survival of induced pluripotent stem cell-derived oligodendrocytes and attenuates longterm teratoma formation in a spinal cord injury model. Biomaterials 83:23–36
Führmann T, Anandakumaran PN, Payne SL, Pakulska MM, Varga BV, Nagy A, Tator C, Shoichet MS (2018) Combined delivery of chondroitinase ABC and human induced pluripotent stem cell-derived neuroepithelial cells promote tissue repair in an animal model of spinal cord injury. Biomed Mater 13:024103
Galgano M, Toshkezi G, Qiu X, Russell T, Chin L, Zhao L-R (2017) Traumatic brain injury: current treatment strategies and future endeavors. Cell Transplant 26:1118–1130
Galindo LT, Filippo TR, Semedo P, Ariza CB, Moreira CM, Camara NO, Porcionatto MA (2011) Mesenchymal stem cell therapy modulates the inflammatory response in experimental traumatic brain injury. Neurol Res Int 2011:564089
Gil V, del Río JA (2012) Analysis of axonal growth and cell migration in 3D hydrogel cultures of embryonic mouse CNS tissue. Nat Protoc 7:268–280
Griffin JW, Hogan MV, Chhabra AB, Deal DN (2013) Peripheral nerve repair and reconstruction. J Bone Joint Surg Am 95:2144–2151

Grinsell D, Keating CP (2014) Peripheral nerve reconstruction after injury: a review of clinical and experimental therapies. Biomed Res Int 2014:698256

Guest JD, Moore SW, Aimetti AA, Kutikov AB, Santamaria AJ, Hofstetter CP, Ropper AE, Theodore N, Ulich TR, Layer RT (2018) Internal decompression of the acutely contused spinal cord: differential effects of irrigation only versus biodegradable scaffold implantation. Biomaterials 185:284–300

Guo Z-Y, Sun X, Xu X-L, Zhao Q, Peng J, Wang Y (2015) Human umbilical cord mesenchymal stem cells promote peripheral nerve repair via paracrine mechanisms. Neural Regen Res 10:651

Hadley MN, Walters BC, Grabb PA, Oyesiku NM, Przybylski GJ, Resnick DK, Ryken TC (2002) Pharmacological therapy after acute cervical spinal cord injury. Neurosurgery 50:S63–S72

Hall ED, Braughler JM (1981) Acute effects of intravenous glucocorticoid pretreatment on the in vitro peroxidation of cat spinal cord tissue. Exp Neurol 73:321–324

Han S, Wang B, Li X, Xiao Z, Han J, Zhao Y, Fang Y, Yin Y, Chen B, Dai J (2016) Bone marrow-derived mesenchymal stem cells in three-dimensional culture promote neuronal regeneration by neurotrophic protection and immunomodulation. J Biomed Mater Res A 104:1759–1769

He L, Zhang Y, Zeng C, Ngiam M, Liao S, Quan D, Zeng Y, Lu J, Ramakrishna S (2009) Manufacture of PLGA multiple-channel conduits with precise hierarchical pore architectures and in vitro/vivo evaluation for spinal cord injury. Tissue Eng Part C Methods 15:243–255

Hobson MI, Green CJ, Terenghi G (2000) VEGF enhances intraneural angiogenesis and improves nerve regeneration after axotomy. J Anat 197:591–605

Hofstetter CP, Holmström NA, Lilja JA, Schweinhardt P, Hao J, Spenger C, Wiesenfeld-Hallin Z, Kurpad SN, Frisén J, Olson L (2005) Allodynia limits the usefulness of intraspinal neural stem cell grafts; directed differentiation improves outcome. Nat Neurosci 8:346–353

Hsieh FY, Lin HH, Hsu SH (2015) 3D bioprinting of neural stem cell-laden thermoresponsive biodegradable polyurethane hydrogel and potential in central nervous system repair. Biomaterials 71:48–57

Hu W, Liu J, Jiang J, Yang F (2016) [Effect of bone marrow mesenchymal stem cells on angiogenesis in rats after brain injury]. Zhong Nan Da Xue Xue Bao Yi Xue Ban 41:489–495

Huang L, Quan X, Liu Z, Ma T, Wu Y, Ge J, Zhu S, Yang Y, Liu L, Sun Z (2015) c-Jun gene-modified Schwann cells: upregulating multiple neurotrophic factors and promoting neurite outgrowth. Tissue Eng A 21:1409–1421

Huang L, Zhu L, Shi X, Xia B, Liu Z, Zhu S, Yang Y, Ma T, Cheng P, Luo K (2018) A compound scaffold with uniform longitudinally oriented guidance cues and a porous sheath promotes peripheral nerve regeneration in vivo. Acta Biomater 68:223–236

Huebner EA, Strittmatter SM (2009) Axon regeneration in the peripheral and central nervous systems. Results Probl Cell Differ 48:339–351

Hutchinson PJ, Corteen E, Czosnyka M, Mendelow AD, Menon DK, Mitchell P, Murray G, Pickard JD, Rickels E, Sahuquillo J, Servadei F, Teasdale GM, Timofeev I, Unterberg A, Kirkpatrick PJ (2006) Decompressive craniectomy in traumatic brain injury: the randomized multicenter RESCUEicp study. Springer, Vienna, pp 17–20. www.RESCUEicp.com

Ianof JN, Anghinah R (2017) Traumatic brain injury: an EEG point of view. Dement Neuropsychol 11:3–5

Inada Y, Morimoto S, Takakura Y, Nakamura T (2004) Regeneration of peripheral nerve gaps with a polyglycolic acid-collagen tube. Neurosurgery 55:640–648

Isaacs J (2010) Treatment of acute peripheral nerve injuries: current concepts. J Hand Surg Am 35: 491–497. quiz 498

Jarrin S, Cabré S, Dowd E (2021) The potential of biomaterials for central nervous system cellular repair. Neurochem Int 144:104971

Jhaveri SJ, Hynd MR, Dowell-Mesfin N, Turner JN, Shain W, Ober CK (2009) Release of nerve growth factor from HEMA hydrogel-coated substrates and its effect on the differentiation of neural cells. Biomacromolecules 10:174–183

Jiang S, Chen W, Zhang Y, Zhang Y, Chen A, Dai Q, Lin S, Lin H (2016) Acupuncture induces the proliferation and differentiation of endogenous neural stem cells in rats with traumatic brain injury. Evid Based Complement Alternat Med 2016:2047412

Jin K, Mao XO, Greenberg DA (2006) Vascular endothelial growth factor stimulates neurite outgrowth from cerebral cortical neurons via Rho kinase signaling. J Neurobiol 66:236–242

Jinadasa S, Boone MD (2016) Controversies in the management of traumatic brain injury. Anesthesiol Clin 34:557–575

Johnson PJ, Tatara A, McCreedy DA, Shiu A, Sakiyama-Elbert SE (2010a) Tissue-engineered fibrin scaffolds containing neural progenitors enhance functional recovery in a subacute model of SCI. Soft Matter 6:5127–5137

Johnson PJ, Tatara A, Shiu A, Sakiyama-Elbert SE (2010b) Controlled release of neurotrophin-3 and platelet-derived growth factor from fibrin scaffolds containing neural progenitor cells enhances survival and differentiation into neurons in a subacute model of SCI. Cell Transplant 19:89–101

Kadoya K, Lu P, Nguyen K, Lee-Kubli C, Kumamaru H, Yao L, Knackert J, Poplawski G, Dulin JN, Strobl H, Takashima Y, Biane J, Conner J, Zhang SC, Tuszynski MH (2016) Spinal cord reconstitution with homologous neural grafts enables robust corticospinal regeneration. Nat Med 22:479–487

Kanekiyo K, Wakabayashi T, Nakano N, Yamada Y, Tamachi M, Suzuki Y, Fukushima M, Saito F, Abe S, Tsukagoshi C (2018) Effects of intrathecal injection of the conditioned medium from bone marrow stromal cells on spinal cord injury in rats. J Neurotrauma 35:521–532

Kim H-J, Lee J-H, Kim S-H (2010) Therapeutic effects of human mesenchymal stem cells on traumatic brain injury in rats: secretion of neurotrophic factors and inhibition of apoptosis. J Neurotrauma 27:131–138

Kim H, Tator CH, Shoichet MS (2011) Chitosan implants in the rat spinal cord: biocompatibility and biodegradation. J Biomed Mater Res A 97:395–404

Kim D-Y, Choi Y-S, Kim S-E, Lee J-H, Kim S-M, Kim Y-J, Rhie J-W, Jun Y-J (2014) In vivo effects of adipose-derived stem cells in inducing neuronal regeneration in Sprague-Dawley rats undergoing nerve defect bridged with polycaprolactone nanotubes. J Korean Med Sci 29:S183–S192

Kim D-K, Nishida H, An SY, Shetty AK, Bartosh TJ, Prockop DJ (2016) Chromatographically isolated CD63+CD81+ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. Proc Natl Acad Sci 113:170

Klein S, Vykoukal J, Felthaus O, Dienstknecht T, Prantl L (2016) Collagen type I conduits for the regeneration of nerve defects. Materials 9:219

Kobolak J, Dinnyes A, Memic A, Khademhosseini A, Mobasheri A (2016) Mesenchymal stem cells: identification, phenotypic characterization, biological properties and potential for regenerative medicine through biomaterial micro-engineering of their niche. Methods 99:62–68

Kokaia Z, Martino G, Schwartz M, Lindvall O (2012) Cross-talk between neural stem cells and immune cells: the key to better brain repair? Nat Neurosci 15:1078–1087

Kringle Pharma Inc. (2019) Phase I/II study of KP-100IT in acute spinal cord injury

Kubiak CA, Grochmal J, Kung TA, Cederna PS, Midha R, Kemp SW (2020) Stem-cell–based therapies to enhance peripheral nerve regeneration. Muscle Nerve 61:449–459

Kukekov VG, Laywell ED, Suslov O, Davies K, Scheffler B, Thomas LB, O'Brien TF, Kusakabe M, Steindler DA (1999) Multipotent stem/progenitor cells with similar properties arise from two neurogenic regions of adult human brain. Exp Neurol 156:333–344

Kumar A, Loane DJ (2012) Neuroinflammation after traumatic brain injury: opportunities for therapeutic intervention. Brain Behav Immun 26:1191–1201

Lampe KJ, Kern DS, Mahoney MJ, Bjugstad KB (2011) The administration of BDNF and GDNF to the brain via PLGA microparticles patterned within a degradable PEG-based hydrogel: protein distribution and the glial response. J Biomed Mater Res A 96:595–607

Lanier ST, Hill JR, Dy CJ, Brogan DM (2021) Evolving techniques in peripheral nerve regeneration. J Hand Surg Am 46:695–701

Laskowski A, Schmidt W, Dinkel K, Martínez-Sánchez M, Reymann KG (2005) bFGF and EGF modulate trauma-induced proliferation and neurogenesis in juvenile organotypic hippocampal slice cultures. Brain Res 1037:78–89

Lavorato A, Raimondo S, Boido M, Muratori L, Durante G, Cofano F, Vincitorio F, Petrone S, Tartara F, Vercelli A (2021) Mesenchymal stem cell treatment perspectives in peripheral nerve regeneration: systematic review. Int J Mol Sci 22:572

Lewandowski G, Steward O (2014) AAVshRNA-mediated suppression of PTEN in adult rats in combination with salmon fibrin administration enables regenerative growth of corticospinal axons and enhances recovery of voluntary motor function after cervical spinal cord injury. J Neurosci 34:9951–9962

Li Z, Guo G, Wang G, Guan C, Yue L (2014) Influence of neural stem cell transplantation on angiogenesis in rats with spinal cord injury. Genet Mol Res 13:6083–6092

Lim TC, Toh WS, Wang L-S, Kurisawa M, Spector M (2012) The effect of injectable gelatin-hydroxyphenylpropionic acid hydrogel matrices on the proliferation, migration, differentiation and oxidative stress resistance of adult neural stem cells. Biomaterials 33:3446–3455

Lin L, Du L (2018) The role of secreted factors in stem cells-mediated immune regulation. Cell Immunol 326:24–32

Liu S, Sandner B, Schackel T, Nicholson L, Chtarto A, Tenenbaum L, Puttagunta R, Müller R, Weidner N, Blesch A (2017) Regulated viral BDNF delivery in combination with Schwann cells promotes axonal regeneration through capillary alginate hydrogels after spinal cord injury. Acta Biomater 60:167–180

Lopes CD, Gonçalves NP, Gomes CP, Saraiva MJ, Pêgo AP (2017) BDNF gene delivery mediated by neuron-targeted nanoparticles is neuroprotective in peripheral nerve injury. Biomaterials 121:83–96

Lopes B, Sousa P, Alvites R, Branquinho M, Sousa AC, Mendonça C, Atayde LM, Luís AL, Varejão ASP, Maurício AC (2022) Peripheral nerve injury treatments and advances: one health perspective. Int J Mol Sci 23:918

Lozano R, Stevens L, Thompson BC, Gilmore KJ, Gorkin R III, Stewart EM, in het Panhuis M, Romero-Ortega M, Wallace GG (2015) 3D printing of layered brain-like structures using peptide modified gellan gum substrates. Biomaterials 67:264–273

Lu K-T, Sun C-L, Wo PYY, Yen H-H, Tang T-H, Ng M-C, Huang M-L, Yang Y-L (2010) Hippocampal neurogenesis after traumatic brain injury is mediated by vascular endothelial growth factor receptor-2 and the Raf/MEK/ERK cascade. J Neurotrauma 28:441–450

Lu P, Wang Y, Graham L, Mchale K, Gao M, Wu D, Brock J, Blesch A, Rosenzweig ES, Havton LA (2012) Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. Cell 150:1264–1273

Lundborg G, Dahlin LB, Danielsen N (1991) Ulnar nerve repair by the silicone chamber technique. Scand J Plast Reconstr Surg Hand Surg 25:79–82

Lundborg G, Rosen B, Abrahamson S, Dahlin L, Danielsen N (1994) Tubular repair of the median nerve in the human forearm: preliminary findings. J Hand Surg 19:273–276

Luo L, He Y, Jin L, Zhang Y, Guastaldi FP, Albashari AA, Hu F, Wang X, Wang L, Xiao J (2021) Application of bioactive hydrogels combined with dental pulp stem cells for the repair of large gap peripheral nerve injuries. Bioact Mater 6:638–654

Ma Y, Dong L, Zhou D, Li L, Zhang W, Zhen Y, Wang T, Su J, Chen D, Mao C (2019) Extracellular vesicles from human umbilical cord mesenchymal stem cells improve nerve regeneration after sciatic nerve transection in rats. J Cell Mol Med 23:2822–2835

Macaya D, Spector M (2012) Injectable hydrogel materials for spinal cord regeneration: a review. Biomed Mater 7:012001

Mackay-Sim A, St John JA (2011) Olfactory ensheathing cells from the nose: clinical application in human spinal cord injuries. Exp Neurol 229:174–180

Mahmood A, Lu D, Chopp M (2004) Marrow stromal cell transplantation after traumatic brain injury promotes cellular proliferation within the brain. Neurosurgery 55:1185–1193

Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science 247:1446–1451
Margul DJ, Park J, Boehler RM, Smith DR, Johnson MA, McCreedy DA, He T, Ataliwala A, Kukushliev TV, Liang J (2016) Reducing neuroinflammation by delivery of IL-10 encoding lentivirus from multiple-channel bridges. Bioeng Transl Med 1:136–148
Marion DW, Darby J, Yonas H (1991) Acute regional cerebral blood flow changes caused by severe head injuries. J Neurosurg 74:407–414
Marmarou A, Lu J, Butcher I, Mchugh GS, Murray GD, Steyerberg EW, Mushkudiani NA, Choi S, Maas AIR (2007) Prognostic value of the Glasgow coma scale and pupil reactivity in traumatic brain injury assessed pre-hospital and on Enrollment: an IMPACT analysis. J Neurotrauma 24: 270–280
Marquardt LM, Ee X, Iyer N, Hunter D, Mackinnon SE, Wood MD, Sakiyama-Elbert SE (2015) Finely tuned temporal and spatial delivery of GDNF promotes enhanced nerve regeneration in a long nerve defect model. Tissue Eng A 21:2852–2864
Mastro-Martínez I, Pérez-Suárez E, Melen G, González-Murillo Á, Casco F, Lozano-Carbonero N, Gutiérrez-Fernández M, Díez-Tejedor E, Casado-Flores J, Ramírez-Orellana M (2015) Effects of local administration of allogenic adipose tissue-derived mesenchymal stem cells on functional recovery in experimental traumatic brain injury. Brain Inj 29:1497–1510
McDonald CM, Carter GT, Fritz RC, Anderson MW, Abresch RT, Kilmer DD (2000) Magnetic resonance imaging of denervated muscle: comparison to electromyography. Muscle Nerve 23: 1431–1434
McKenzie IA, Biernaskie J, Toma JG, Midha R, Miller FD (2006) Skin-derived precursors generate myelinating Schwann cells for the injured and dysmyelinated nervous system. J Neurosci 26: 6651–6660
Meena P, Kakkar A, Kumar M, Khatri N, Nagar RK, Singh A, Malhotra P, Shukla M, Saraswat SK, Srivastava S, Datt R, Pandey S (2021) Advances and clinical challenges for translating nerve conduit technology from bench to bed side for peripheral nerve repair. Cell Tissue Res 383:617–644
Meyer C, Stenberg L, Gonzalez-Perez F, Wrobel S, Ronchi G, Udina E, Suganuma S, Geuna S, Navarro X, Dahlin LB (2016) Chitosan-film enhanced chitosan nerve guides for long-distance regeneration of peripheral nerves. Biomaterials 76:33–51
Mimura T, Dezawa M, Kanno H, Sawada H, Yamamoto I (2004) Peripheral nerve regeneration by transplantation of bone marrow stromal cell—derived Schwann cells in adult rats. J Neurosurg 101:806–812
Mo L, Yang Z, Zhang A, Li X (2010) The repair of the injured adult rat hippocampus with NT-3-chitosan carriers. Biomaterials 31:2184–2192
Modrak M, Talukder MAH, Gurgenashvili K, Noble M, Elfar JC (2020) Peripheral nerve injury and myelination: potential therapeutic strategies. J Neurosci Res 98:780–795
Mokalled MH, Patra C, Dickson AL, Endo T, Stainier DY, Poss KD (2016) Injury-induced ctgfa directs glial bridging and spinal cord regeneration in zebrafish. Science 354:630–634
Mokarram N, Dymanus K, Srinivasan A, Lyon JG, Tipton J, Chu J, English AW, Bellamkonda RV (2017) Immunoengineering nerve repair. Proc Natl Acad Sci 114:E5077–E5084
Molcanyi M, Riess P, Bentz K, Maegele M, Hescheler J, Schäfke B, Trapp T, Neugebauer E, Klug N, Schäfer U (2007) Trauma-associated inflammatory response impairs embryonic stem cell survival and integration after implantation into injured rat brain. J Neurotrauma 24:625–637
Moppett IK (2007) Traumatic brain injury: assessment, resuscitation and early management. Br J Anaesth 99:18–31
Moshayedi P, Nih LR, Llorente IL, Berg AR, Cinkornpumin J, Lowry WE, Segura T, Carmichael ST (2016) Systematic optimization of an engineered hydrogel allows for selective control of human neural stem cell survival and differentiation after transplantation in the stroke brain. Biomaterials 105:145–155
Nagashima K, Miwa T, Soumiya H, Ushiro D, Takeda-Kawaguchi T, Tamaoki N, Ishiguro S, Sato Y, Miyamoto K, Ohno T (2017) Priming with FGF2 stimulates human dental pulp cells to

promote axonal regeneration and locomotor function recovery after spinal cord injury. Sci Rep 7:1–12
Nakano N, Nakai Y, Seo T-B, Yamada Y, Ohno T, Yamanaka A, Nagai Y, Fukushima M, Suzuki Y, Nakatani T (2010) Characterization of conditioned medium of cultured bone marrow stromal cells. Neurosci Lett 483:57–61
Namiki JUN, Kojima A, Tator CH (2000) Effect of brain-derived neurotrophic factor, nerve growth factor, and neurotrophin-3 on functional recovery and regeneration after spinal cord injury in adult rats. J Neurotrauma 17:1219–1231
Neal RA, McClugage SG III, Link MC, Sefcik LS, Ogle RC, Botchwey EA (2009) Laminin nanofiber meshes that mimic morphological properties and bioactivity of basement membranes. Tissue Eng Part C Methods 15:11–21
Neal RA, Tholpady SS, Foley PL, Swami N, Ogle RC, Botchwey EA (2012) Alignment and composition of laminin–polycaprolactone nanofiber blends enhance peripheral nerve regeneration. J Biomed Mater Res A 100:406–423
Ng SY, Lee AYW (2019) Traumatic brain injuries: pathophysiology and potential therapeutic targets. Front Cell Neurosci 13:528
Nguyen HX, Hooshmand MJ, Saiwai H, Maddox J, Salehi A, Lakatos A, Nishi RA, Salazar D, Uchida N, Anderson AJ (2017) Systemic neutrophil depletion modulates the migration and fate of transplanted human neural stem cells to rescue functional repair. J Neurosci 37:9269–9287
Nisbet DR, Rodda AE, Horne MK, Forsythe JS, Finkelstein DI (2009) Neurite infiltration and cellular response to electrospun polycaprolactone scaffolds implanted into the brain. Biomaterials 30:4573–4580
Nunes MC, Roy NS, Keyoung HM, Goodman RR, McKhann G 2nd, Jiang L, Kang J, Nedergaard M, Goldman SA (2003) Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. Nat Med 9:439–447
O'Shea TM, Burda JE, Sofroniew MV (2017) Cell biology of spinal cord injury and repair. J Clin Invest 127:3259–3270
Park J, Decker JT, Smith DR, Cummings BJ, Anderson AJ, Shea LD (2018) Reducing inflammation through delivery of lentivirus encoding for anti-inflammatory cytokines attenuates neuropathic pain after spinal cord injury. J Control Release 290:88–101
Paskal AM, Paskal W, Pietruski P, Wlodarski PK (2019) Polyethylene glycol: the future of posttraumatic nerve repair? Systemic review. Int J Mol Sci 20:1478
Patel K, Sun D (2016) Strategies targeting endogenous neurogenic cell response to improve recovery following traumatic brain injury. Brain Res 1640:104–113
Pawar K, Prang P, Müller R, Caioni M, Bogdahn U, Kunz W, Weidner N (2015) Intrinsic and extrinsic determinants of central nervous system axon outgrowth into alginate-based anisotropic hydrogels. Acta Biomater 27:131–139
Pearn ML, Niesman IR, Egawa J, Sawada A, Almenar-Queralt A, Shah SB, Duckworth JL, Head BP (2017) Pathophysiology associated with traumatic brain injury: current treatments and potential novel therapeutics. Cell Mol Neurobiol 37:571–585
Pearse DD, Bastidas J, Izabel SS, Ghosh M (2018) Schwann cell transplantation subdues the pro-inflammatory innate immune cell response after spinal cord injury. Int J Mol Sci 19:2550
Przekora A, Juszkiewicz L (2020) The effect of autologous adipose tissue–derived mesenchymal stem cells' therapy in the treatment of chronic posttraumatic spinal cord injury in a domestic ferret patient. Cell Transplant 29:0963689720928982
Qu C, Xiong Y, Mahmood A, Kaplan DL, Goussev A, Ning R, Chopp M (2009) Treatment of traumatic brain injury in mice with bone marrow stromal cell–impregnated collagen scaffolds. J Neurosurg 111:658–665
Rayner M, Brown H, Wilcox M, Phillips J, Quick T (2020) Quantifying regeneration in patients following peripheral nerve injury. J Plast Reconstr Aesthet Surg 73:201–208
Reilly PL (2001) Brain injury: the pathophysiology of the first hours. 'Talk and Die revisited'. J Clin Neurosci 8:398–403

Reshamwala R (2020) Novel surgical approaches for transplanting three-dimensional constructs of olfactory ensheathing cells to repair the injured spinal cord. PhD, Griffith University

Reshamwala R, Shah M, St John J, Ekberg J (2019) Survival and integration of transplanted olfactory ensheathing cells are crucial for spinal cord injury repair: insights from the last 10 years of animal model studies. Cell Transplant 28:132S–159S

Reshamwala R, Shah M, Belt L, Ekberg JAK, St John JA (2020) Reliable cell purification and determination of cell purity: crucial aspects of olfactory ensheathing cell transplantation for spinal cord repair. Neural Regen Res 15:2016–2026

Rhea EM, Banks WA (2019) Role of the blood-brain barrier in central nervous system insulin resistance. Front Neurosci 13:521

Richardson RM, Holloway KL, Bullock MR, Broaddus WC, Fillmore HL (2006) Isolation of neuronal progenitor cells from the adult human neocortex. Acta Neurochir (Wien) 148:773–777

Riess P, Molcanyi M, Bentz K, Maegele M, Simanski C, Carlitscheck C, Schneider A, Hescheler J, Bouillon B, Schäfer U (2007) Embryonic stem cell transplantation after experimental traumatic brain injury dramatically improves neurological outcome, but may cause tumors. J Neurotrauma 24:216–225

Rolfe A, Sun D (2015) Frontiers in neuroengineering stem cell therapy in brain trauma: implications for repair and regeneration of injured brain in experimental TBI models. In: Kobeissy FH (ed) Brain neurotrauma: molecular, neuropsychological, and rehabilitation aspects. CRC Press/ Taylor & Francis, Boca Raton. © 2015 by Taylor & Francis Group, LLC

Rosenzweig ES, Brock JH, Lu P, Kumamaru H, Salegio EA, Kadoya K, Weber JL, Liang JJ, Moseanko R, Hawbecker S (2018) Restorative effects of human neural stem cell grafts on the primate spinal cord. Nat Med 24:484–490

Roy NS, Benraiss A, Wang S, Fraser RA, Goodman R, Couldwell WT, Nedergaard M, Kawaguchi A, Okano H, Goldman SA (2000) Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. J Neurosci Res 59:321–331

Ryabov S, Zvyagintseva M, Yadgarov MY, Bazanovich S, Smirnov V (2020) Comparison of the efficiency of systemic and local cell therapy with human umbilical cord blood mononuclear cells in rats with severe spinal cord injury. Bull Exp Biol Med 168:552–555

Saremi J, Mahmoodi N, Rasouli M, Ranjbar FE, Mazaheri EL, Akbari M, Hasanzadeh E, Azami M (2021) Advanced approaches to regenerate spinal cord injury: the development of cell and tissue engineering therapy and combinational treatments. Biomed Pharmacother 146:112529

Seddon H (1943) Three types of nerve injury. Brain 66:237–288

Shah SR, Bindra R, Griffin JW (2010) Irreducible dislocation of the thumb interphalangeal joint with digital nerve interposition: case report. J Hand Surg Am 35:422–424

Shakhbazau A, Kawasoe J, Hoyng SA, Kumar R, van Minnen J, Verhaagen J, Midha R (2012) Early regenerative effects of NGF-transduced Schwann cells in peripheral nerve repair. Mol Cell Neurosci 50:103–112

Shetty AK, Mishra V, Kodali M, Hattiangady B (2014) Blood brain barrier dysfunction and delayed neurological deficits in mild traumatic brain injury induced by blast shock waves. Front Cell Neurosci 8:232

Shi H, Li X, Yang J, Zhao Y, Xue C, Wang Y, He Q, Shen M, Zhang Q, Yang Y (2019) Bone marrow-derived neural crest precursors improve nerve defect repair partially through secreted trophic factors. Stem Cell Res Ther 10:1–15

Shlosberg D, Benifla M, Kaufer D, Friedman A (2010) Blood–brain barrier breakdown as a therapeutic target in traumatic brain injury. Nat Rev Neurol 6:393–403

Silva NA, Sousa N, Reis RL, Salgado AJ (2014) From basics to clinical: a comprehensive review on spinal cord injury. Prog Neurobiol 114:25–57

Skardelly M, Gaber K, Burdack S, Scheidt F, Hilbig H, Boltze J, Förschler A, Schwarz S, Schwarz J, Meixensberger J (2011) Long-term benefit of human fetal neuronal progenitor cell transplantation in a clinically adapted model after traumatic brain injury. J Neurotrauma 28:401–414

Smith DR, Margul DJ, Dumont CM, Carlson MA, Munsell MK, Johnson M, Cummings BJ, Anderson AJ, Shea LD (2019) Combinatorial lentiviral gene delivery of pro-oligodendrogenic factors for improving myelination of regenerating axons after spinal cord injury. Biotechnol Bioeng 116:155–167

Stenberg L, Kodama A, Lindwall-Blom C, Dahlin LB (2016) Nerve regeneration in chitosan conduits and in autologous nerve grafts in healthy and in type 2 diabetic Goto–Kakizaki rats. Eur J Neurosci 43:463–473

Stenberg L, Stößel M, Ronchi G, Geuna S, Yin Y, Mommert S, Mårtensson L, Metzen J, Grothe C, Dahlin LB (2017) Regeneration of long-distance peripheral nerve defects after delayed reconstruction in healthy and diabetic rats is supported by immunomodulatory chitosan nerve guides. BMC Neurosci 18:1–27

Steward O, Sharp KG, Yee KM (2014) Long-distance migration and colonization of transplanted neural stem cells. Cell 156:385–387

Stiefel MF, Tomita Y, Marmarou A (2005) Secondary ischemia impairing the restoration of ion homeostasis following traumatic brain injury. J Neurosurg 103:707–714

Strandberg EJ, Mozaffar T, Gupta R (2007) The role of neurodiagnostic studies in nerve injuries and other orthopedic disorders. J Hand Surg Am 32:1280–1290

Sunderland S (1951) A classification of peripheral nerve injuries producing loss of function. Brain 74:491–516

Tajdaran K, Chan K, Gordon T, Borschel GH (2019) Matrices, scaffolds, and carriers for protein and molecule delivery in peripheral nerve regeneration. Exp Neurol 319:112817

Takahashi H, Yamazaki M, Okawa A, Sakuma T, Kato K, Hashimoto M, Hayashi K, Furuya T, Fujiyoshi T, Kawabe J (2012) Neuroprotective therapy using granulocyte colony-stimulating factor for acute spinal cord injury: a phase I/IIa clinical trial. Eur Spine J 21:2580–2587

Taras JS, Jacoby SM, Lincoski CJ (2011) Reconstruction of digital nerves with collagen conduits. J Hand Surg Am 36:1441–1446

Tate CC, Shear DA, Tate MC, Archer DR, Stein DG, Laplaca MC (2009) Laminin and fibronectin scaffolds enhance neural stem cell transplantation into the injured brain. J Tissue Eng Regen Med 3:208–217

Taylor D, Gercel-Taylor C (2013) The origin, function, and diagnostic potential of RNA within extracellular vesicles present in human biological fluids. Front Genet 4:142

Tedeschi A, Dupraz S, Laskowski CJ, Xue J, Ulas T, Beyer M, Schultze JL, Bradke F (2016) The calcium channel subunit Alpha2delta2 suppresses axon regeneration in the adult CNS. Neuron 92:419–434

Thau-Zuchman O, Shohami E, Alexandrovich AG, Leker RR (2010) Vascular endothelial growth factor increases neurogenesis after traumatic brain injury. J Cereb Blood Flow Metab 30:1008–1016

Theodore N, Hlubek R, Danielson J, Neff K, Vaickus L, Ulich TR, Ropper AE (2016) First human implantation of a bioresorbable polymer scaffold for acute traumatic spinal cord injury: a clinical pilot study for safety and feasibility. Neurosurgery 79:E305–E312

Thomas AM, Seidlits SK, Goodman AG, Kukushliev TV, Hassani DM, Cummings BJ, Anderson AJ, Shea LD (2014) Sonic hedgehog and neurotrophin-3 increase oligodendrocyte numbers and myelination after spinal cord injury. Integr Biol 6:694–705

Torres-Espín A, Hernández J, Navarro X (2013) Gene expression changes in the injured spinal cord following transplantation of mesenchymal stem cells or olfactory ensheathing cells. PLoS One 8:e76141

Tuszynski MH, Wang Y, Graham L, Gao M, Wu D, Brock J, Blesch A, Rosenzweig ES, Havton LA, Zheng B (2014) Neural stem cell dissemination after grafting to CNS injury sites. Cell 156: 388–389

Tysseling-Mattiace VM, Sahni V, Niece KL, Birch D, Czeisler C, Fehlings MG, Stupp SI, Kessler JA (2008) Self-assembling nanofibers inhibit glial scar formation and promote axon elongation after spinal cord injury. J Neurosci 28:3814–3823

Urabe T, Zhao Q, Danielsen N, Lundborg G (1996) Regeneration across a partial defect in rat sciatic nerve encased in a silicone chamber. Scand J Plast Reconstr Surg Hand Surg 30:7–15
Valenzuela V, Onate M, Hetz C, Court FA (2016) Injury to the nervous system: a look into the ER. Brain Res 1648:617–625
Valls-Sole J, Castillo CD, Casanova-Molla J, Costa J (2011) Clinical consequences of reinnervation disorders after focal peripheral nerve lesions. Clin Neurophysiol 122:219–228
Vertex Pharmaceuticals Inc. (2018) Study to assess the efficacy and safety of VX-210 in subjects with acute traumatic cervical spinal cord injury
Vespa P (2005) Continuous EEG monitoring for the detection of seizures in traumatic brain injury, infarction, and intracerebral hemorrhage: "to detect and protect". J Clin Neurophysiol 22:99–106
Vespa PM, Nuwer MR, Nenov V, Ronne-Engstrom E, Hovda DA, Bergsneider M, Kelly DF, Martin NA, Becker DP (1999) Increased incidence and impact of nonconvulsive and convulsive seizures after traumatic brain injury as detected by continuous electroencephalographic monitoring. J Neurosurg 91:750–760
Vijayavenkataraman S (2020) Nerve guide conduits for peripheral nerve injury repair: a review on design, materials and fabrication methods. Acta Biomater 106:54–69
Vismara I, Papa S, Rossi F, Forloni G, Veglianese P (2017) Current options for cell therapy in spinal cord injury. Trends Mol Med 23:831–849
Waitayawinyu T, Parisi DM, Miller B, Luria S, Morton HJ, Chin SH, Trumble TE (2007) A comparison of polyglycolic acid versus type 1 collagen bioabsorbable nerve conduits in a rat model: an alternative to autografting. J Hand Surg Am 32:1521–1529
Wang Y, Cooke MJ, Morshead CM, Shoichet MS (2012) Hydrogel delivery of erythropoietin to the brain for endogenous stem cell stimulation after stroke injury. Biomaterials 33:2681–2692
Wang S, Cheng H, Dai G, Wang X, Hua R, Liu X, Wang P, Chen G, Yue W, An Y (2013) Umbilical cord mesenchymal stem cell transplantation significantly improves neurological function in patients with sequelae of traumatic brain injury. Brain Res 1532:76–84
Wang F, Gao ZY, Zhang T, Xu SY, Wang D, Li HP, He XJ (2016) [Study of olfactory ensheathing cells transplantation and treadmill training on improving hindlimb motor function of spinal cord injury rats]. Zhongguo Gu Shang 29:928–938
Wang Y, Tan H, Hui X (2018) Biomaterial scaffolds in regenerative therapy of the central nervous system. Biomed Res Int 2018:7848901–7848901
Werner C, Engelhard K (2007) Pathophysiology of traumatic brain injury. Br J Anaesth 99:4–9
Wilems TS, Pardieck J, Iyer N, Sakiyama-Elbert SE (2015) Combination therapy of stem cell derived neural progenitors and drug delivery of anti-inhibitory molecules for spinal cord injury. Acta Biomater 28:23–32
Windrem MS, Roy NS, Wang J, Nunes M, Benraiss A, Goodman R, McKhann GM 2nd, Goldman SA (2002) Progenitor cells derived from the adult human subcortical white matter disperse and differentiate as oligodendrocytes within demyelinated lesions of the rat brain. J Neurosci Res 69: 966–975
Woodhall E, West AK, Chuah MI (2001) Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. Mol Brain Res 88:203–213
Xin H, Katakowski M, Wang F, Qian J-Y, Liu XS, Ali MM, Buller B, Zhang ZG, Chopp M (2017) MicroRNA-17–92 cluster in exosomes enhance neuroplasticity and functional recovery after stroke in rats. Stroke 48:747–753
Xiong Y, Mahmood A, Chopp M (2009) Emerging treatments for traumatic brain injury. Expert Opin Emerg Drugs 14:67–84
Xu P, Rosen KM, Hedstrom K, Rey O, Guha S, Hart C, Corfas G (2013) Nerve injury induces glial cell line-derived neurotrophic factor (gdnf) expression in Schwann cells through purinergic signaling and the pkc-pkd pathway. Glia 61:1029–1040
Xu B, Zhang Y, Du X-F, Li J, Zi H-X, Bu J-W, Yan Y, Han H, Du J-L (2017) Neurons secrete miR-132-containing exosomes to regulate brain vascular integrity. Cell Res 27:882–897

Yan Y, Ma T, Gong K, Ao Q, Zhang X, Gong Y (2014) Adipose-derived mesenchymal stem cell transplantation promotes adult neurogenesis in the brains of Alzheimer's disease mice. Neural Regen Res 9:798

Yasuhara T, Kameda M, Sasaki T, Tajiri N, Date I (2017) Cell therapy for Parkinson's disease. Cell Transplant 26:1551–1559

Yi S, Xu L, Gu X (2019) Scaffolds for peripheral nerve repair and reconstruction. Exp Neurol 319: 112761

Zhang Y, Ang BT, Xiao ZC, Ng I (2009) DNA vaccination against neurite growth inhibitors to enhance functional recovery following traumatic brain injury. In: Steiger HJ (ed) Acta neurochirurgica supplements, 2009//. Springer, Vienna, pp 347–351

Zhang Y, Chopp M, Meng Y, Katakowski M, Xin H, Mahmood A, Xiong Y (2015) Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. J Neurosurg 122:856–867

Zhang S, Li J, Jiang H, Gao Y, Cheng P, Cao T, Li D, Wang J, Song Y, Liu B (2018) Dorsal root ganglion maintains stemness of bone marrow mesenchymal stem cells by enhancing autophagy through the AMPK/mTOR pathway in a coculture system. Stem Cells Int 2018:8478953

Zhu X, Lee J, Wong J, Tan WL, Feng Z, Wang T, Xiao Z, Ng I (2007) Pre-stroke DNA immunization against neurite growth inhibitors is beneficial to the recovery from focal cerebral ischemia in rats. Neural Regen Res 2:513–518

Ziemba AM, Gilbert RJ (2017) Biomaterials for local, controlled drug delivery to the injured spinal cord. Front Pharmacol 8:245

Prenatal Interventions for the Treatment of Congenital Disorders

12

Kshitiz Singh

12.1 Introduction

Regenerative medicine definition includes the use of diverse approaches to repair, replace or restore the functional loss of cells, tissues, or organs due to any etiology (Greenwood et al. 2006). Treating diseases as early as possible by correcting the defective tissues or replacing the defective cells is the primary concept behind regenerative medicine. The intent of treating the diseases before the onset of irreversible damage to the body has been the reason for the early intervention at the fetal stage. Moreover, the development of early diagnostic methodologies—imaging and molecular diagnosis—has provided us the opportunity to detect the disease-causing events early and consequently, intervene promptly to ameliorate the diseases.

There has been significant development in the field of in utero treatment approaches. On PubMed search, 29,665 search results appear for this systematic search (Table 12.1 and Fig. 12.1). About 70% of these articles have been published in this century (since 2000). With the development of gene replacement and gene editing technologies (targeted nucleases, base editors), the potential ability to correct severe and debilitating diseases are better than any time in the past.

Certain characteristics inherent to the fetus make it an ideal candidate for early intervention and modern advanced therapeutic strategies, for example, gene therapy and gene editing. Small size, less developed biological barriers (e.g., blood–brain barrier), accessible progenitor, and stem cells and immature immune response are some of the properties of the fetus which we can utilize for treating fatal genetic disorders (Flake 2003).

K. Singh (✉)
Queen's University, Kingston, ON, Canada
e-mail: kshitiz.singh@queensu.ca

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_12

Table 12.1 PubMed search results for in utero transplantation, gene therapy, and gene editing

Aim of search	Search term	Website	No. of search results
Identify literature of in utero transplantation, gene therapy and gene editing	(((transplantation) OR (gene therapy)) OR (gene editing)) AND ((fetal) OR ("in utero"))	PubMed Search formatted link: https://pubmed.ncbi.nlm.nih.gov/?term=%28%28%28transplantation%29+OR+%28gene+therapy%29%29+OR+%28gene+editing%29%29+AND+%28%28fetal%29+OR+%28%22in+utero%22%29%29&ac=no&sort=relevance	29,665 (Accessed on March 13, 2022)

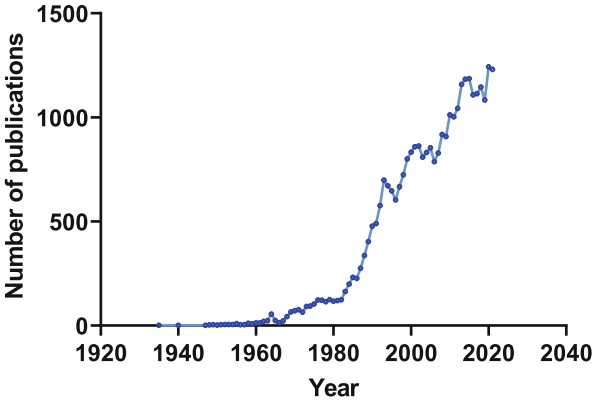


Fig. 12.1 Number of publications between 1935 and 2021 referring to fetal gene therapy and transplantation (PubMed search in Table 12.1)

The small size of the fetus allows us to maximize the dose per unit weight of the recipient and also provides a practical advantage in terms of the pharmaceutical dose we can deliver. For example, a fetus weighs 600 g compared to a 60 kg adult. So, less dose is needed to treat a fetus than an adult human, especially for advanced biological therapies, such as gene and cell therapy, where mass manufacturing is challenging. With the same amount of biological therapeutic product, we can treat more patients. Along similar lines, in a fetus, progenitor and stem cells are more prevalent and if we can correct these cells with advanced therapeutics, we can treat the diseases for the lifetime of the patient. Moreover, immunological and physical barriers are not well-developed in the fetus, which can allow us to deliver therapeutics early and help protect the patient when it comes out from the protective environment of the mother's womb.

The aim of this chapter is to summarize the preclinical research for in utero approaches (prenatal surgery, cell therapy, and gene therapy) for the treatment of genetic diseases.

12.2 Technological Advances in Early Diagnosis

Cutting-edge diagnostic, minimally invasive surgical approaches, and research methodologies reveal the mechanisms and etiologies of the diseases. Barcoded cells tracking helps in defining nesting locations of cells within the fetus (Wang et al. 2021a). Tracing developing fetal cells and identifying the trajectory of cells after transplantation are significant for the design of rational therapeutics. Barcoding the cells can be one such approach, as next-generation sequencing approaches have raised the sensitivity and specificity of detecting the minuscule amounts of nucleic acids (Wang et al. 2021b). Progress in predictive technology for the quality control of isolation of specific cell types opens prospects for future clinical applications (Zia et al. 2021). Several surgical and minimally invasive methods (e.g., fetoscopy) are already being used in the fetus to overcome severe anatomic and inflammatory anomalies, such as heart syndromes, twin-to-twin transfusion syndrome, myelomeningocele, and sacrococcygeal teratoma (Lin et al. 2021).

Early diagnostic methodologies such as non-invasive prenatal detection of disease-causing mutations in maternal plasma using digital droplet Polymerase Chain Reaction (PCR) (D'Aversa et al. 2022), next-generation sequencing, prenatal rapid exome sequencing (Mellis et al. 2022; Wei et al. 2022), copy-number variation detection (Cai et al. 2021), DNA methylation studies (reference-free and reference-based cell type estimation) (Dieckmann et al. 2022), non-invasive prenatal fetal blood group testing and coelocentesis will help in early detection of severe and incurable disorders (Clausen et al. 2022; Giambona et al. 2022; Wu et al. 2022). Couples (one or both) harboring chimeric heterozygous mutations may specifically be benefitted from early preimplantation genetic testing. In the study by Li et al. (2022a), the authors screened the embryos of a couple with heterozygous pathogenic mutation for osteogenesis imperfecta and then, implanted the wild-type embryo. Certain syndromes, such as Aicardi syndrome lack genetic tests to confirm the diagnosis of the disease, the development of early diagnostic methodologies, and mechanistic understanding may guide novel treatment strategies (Pomar et al. 2022). An increase in accessibility of modern fetal diagnostic and therapeutic approaches will allow for expanding the treatments to more patients in the future (Poojari et al. 2022).

12.3 Prenatal Pharmacotherapy

Fetal alcohol spectrum disorders (FASD) are a common cause of physical, cognitive, and behavioral abnormalities. A study into the mechanisms of this is divulging that early interventions based on the principles of central nervous system development and regeneration can ameliorate the damage (Gomez and Abdul-Rahman 2021). Administration of choline prenatally has shown to reduce the effects of prenatal alcohol exposure and leads to improved cognitive and behavioral outcomes (Wozniak et al. 2020).

For prenatally detected cardiac rhabdomyomas, transplacental sirolimus administration has been explored as a safe therapeutic option. However, there is a risk of sirolimus-associated growth restriction (Wozniak et al. 2020).

12.4 Prenatal Surgery

In utero surgery is commonly performed for photocoagulation of placental anastomosis in twin-twin transfusion syndrome to allow for equal sharing of blood between the twins (Codsi and Audibert 2019). Additionally, fetal surgery can be a potential option for the diseases like severe congenital diaphragmatic hernia with ineffective postnatal treatments and potential preclinical evidence of benefit by early surgical intervention. For example, it has been advocated that prenatal myelomeningocele repair in the carefully selected patient can have improved functional outcomes (Adzick et al. 2011; Peranteau and Adzick 2016). Despite vigilant patient selection and many parents opting for prenatal surgical intervention, a small group of parents chooses to terminate of pregnancy because of the risk of unexpected outcomes (Crombag et al. 2021). As more fetal therapy centers are conducting additional studies to catalog beneficial outcomes and the risk of complications, the results will support the decision process of stakeholders, especially parents (da Rocha et al. 2021; Lillegard et al. 2022; Moehrlen et al. 2021; Vonzun et al. 2021). Improved surgical approaches, for example, fetoscopic repair can reduce the risks associated with open repair (Cortes et al. 2021). However, premature delivery might be a risk for such interventions (Diehl et al. 2021).

Prenatal fetoscopic surgeries can have improved outcomes in congenital diaphragmatic hernia, fetal lower urinary tract obstruction, and ultrasound-guided fetal aortic valvuloplasty for hypoplastic left heart syndrome (Codsi and Audibert 2019). Moreover, in fetal lower umbilical tract obstruction, prenatal cystoscopy and fetal vesicoamniotic shunt did not show a difference in perinatal survival. The complication of urological fistula was found in 10% of patients, who underwent laser ablation. Technological advances, which can help direct the laser energy accurately in all fetal-placental positions, may reduce fistula-related complications (Vinit et al. 2020). Balanced, paired use of drugs, and surgery can be therapeutically synergistic; for example, the use of sildenafil and fetal tracheal occlusion has preclinically been shown to be beneficial in congenital diaphragmatic hernia (Russo et al. 2022).

In addition, knowledge gained from preclinical research in large animal models can help in improved outcomes of these novel surgical procedures (Coons et al. 2021).

12.5 Prenatal Cell Therapy

Prenatal mesenchymal, stromal, and epithelial stem cells transplantation is a valuable methodology for treating congenital disorders. Recently intraamniotic injection of amniotic mesenchymal stem cells can bring partial closure of spina bifida and improve functional outcomes (Kunpalin et al. 2021; Shieh et al. 2019).

Human amnion-derived epithelial cells (hAEC) possess stem cell-like properties and are capable to be differentiated into different cell types. In vitro, these cells have been differentiated into different cell types—hepatocyte-like cells, insulin-producing cells, islet-like cells, corneal epithelial-like cells, neural cells, osteogenic cells, epidermal cells, Schwann-like cells, and cardiomyocyte-like cells (Zhang and Lai 2020).

Alpha thalassemia major is one of the common monogenic disorders. Early diagnosis by percutaneous umbilical cord blood sampling, followed by in utero blood transfusion can produce favorable outcomes (MacKenzie et al. 2021; Hui et al. 2022; Horvei et al. 2021; Demirci et al. 2021). Postnatally, the patients can be managed with multiple transfusions or stem cell transplantation. Moreover, preclinical studies have shown successful transplantation of prenatal hematopoietic stem cells (Peranteau et al. 2002).

Fetoplacental extracellular vesicles (fEV) have been found to downregulate innate and adaptive immunity. In utero exposure to allogenic exosomes reduced the lymphocytic reaction to the allogenic antigens, however, it did not result in the tolerance to allogenic graft (Chen 2021a). In another study, transamniotic stem cell therapy using mesenchymal stem cells has been shown to reverse some of the effects of intrauterine growth retardation (IUGR) in a rat model (Labuz et al. 2022). Discoveries into the behavior of fetal macrophages can help in diving into novel treatment approaches. For example, fetal $CD116^{+}CD64^{-}$ macrophage precursors thrive better than the adult counterparts in perinatal lung alveoli. Fetal mesenchymal stromal cell extracellular vesicle can be beneficial in preventing preeclampsia-associated lung injury and ventilation-associated lung injury (Taglauer et al. 2022; Horie et al. 2021).

In utero mesenchymal stem cell therapy is also being investigated as a therapeutic option to prevent spontaneous abortions, hypothetically benefitting by inhibition of excessive complement activation and promoting the balance of angiogenic factors (Shahgaldi et al. 2022).

12.6 Prenatal Gene Therapy and Gene Editing

Gene therapy and gene editing (e.g., CRISPR-Cas9 system) approaches involve the administration of genetic material to modify the expression of genes and thus treat the diseased cells and tissues. Such molecules can be delivered prenatally by diverse routes of administration—vitelline vein, intraamniotic (Alapati et al. 2019), and intracerebral route (MacKenzie 2018).

CRISPR/Cas9 nucleases have shown to be therapeutically beneficial in diseases, for example, type 1 tyrosinemia and Surfactant protein C (SFTPC) deficiency. Moreover, recently base editors in the fetus have shown therapeutic benefits (Singh et al. 2021). Prime editing can be beneficial to treat those mutations which are not amenable to CRISPR-Cas9 nucleases or base editors (Anzalone et al. 2019).

Prenatal gene therapy and gene editing approaches can be beneficial in several genetic and metabolic diseases: sickle cell disease and thalassemia (Li et al. 2022b), spinal muscular atrophy (Rashnonejad et al. 2019), type 1 tyrosinemia (Singh et al. 2021; Rossidis et al. 2018), mucopolysaccharidoses (Bose et al. 2021), and surfactant protein C deficiency (Alapati et al. 2019). Transamniotic fetal immunotherapy can also be beneficial for some diseases (Whitlock et al. 2022).

12.7 Future Direction and Implications

The use of viral vectors for gene therapy and gene editing has been a concern among caregivers and patients. However, alternative delivery strategies such as lipid and synthetic polymer nanoparticles for the delivery of gene therapy and gene editing molecules have helped in removing some of the viral vector side effects, such as viral genome integration events (Ricciardi et al. 2018). The development of targeted fetal and tissue specific promoters to increase the specificity of gene expression, especially for gene editors, can also help in alleviating viral vectors associated adverse events (Singh et al. 2018).

Arguably, it has been considered that exposure of the fetus to foreign antigens can induce tolerability; however, the response of the fetus can vary with the type of antigenic exposure, and consequently, the results can be immunogenic or tolerogenic (Chen 2021b; Mold and McCune 2012). Therefore, careful preclinical investigation for the safety of the therapeutic molecules or cells should be the primary aspect of evaluation. Some large animal gene therapy with Adeno-associated viral vector (AAV) studies have observed an increase in the incidence of premature labor, compared to saline control injection. However, it is still unclear whether such premature labor is a result of specific viral vector formulations, contaminants, or species-specific response, since, other in utero studies in large animal models such as sheep, rhesus has been successful (Finkel and Lorson 2022). Systemic infusion of human amniotic epithelial cells can carry a risk of embolism, so further development of methodologies for safe transfusion (e.g., intraportal infusion) may ameliorate this risk (Tanaka et al. 2022).

High-throughput and high-resolution profiling of stem and progenitor cells by technological advances can increase the probability of divulging mechanisms of diseases and effects of gene therapy approaches (Vanuytsel et al. 2022). The role of immune checkpoint modulators (PD-1, Tim-3, VISTA) has been identified in determining the pregnancy outcome (Zhao et al. 2022). This new knowledge will help in unraveling the side effects associated with prenatal approaches and thus control the adverse outcomes of the diseases. Furthermore, awareness among parents and genetic counselors about the safety and efficacy of fetal treatments can increase

the acceptance of new fetal therapies (Scott et al. 2022). Interdisciplinary discoveries, targeted development of devices for targeted surgical and radiation delivery, robotics for precise surgical intervention, and understanding of biology, gene delivery and therapeutic vectors will pave way for prenatal therapy of several diseases, which are currently untreatable or have limited treatments. Additionally, interdisciplinarity should not only be applied in the context of medical technologies; however, considerations regarding the societal and ethical implications, along with maternal-fetal health, should be paramount (Brown and Koenig 2021).

References

Adzick NS, Thom EA, Spong CY, Brock JW, Burrows PK, Johnson MP, Howell LJ, Farrell JA, Dabrowiak ME, Sutton LN, Gupta N, Tulipan NB, D'Alton ME, Farmer DL (2011) A randomized trial of prenatal versus postnatal repair of myelomeningocele. N Engl J Med 364: 993–1004

Alapati D, Zacharias WJ, Hartman HA, Rossidis AC, Stratigis JD, Ahn NJ, Coons B, Zhou S, Li H, Singh K, Katzen J, Tomer Y, Chadwick AC, Musunuru K, Beers MF, Morrisey EE, Peranteau WH (2019) In utero gene editing for monogenic lung disease. Sci Transl Med 11. https://doi.org/10.1126/scitranslmed.aav8375

Anzalone AV, Randolph PB, Davis JR, Sousa AA, Koblan LW, Levy JM, Chen PJ, Wilson C, Newby GA, Raguram A, Liu DR (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576:149–157

Bose SK, White BM, Kashyap MV, Dave A, De Bie FR, Li H, Singh K, Menon P, Wang T, Teerdhala S, Swaminathan V, Hartman HA, Jayachandran S, Chandrasekaran P, Musunuru K, Jain R, Frank DB, Zoltick P, Peranteau WH (2021) In utero adenine base editing corrects multi-organ pathology in a lethal lysosomal storage disease. Nat Commun 12:4291

Brown JEH, Koenig BA (2021) Ethical, legal, and social implications of fetal gene therapy. Clin Obstet Gynecol 64:933–940

Cai M, Fu X, Xu L, Lin N, Huang H (2021) renatal diagnosis of 17p11.2 copy number abnormalities associated with Smith–Magenis and Potocki–Lupski syndromes in fetuses. Front Genet 12. https://www.frontiersin.org/article/10.3389/fgene.2021.779237

Chen J-C (2021a) Immunological consequences of in utero exposure to foreign antigens. Front Immunol 12:638435

Chen JC (2021b) Immunological consequences of in utero exposure to foreign antigens. Front Immunol 12:1227

Clausen FB, Hellberg Å, Bein G, Bugert P, Schwartz D, Drnovsek TD, Finning K, Guz K, Haimila K, Henny C, O'Brien H, Orzinska A, Sørensen K, Thorlacius S, Wikman A, Denomme GA, Flegel WA, Gassner C, de Haas M, Hyland C, Ji Y, Lane WJ, Nogués N, Olsson ML, Peyrard T, van der Schoot CE, Weinstock C, Legler T (2022) Recommendation for validation and quality assurance of non-invasive prenatal testing for foetal blood groups and implications for IVD risk classification according to EU regulations. Vox Sang 117:157–165

Codsi E, Audibert F (2019) Fetal surgery: past, present, and future perspectives. J Obstet Gynaecol Can 41:S287–S289

Coons BE, Lawrence KM, Didier R, Sridharan A, Moon JK, Rossidis AC, Baumgarten HD, Kim AG, Mejaddam AY, Ozawa K, De Bie F, Davey M, Flake AW (2021) Fetoscopic insufflation modeled in the extrauterine environment for neonatal development (EXTEND): fetoscopic insufflation is safe for the fetus. J Pediatr Surg 56:170–179

Cortes MS, Chmait RH, Lapa DA, Belfort MA, Carreras E, Miller JL, Samaha RBB, Gonzalez GS, Gielchinsky Y, Yamamoto M, Persico N, Santorum M, Otaño L, Nicolaou E, Yinon Y, Faig-Leite F, Brandt R, Whitehead W, Maiz N, Baschat A, Kosinski P, Nieto-Sanjuanero A,

Chu J, Kershenovich A, Nicolaides KH (2021) Experience of 300 cases of prenatal fetoscopic open spina bifida repair: report of the International Fetoscopic Neural Tube Defect Repair Consortium. Am J Obstet Gynecol 225(678):e1–678.e11

Crombag N, Sacco A, Stocks B, De Vloo P, van der Merwe J, Gallagher K, David A, Marlow N, Deprest J (2021) 'We did everything we could'—a qualitative study exploring the acceptability of maternal-fetal surgery for spina bifida to parents. Prenat Diagn 41:910–921

D'Aversa E, Breveglieri G, Boutou E, Balassopoulou A, Voskaridou E, Pellegatti P, Guerra G, Scapoli C, Gambari R, Borgatti M (2022) Droplet digital PCR for non-invasive prenatal detection of fetal single-gene point mutations in maternal plasma. Int J Mol Sci 23:2819

da Rocha LSN, Bunduki V, de Amorim Filho AG, Cardeal DD, Matushita H, Fernandes HS, Nani FS, de Francisco RPV, de Carvalho MHB (2021) Open fetal myelomeningocele repair at a university hospital: surgery and pregnancy outcomes. Arch Gynecol Obstet 304:1443–1454

Demirci S, Leonard A, Essawi K, Tisdale JF (2021) CRISPR-Cas9 to induce fetal hemoglobin for the treatment of sickle cell disease. Mol Ther Methods Clin Dev 23:276–285

Dieckmann L, Cruceanu C, Lahti-Pulkkinen M, Lahti J, Kvist T, Laivuori H, Sammallahti S, Villa PM, Suomalainen-König S, Rancourt RC, Plagemann A, Henrich W, Eriksson JG, Kajantie E, Entringer S, Braun T, Räikkönen K, Binder EB, Czamara D (2022) Reliability of a novel approach for reference-based cell type estimation in human placental DNA methylation studies. Cell Mol Life Sci 79:115

Diehl D, Belke F, Kohl T, Axt-Fliedner R, Degenhardt J, Khaleeva A, Oehmke F, Faas D, Ehrhardt H, Kolodziej M, Uhl E, Windhorst AC, Neubauer BA (2021) Fully percutaneous fetoscopic repair of myelomeningocele: 30-month follow-up data. Ultrasound Obstet Gynecol 57:113–118

Finkel RS, Lorson CL (2022) Friend or Foe(tal): challenges in development of a large animal model for pre-clinical fetal gene therapy. Gene Ther 29(6):316–318. https://www.nature.com/articles/s41434-022-00327-4

Flake AW (2003) Surgery in the human fetus: the future. J Physiol 547:45–51

Giambona A, Vinciguerra M, Leto F, Cassarà F, Cucinella G, Cigna V, Orlandi E, Piccione M, Picciotto F, Maggio A (2022) Very early prenatal diagnosis of Cockayne's syndrome by coelocentesis. J Obstet Gynaecol 42(5):1524–1531

Gomez DA, Abdul-Rahman OA (2021) Fetal alcohol spectrum disorders: current state of diagnosis and treatment. Curr Opin Pediatr 33:570–575

Greenwood HL, Singer PA, Downey GP, Martin DK, Thorsteinsdóttir H, Daar AS (2006) Regenerative medicine and the developing world. PLoS Med 3:e381

Horie S, Gonzalez H, Brady J, Devaney J, Scully M, O'Toole D, Laffey JG (2021) Fresh and cryopreserved human umbilical-cord-derived mesenchymal stromal cells attenuate injury and enhance resolution and repair following ventilation-induced lung injury. Int J Mol Sci 22:12842

Horvei P, MacKenzie T, Kharbanda S (2021) Advances in the management of α-thalassemia major: reasons to be optimistic. Hematology 2021:592–599

Hui PW, Pang P, Tang MHY (2022) 20 years review of antenatal diagnosis of haemoglobin Bart's disease and treatment with intrauterine transfusion. Prenat Diagn 42(9):1155–1161. https://doi.org/10.1002/pd.6125

Kunpalin Y, Subramaniam S, Perin S, Gerli MFM, Bosteels J, Ourselin S, Deprest J, De Coppi P, David AL (2021) Preclinical stem cell therapy in fetuses with myelomeningocele: a systematic review and meta-analysis. Prenat Diagn 41:283–300

Labuz DF, Whitlock AE, Kycia I, Zurakowski D, Fauza DO (2022) Intrauterine Growth Restriction (IUGR) as a potential target for transamniotic stem cell therapy. J Pediatr Surg 57(6):999–1003. S0022-3468(22)00141–5

Li C, Hou R, Liu C, Li H, Li-Ling J, Lyu Y (2022a) [Identification of pathogenic variant and preimplantation genetic testing for a Chinese family affected with osteogenesis imperfecta]. Zhonghua Yi Xue Yi Chuan Xue Za Zhi 39:21–25

Li L et al (2022b) Genetic correction of concurrent α- and β-thalassemia patient-derived pluripotent stem cells by the CRISPR-Cas9 technology | EndNote Click. Stem Cell Res Ther 13:102.

https://click.endnote.com/viewer?doi=10.1186%2Fs13287-022-02768-5&token=WzI1NzA4MzIsIjEwLjExOD Y vc z E z M j g 3 L T A y M i 0 w M j c 2 O C 0 1 I l 0 . W j 2 jLY6HvwTBLkZZWuwVTsbQWkA

Lillegard JB, Eyerly-Webb SA, Watson DA, Bahtiyar MO, Bennett KA, Emery SP, Fisher A, Goldstein RB, Goodnight WH, Lim F-Y, McCullough LB, Moehrlen U, Moldenhauer JS, Moon-Grady AJ, Ruano R, Skupski DW, Treadwell MC, Tsao K, Wagner AJ, Zaretsky MV (2022) Placental location in maternal-fetal surgery for myelomeningocele. Fetal Diagn Ther 49(3):117–124. https://doi.org/10.1159/000521379

Lin T-Y, Wataganara T, Shaw SW (2021) From non-invasive to invasive fetal therapy: a comprehensive review and current update. Taiwan J Obstet Gynecol 60:595–601

MacKenzie TC (2018) Future AAVenues for in utero gene therapy. Cell Stem Cell 23:320–321

MacKenzie TC, Amid A, Angastiniotis M, Butler C, Gilbert S, Gonzalez J, Keller RL, Kharbanda S, Kirby-Allen M, Koenig BA, Kyono W, Lal A, Lianoglou BR, Norton ME, Ogasawara KK, Panchalee T, Rosner M, Schwab M, Thompson A, Waye JS, Vichinsky E (2021) Consensus statement for the perinatal management of patients with α thalassemia major. Blood Adv 5:5636–5639

Mellis R, Eberhardt R, Hamilton S, PAGE Consortium, McMullan D, Kilby M, Maher E, Hurles M, Giordano J, Aggarwal V, Goldstein D, Wapner R, Chitty L (2022) Fetal exome sequencing for isolated increased nuchal translucency: should we be doing it? BJOG Int J Obstet Gynaecol 129:52–61

Moehrlen U, Ochsenbein N, Vonzun L, Mazzone L, Horst M, Schauer S, Wille DA, Hagmann C, Kottke R, Grehten P, Casanova B, Strübing N, Moehrlen T, Tharakan S, Padden B, Bassler D, Zimmermann R, Meuli M (2021) Fetal surgery for spina bifida in Zurich: results from 150 cases. Pediatr Surg Int 37:311–316

Mold JE, McCune JM (2012) Mccune. In: Advances in immunology. Elsevier, pp 73–111

Peranteau WH, Adzick NS (2016) Prenatal surgery for myelomeningocele. Curr Opin Obstet Gynecol 28:111–118

Peranteau WH, Hayashi S, Hsieh M, Shaaban AF, Flake AW (2002) High-level allogeneic chimerism achieved by prenatal tolerance induction and postnatal nonmyeloablative bone marrow transplantation. Blood 100:2225–2234

Pomar L et al (2022) Prenatal diagnosis of Aicardi syndrome based on a suggestive imaging pattern: a multicenter case-series. Prenat Diagn 42(4):484–494. Wiley Online Library. https://obgyn.onlinelibrary.wiley.com/doi/10.1002/pd.6085

Poojari VG, Paladugu S, Vasudeva A, Mundkur A, Pai MV, Kumar P (2022) We need to improve prenatal screening practices in primary obstetric care: a representative data from a fetal medicine unit in coastal Karnataka. J Obstet Gynaecol India 72:19–25

Rashnonejad A, Amini Chermahini G, Gündüz C, Onay H, Aykut A, Durmaz B, Baka M, Su Q, Gao G, Özkınay F (2019) Fetal gene therapy using a single injection of recombinant AAV9 rescued SMA phenotype in mice. Mol Ther 27:2123–2133

Ricciardi AS, Bahal R, Farrelly JS, Quijano E, Bianchi AH, Luks VL, Putman R, López-Giráldez F, Coşkun S, Song E, Liu Y, Hsieh W-C, Ly DH, Stitelman DH, Glazer PM, Saltzman WM (2018) In utero nanoparticle delivery for site-specific genome editing. Nat Commun 9:2481. https://doi.org/10.1038/s41467-018-04894-2

Rossidis AC, Stratigis JD, Chadwick AC, Hartman HA, Ahn NJ, Li H, Singh K, Coons BE, Li L, Lv W, Zoltick PW, Alapati D, Zacharias W, Jain R, Morrisey EE, Musunuru K, Peranteau WH (2018) In utero CRISPR-mediated therapeutic editing of metabolic genes. Nat Med 24:1513–1518

Russo FM, Da Cunha MGMCM, Jimenez J, Lesage F, Eastwood MP, Toelen J, Deprest J (2022) Complementary effect of maternal sildenafil and fetal tracheal occlusion improves lung development in the rabbit model of congenital diaphragmatic hernia. Ann Surg 275:e586–e595

Scott DA, Gofin Y, Berry AM, Adams AD (2022) Underlying genetic etiologies of congenital diaphragmatic hernia. Prenat Diagn 42(3):373–386. https://doi.org/10.1002/pd.6099

Shahgaldi S, Rezaei Kahmini F, Moazzeni SM (2022) Mesenchymal stem cell therapy attenuates complement C3 deposition and improves the delicate equilibrium between angiogenic and anti-angiogenic factors in abortion-prone mice. Mol Immunol 141:246–256

Shieh HF, Tracy SA, Hong CR, Chalphin AV, Ahmed A, Rohrer L, Zurakowski D, Fauza DO (2019) Transamniotic stem cell therapy (TRASCET) in a rabbit model of spina bifida. J Pediatr Surg 54:293–296

Singh K, Evens H, Nair N, Rincón MY, Sarcar S, Samara-Kuko E, Chuah MK, VandenDriessche T (2018) Efficient in vivo liver-directed gene editing using CRISPR/Cas9. Mol Ther 26:1241–1254

Singh K et al (2021) In utero lipid nanoparticle delivery of CRISPR technology to correct hereditary tyrosinemia type ASGCT annual meeting abstracts. Mol Ther 29:1–427

Taglauer ES et al (2022) Antenatal mesenchymal stromal cell extracellular vesicle therapy prevents preeclamptic lung injury in mice. Am J Respir Cell Mol Biol 66(1):86–95. https://www.atsjournals.org/doi/10.1165/rcmb.2021-0307OC

Tanaka M, Tokodai K, Sato M, Yamada S, Okita H, Ito T, Saito M, Hoshiai T, Miyagi S, Miki T, Unno M, Kamei T, Goto M (2022) Distribution of amniotic epithelial cells after intraportal infusion in a rat model. Transplant Proc 54(2):513–515. https://doi.org/10.1016/j.transproceed.2021.09.077

Vanuytsel K, Yeung AK, Dowrey TW, Murphy GJ, Belkina AC (2022) Comprehensive phenotyping of hematopoietic stem and progenitor cells in the human fetal liver. Cytometry A. https://doi.org/10.1002/cyto.a.24540

Vinit N, Gueneuc A, Bessières B, Dreux S, Heidet L, Salomon R, Lapillonne A, De Bernardis G, Salomon LJ, Stirnemann JJ, Blanc T, Ville Y (2020) Fetal cystoscopy and vesicoamniotic shunting in lower urinary tract obstruction: long-term outcome and current technical limitations. Fetal Diagn Ther 47:74–83

Vonzun L, Kahr M, Noll F, Mazzone L, Moehrlen U, Meuli M, Hüsler M, Krähenmann F, Zimmermann R, Ochsenbein-Kölble N (2021) Systematic classification of maternal and fetal intervention-related complications following open fetal myelomeningocele repair—results from a large prospective cohort. BJOG Int J Obstet Gynaecol 128:1184–1191

Wang M-Y, Zhou Y, Lai G-S, Huang Q, Cai W-Q, Han Z-W, Wang Y, Ma Z, Wang X-W, Xiang Y, Fang S-X, Peng X-C, Xin H-W (2021a) DNA barcode to trace the development and differentiation of cord blood stem cells (Review). Mol Med Rep 24:849

Wang M-Y, Zhou Y, Lai G-S, Huang Q, Cai W-Q, Han Z-W, Wang Y, Ma Z, Wang X-W, Xiang Y, Fang S-X, Peng X-C, Xin H-W (2021b) DNA barcode to trace the development and differentiation of cord blood stem cells (Review). Mol Med Rep 24:1–10

Wei X, Zhou X, Zhou J, Zou G, Yang Y, Zhou F, Xiong S, Chen J, Sun L (2022) The value of exome sequencing in thoracoamniotic shunt for severe pleural effusion with fetal hydrops: a retrospective clinical study. Fetal Diagn Ther. https://doi.org/10.1159/000521212

Whitlock AE, Labuz DF, Kycia I, Zurakowski D, Fauza DO (2022) Passive perinatal immunotherapy via transamniotic antibody delivery. J Pediatr Surg 57:52–55

Wozniak JR, Fink BA, Fuglestad AJ, Eckerle JK, Boys CJ, Sandness KE, Radke JP, Miller NC, Lindgren C, Brearley AM, Zeisel SH, Georgieff MK (2020) Four-year follow-up of a randomized controlled trial of choline for neurodevelopment in fetal alcohol spectrum disorder. J Neurodev Disord 12:9

Wu Q, Zheng L, Huang H, Lin H, Lin X, Xu L, Chen R, Lin D, Chen G (2022) Rapid and label-free prenatal detection of Down's syndrome using body fluid surface enhanced Raman spectroscopy. J Biomed Nanotechnol 18:243–250

Zhang Q, Lai D (2020) Application of human amniotic epithelial cells in regenerative medicine: a systematic review. Stem Cell Res Ther 11:439

Zhao S-J, Muyayalo KP, Luo J, Huang D, Mor G, Liao A-H (2022) Next generation of immune checkpoint molecules in maternal-fetal immunity. Immunol Rev 308(1):40–54. https://doi.org/10.1111/imr.13073

Zia S, Martini G, Pizzuti V, Maggio A, Simonazzi G, Reschiglian P, Bonsi L, Alviano F, Roda B, Zattoni A (2021) A new predictive technology for perinatal stem cell isolation suited for cell therapy approaches. Micromachines 12:782

Understanding LncRNAs in Biomaterials Development for Osteointegration

13

Yuyu Zhao, Long Bai, Xiaohong Yao, Ruiqiang Hang, and Yin Xiao

13.1 Osseointegration Processes

Loosening of intraosseous implants has been a persistent concern, which accounts for the failure of most implant replacements. This issue is primarily caused by the deficient osseointegration between the host bone and implant. Osseointegration has initially been defined as "the direct connection of structure and function between the bone tissue and implant surface" (Listgarten et al. 1991). In 2012, Zarb et al. redefined osseointegration as "a time-varying healing process in which the material is progressively and rigidly bonded to the bone tissue and stably retained at the implantation site during functional loading at the interface" (Zarb and Koka 2012). This definition explains that osseointegration is a time-variant sophisticated healing process that includes four distinct but overlapping stages: blood clot formation, immune response, angiogenesis, and osteogenesis (Chen et al. 2016, 2017; Bai et al. 2018a, b, 2021a, b). Figure 13.1 illustrates the different stages of osseointegration and cellular repsonses in each stage.

Y. Zhao · X. Yao · R. Hang
Shanxi Key Laboratory of Biomedical Metal Materials, College of Materials Science and Engineering, Taiyuan University of Technology, Taiyuan, China

L. Bai
Key Laboratory for Ultrafine Materials of Ministry of Education, East China University of Science and Technology, Shanghai, China

Y. Xiao (✉)
Centre of Biomedical Technology, Queensland University of Technology, Brisbane, QLD, Australia

Australia-China Centre for Tissue Engineering and Regenerative Medicine, Queensland University of Technology, Brisbane, QLD, Australia
e-mail: yin.xiao@qut.edu.au

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_13

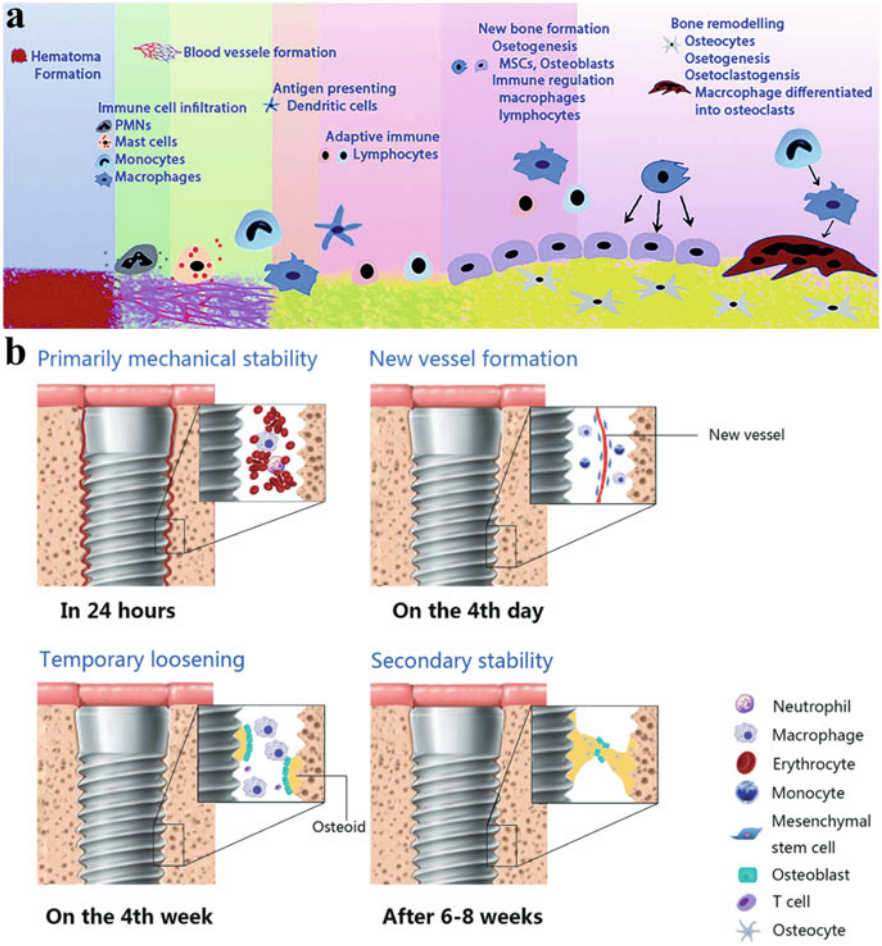


Fig. 13.1 Various stages of osseointegration. (**a**) Different stages of osseointegration and the involvement of diverse cells in each stage (Chen et al. 2017). (Copyright Royal Society of Chemistry Ltd); (**b**) Timeline of implant osseointegration and events occurring around the implant over time (Wang et al. 2016). (Copyright Wiley)

13.1.1 Blood Clot Formation

The implant surface comes into contact with blood, thus immediately triggering the blood clot formation. The hematomas are composed of activated platelet plugs and reinforced by a three-dimensional fibrin fibrous network. After implantation, plasma proteins adsorb to the implant surfaces, and blood-derived platelets are subsequently activated at the interface. Activated platelets mediate the expression of GPIIb/IIIa (also known as integrin αIIbβ3), glycoprotein-1 (TSP-1), and P-selectin (Nurden et al. 2008). As the receptor for fibrinogen and von Willebrand factor (vWF), GPIIb/

IIIa can further trigger peripheral platelet activation (Du et al. 1991). TSP-1 is an adhesion glycoprotein that regulates the interactions of cell-cell and cell-matrix. It couples with fibrinogen, fibronectin, laminin, collagen V, and integrin αV/β1 and takes a vital role in the induction of platelet aggregation (Browder et al. 2000). In addition, P-selectin can mediate platelet-fibrin and platelet-platelet combination to enhance platelet aggregation (Blair and Flaumenhaft 2009). Upregulated expression of the above three signaling molecules (GPIIb/IIIa, TSP-1, and P-selectin) ultimately results in elevated local thrombin activation (Sivaraman and Latour 2011). Thrombin converts free fibrinogen in the blood into fibrin monomers (Roy et al. 2007). The self-polymerization of fibrin monomers through the semi-interlaced and double-chain intertwined modes constructs the protofibrils, which are subsequently linked and polymerized into fibril bundles with non-covalent bonds and eventually cross-linked to form a three-dimensional fibrin fiber structure (Chan et al. 2015).

The thickness and density of the clot fibrous networks are adjustable and intimately related to the degree of the platelet activation which can be effectively dominated by the various physicochemical properties of implant surfaces. Meanwhile, large amounts of growth factors and cytokines (vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), endothelial growth factor (EGF), platelet-derived growth factor (PDGF), etc.) are progressively secreted by the activated platelets into the ambient environment, (Burkhardt et al. 2017; Anitua et al. 2006; Andrae et al. 2008; Kim et al. 2010). These mediators will inevitably have a positive impact on osseointegration.

13.1.2 Immune Responses, Angiogenesis, and Osteogenesis

During blood clot formation, multiple immune cells like T cells, B cells, macrophages (MΦs), and neutrophils, are wrapped in the fibrin fibrous network scaffold, which clean the traumatic sites from pathogenic inducements, cellular debris, and apoptotic cells (Bai et al. 2021b; Herter et al. 2014; Eming et al. 2007; Shahneh et al. 2021). Therefore, the inflammatory phase mediated by immune cells ensues after the hematoma formation, starts within the initial 12 h, and usually ends around 7 days (Oryan et al. 2013). Among the immune cells, MΦs take pivotal roles in the immune responses and are one of the main effector cells in the inflammation responses (Miron and Bosshardt 2016). MΦs have multiple phenotypes, of which the un-activated MΦs are called M0 phenotype. MΦs are highly plastic and can be activated to the two typical phenotypes (M1 and M2) (Chen et al. 2017). The M1 phenotype of MΦs is known to intensify inflammation responses by secreting inflammatory cytokines like interleukin-1β (IL-1β), IL-6, and tumor necrosis factor-α (TNFα). Although initial inflammatory reactions are inevitable, a prolonged presence of inflammation and M1 polarization gives rise to the fibrous encapsulation formation around implants, which results in insufficient osseointegration and implant loosening (Chen et al. 2016). On the contrary, the M2 phenotype of MΦs alleviates inflammation by producing IL-4, IL-10, and expression of differentiation marker 206 (CD206). Additionally, M2-phenotypic MΦs can generate a favorable

osteoimmune microenvironment by releasing growth factors (e.g., VEGFA, TGF-β1, and bone morphogenetic protein-2 (BMP2)), which in turn promote osteogenic cell growth and differentiation (Chen et al. 2016, 2017; Bai et al. 2018a, b).

After acute inflammation subsides, fibroblasts, endothelial cells, osteoblasts, and mesenchymal stem cells are converged to the traumatic sites, remodeling the early temporary cell-adhesion matrix and progressively replacing it with a collagen-rich matrix to restore tissue homeostasis and implement tissue repair (Gurtner et al. 2008). In parallel with the action of MΦs, angiogenesis is gradually initiated within 24 h after implantation (Wang et al. 2016). Angiogenesis, a biological procedure of the new vasculature generation and the formation of capillaries from existing vessels, involves the sprouting, growth, and maturation of new blood vessels (Nagy et al. 2008). Endothelial progenitor cells (EPC) or stem cells are recruited and then proliferate, differentiate, and reconstitute to form new blood vessels. Microvasculature grows by outgrowth over existing vessels to form new vascular branches due to the proliferation, migration, and reconstruction of mature endothelial cells in existing vessels. The new reconstructed vasculature network can not only sustain oxygen and nutrient exchange for new bone formation, and bring growth factors and chemokines to the bone-implant interface, but also guide the transportation of calcium and phosphate for the mineralization and remove metabolic waste.

After blood clot formation, immunomodulation, and revascularization, osseointegration is ultimately accomplished through the accumulation of the de novo bone on implant surfaces. As the main functional cells of new bone production, osteoblasts migrate to the bone-implant interface and upregulate the synthesis, secretion, and mineralization of extracellular matrix by expressing various osteogenic molecules and mediators (e.g., BMP-2, TGF-β1, osteoprotegerin (OPG), osteopontin (OPN), and osteocalcin (OCN)), eventually forming new bone. In general, newly formed bone appears around the implants within 5–7 days after implant placement, and the calcification of the bone matrix occurs simultaneously at the peri-implant (Marco et al. 2005). After 6 weeks, the new bone around the implant (contact osteogenesis) is interconnected with the new-formed bone adjacent to the host bone tissue (distant osteogenesis) (Wang et al. 2016; Davie 2003). Eight to twelve weeks later, mature lamellar bone is detected around the implant surface, thus completing osseointegration at the interface (Wang et al. 2016; Kolar et al. 2010).

In the whole process of osseointegration, the role of the blood clot is extremely pivotal as its regulating effects on the early immune responses and angio/osteogenesis through multiple action mechanisms. A study indicated that the hematoma around the alkali-treated titanium (Ti) implants was applied as a natural scaffold, which exhibited the exceptional properties that hastened the peri-implant angiogenesis and regeneration process (Burkhardt et al. 2017). The adaptable clots formed on the micro-structured Ti implant surfaces could stimulate stem cell recruitment, migration, and differentiation, thus promoting bone repair (Yang et al. 2016). The initial hematoma induced by smaller diameter TiO_2 nanotube arrays on the Ti implant surface could facilitate the conversion of MΦs from pro-inflammatory M1 phenotype to anti-inflammatory and pro-healing M2 phenotype, inhibit

inflammation, thereby promoting subsequent vascularization and new bone formation (Bai et al. 2021b). In addition, endeavors have been carried out to expound the crucial effects of mRNAs and long non-coding RNAs (LncRNAs) within the blood clot on immune responses, angio/osteo-genesis, and osseointegration (Bai et al. 2021a, b). Consequently, the physicochemical properties of the implant surfaces modulate the features of the initial blood clot, which regulate the subsequent different stages of osseointegration through various mechanisms. This chapter focuses on the roles of LncRNA profiles within the implant-mediated blood clots on osseointegration.

13.2 LncRNAs and Their Functions

13.2.1 Characteristics and Classification of LncRNAs

LncRNAs are a category of non-coding RNA molecules with transcripts longer than 200 nucleotides, which generally do not encode proteins but participate in protein-coding gene regulation at multiple aspects (mainly chromatin remodeling, transcriptional control, and post-transcriptional processing) in the form of RNA (Mercer et al. 2009; Kapranov et al. 2007). LncRNAs are tissue-specific and spatiotemporal specific. LncRNA expression is distinct among diverse tissues and organs and the LncRNA profile expression levels in the same tissue or organ may also change at different growth stages. Most LncRNAs possess conserved secondary structures, splicing forms, and subcellular localization, which are important for LncRNAs to perform their functions. However, the functional mechanisms of LncRNAs are more intractable to deduce than the microRNAs (miRNAs) and proteins, and it is currently impossible to infer their functions based on sequence or structure alone. In the mammalian genome, the transcripts of LncRNAs can account for up to 4–9% of total RNA sequences (the corresponding percentage of protein-coding RNAs is 1%). The LncRNAs are another treasure trove for gene function research.

LncRNAs are characterized by multiple types, modes of action, and quantities. Depending on the LncRNA locations on the genome relative to protein-coding genes, they are classified into five types: sense, antisense, bidirectional, intronic, and intergenic (Mercer et al. 2009). These location relationships are correlated with the functions of LncRNAs. Additionally, based on the LncRNA functions, they are divided into the signal molecule, decoy molecule, guide molecule, scaffold molecule, and so on (Wang and Chang 2011).

13.2.2 Biological Functions of LncRNAs

LncRNAs were originally regarded as the “noise” of genome transcription and by-products of RNA polymerase (RNAP) II transcription, which did not execute biological functions. Nevertheless, numerous researches have revealed a wide range of functions for LncRNAs in various biological fields including roles in

chromosome dynamics, telomere biology, and subcellular-structural organization (Mercer et al. 2009; Amaral and Mattick 2008). Specifically, LncRNAs participate in a variety of critical genetic-biological processes including chromosome silencing, genomic imprinting, chromatin remodeling, transcription activation, transcription interference, and intranuclear transportation. The processes subsequently regulate multiple aspects of cell activities and homeostasis such as survival, proliferation, metabolism, differentiation, subcellular structure and localization, and genomic stability, ultimately affecting miscellaneous physiological and pathological developments, such as neuronal disorders, immune responses, and cancer (Wapinski and Chang 2011; Statello et al. 2020).

13.2.3 Action Mechanisms of LncRNAs

The regulating effects of LncRNAs are fundamentally reflected in their involvement in manipulating the expression of surrounding protein-coding genes. The action mechanisms of LncRNAs for the multilevel modulation of gene expression are generally focused on three aspects (epigenetic regulation, transcriptional regulation, and post-transcriptional regulation) (Mercer et al. 2009). The following text describes the regulatory mechanisms of LncRNAs at different levels, and Fig. 13.2 shows the schematic diagram of the regulatory mechanisms of LncRNAs.

13.2.3.1 Epigenetic Regulation

In epigenetic regulation, LncRNAs are mainly involved in mediating chromatin remodeling. LncRNAs can recruit the remodeling/modification complexes of chromatin to the specialized loci, altering DNA/RNA methylation/hydroxymethylation, histone acetylation/methylation, RNA polymerase accessibility, and chromosome

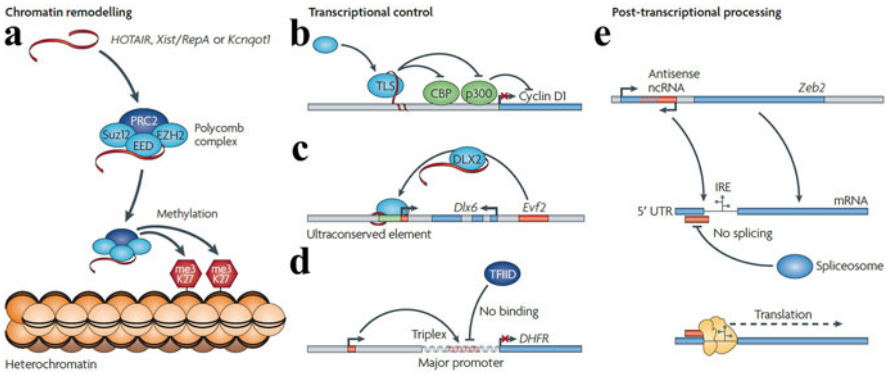


Fig. 13.2 Schematic representation of the action mechanisms of LncRNAs in regulating the transcription and translation of local protein-coding genes at multiple levels (Mercer et al. 2009). (Copyright Springer Nature) (**a**) LncRNAs participate in chromatin remodeling; (**b**–**d**) LncRNAs take regulatory effects in the transcriptional control; (**e**) LncRNAs regulate the mRNA splice in the post-transcriptional process

structure and modification states, which in turn regulate the expression of correlated genes. The development of certain diseases such as cancer is closely related to the DNA/RNA methylation mutations, while alterations in chromatin modification status also usually influence the expression of relevant genes, most commonly the epigenetic factor modifications such as H3K4me3, H3K9me2, H3K27me3, and H4K20me3 that occur in the promoter region. Generally, the chromatin rich in activated histone modifications (e.g., H3K4me3, H3K36me3, and histone acetylation) is in the open status; and the chromatin rich in restrained histone modifications (H3K9me3, H3K27me3, H4K20me3, DNA methylation, etc.) is in the closed status. Alteration of chromatin activity can promote or inhibit transcription and control gene expression. For example, the LncRNAs HOTAIR, Xist/RepA, or Kcnqot1, respectively, recruits the polycomb-complexes to the HoxD locus, X chromosome, or Kcnq1 region, where they trimethylate H3K27me3 to guide heterochromatin formation and silence gene transcription (Fig. 13.2a) (Rinn et al. 2007).

13.2.3.2 Transcriptional Regulation

The prevalent coordination with promoters, enhancers, and transcription factors is the central action mechanism of LncRNAs in the transcription regulation (Guenther et al. 2007; Wang et al. 2008; Feng et al. 2006; Martianov et al. 2007; Ashe et al. 1997). Proximal promoters are transcribed into LncRNAs thus recruiting the RNA-binding proteins and engaging them in the transcription procedures. For example, the transcription of LncRNAs connected with the promoter of cyclin D1 gene can be initiated by DNA damage signals, thereby acting synergistically to recruit the RNA-binding protein TLS and regulate its activities (Fig. 13.2b) (Wang et al. 2008). Afterwards, TLS suppresses the histone acetyltransferase activities of CREB-binding protein (CBP) and p300 to repress cyclin D1 transcription. As essential regulatory mediators in the process of gene transcription, transcription factors can bind to RNAs and regulate RNA transcription, localization, and stability. Meanwhile, LncRNAs are the vital ligands or co-factors that can regulate transcription factor activities. The binding between the LncRNAs and transcription factors can generate specific complexes to regulate gene transcription. LncRNA Evf2 is transcribed from an ultra-conservative distal enhancer and can recruit transcription factor DLX2 to control its activities as a co-activator, thus handling the transcription of adjoining protein-coding genes (like Dlx6) (Fig. 13.2c) (Feng et al. 2006). In addition, LncRNAs exhibit the functions of promoting enhancer cyclization and regulating insulators (suppressing enhancer function) to activate gene expression (Ashe et al. 1997; Witham et al. 2013). LncRNAs can affect RNAP II activities in a variety of ways, including regulating promoter choice by interacting with the initiation complexes. A LncRNA transcribed from the upstream domain of the dihydrofolate reductase (DHFR) site (minor promoter) can form a triplex in the major promoter of DHFR to obstruct the combination of transcriptional factor TFIID, consequently silencing DHFR gene transcription (Fig. 13.2d) (Martianov et al. 2007). Additionally, the LncRNA transcription can interfere with the expression of adjacent protein-coding genes (Mercer et al. 2009). When upstream LncRNAs are transcribed, they cross the promoter regions of neighboring target

genes, interfering with the binding between the transcription factors and promoters of the target genes, thereby inhibiting the target gene expression.

13.2.3.3 Post-transcriptional Regulation

Recognition of complementary sequences by LncRNAs allows for highly specific interactions with mRNAs, which facilitate the regulation of various procedures in mRNA post-transcriptional processes that include variable splice, editing, transportation, translation, and degradation (Mercer et al. 2009). These procedures are important for gene function polymorphism. Most genomes of mammals express antisense transcripts and the transcripts constitute a category of LncRNAs (antisense LncRNAs) which are especially proficient at modulating mRNA dynamics (He et al. 2008). In the process of mRNA variable splicing regulation, antisense LncRNAs bind to mRNA complementary regions and influence the recruitment of spliceosomes at specific splice sites to control the mRNA splicing process, and also have an impact on RNA editing. Antisense LncRNAs can obscure pivotal cis-elements in mRNA by forming RNA duplexes, as in the instance of Zeb2 antisense RNA (Beltran et al. 2008). An antisense LncRNA can obscure the 5′ splice locus in the zinc finger homeobox mRNA Zeb2, avoiding spliceosome recognition and allowing intron retention (Fig. 13.2e). Subsequently, the internal ribosome entry site (IRE) in the retained intron is recognized by translation machinery that then binds to the ribosomes to implement high-efficiency Zeb2 translation and synthesis.

There are still numerous unknown functions of LncRNAs awaiting to be explored. Some antisense LncRNAs can also interact with mRNAs to play regulatory roles during mRNA intranuclear transportation and intracellular localization. For instance, the LncRNA NRON has been demonstrated to control the intranuclear transportation of transcription factor NFAT (Willingham et al. 2005), while it is observed that a large number of LncRNAs are localized in the cell cytoplasm (Kapranov et al. 2007).

13.3 LncRNAs in the Blood Clot and Osseointegration

Given that LncRNAs are burgeoning crucial mediators in gene transcription regulation, their significant roles in osseointegration are conceivable. This section will introduce the influence of LncRNAs within the blood clots mediated by nano-dimensional implant surfaces on osseointegration (Bai et al. 2021a).

13.3.1 Blood Clot Feature and Osseointegration Mediated by Nano-dimensional Implant Surfaces

Highly ordered titania nanotube arrays (TNAs) with different diameters have been applied to study coagulation and osteointegration. In this study, the TNAs with average diameters of 15 (TNA 15), 60 (TNA 60), and 120 nm (TNA 120) were used

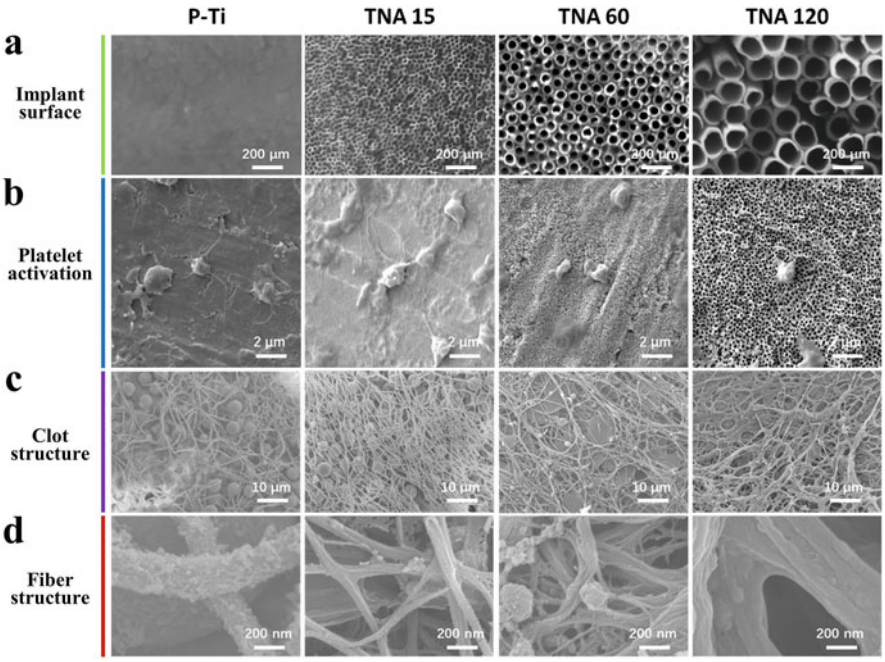


Fig. 13.3 Platelet activation and blood clot feature meditated by different TNAs (Bai et al. 2021a). (Copyright Multidisciplinary Digital Publishing Institute) (**a**) Surface morphology of P-Ti and TNAs; (**b**) Platelet activation; (**c**, **d**) Blood clot and fibrin fiber features. P-Ti: pure titanium, TNA 15: 15 nm diameter titania nanotube array, TNA 60: 60 nm diameter titania nanotube array, TNA 120: 120 nm diameter titania nanotube array

to study their nano-scaled effects (Fig. 13.3a). Platelet-rich plasma was dropped on implant surfaces and subsequently incubated for 30 min to observe the platelet morphology adhered to the surfaces. As shown in Fig. 13.3b, more platelets with a significant activation morphology attached to the TNA15 surface and extended a large spreading area and plentiful pseudopodia. The blood clot morphology on the implant surfaces was observed after 24 h of implantation. The dense and thin fibrin fibers interspersed blood clots formed on TNA 15 surface, while the incompact and thick fibrin fibers decorated blood clots were observed on others, particularly on the TNA 120 surface (Fig. 13.3c, d). In addition, numerous plasma proteins adsorbed to TNA 15 surface, covering and sealing the nanotubes. Therefore, the blood clot features can be modulated by the different surface nano-scales of implants. Smaller diameter TNA (15 nm) facilitates obvious platelet activation, thus leading to the appropriate blood clot properties.

The blood colt mediated by TNA 15 surface could modulate immune responses of MΦs, downregulated inflammation, drove MΦs to pro-healing M2 polarization phenotype, and manipulate a favorable osteoimmunomodulatory environment (Bai et al. 2021b). A large number of activated immune cells and platelets were recruited

in the hematomas formed on TNA 15 surface, indicating coordinated effects of blood clots and immune responses during the early healing phase. Additionally, the smaller diameter TNA (15 nm) directly inhibited the MΦ inflammatory responses, guided a rapid M1-to-M2 phenotype transition of MΦs, and promoted MΦs to elevate the transcription of growth factors and mediators that are conducive to angio/osteogenesis (Bai et al. 2021b, 2018c). The direct promotion effects of TNA 15 on neovascularization were also significant (Bai et al. 2018c). Nano-scaled effects of the implant surfaces exhibit a significant impact on all stages of osseointegration. In particular, the features of initial blood clots mediated by implants play a crucial role in subsequent stages. Smaller diameter TNA (15 nm)-mediated clot is more conducive to new bone formation and osseointegration.

13.3.2 LncRNAs in Blood Clots Mediated by Nano-dimensional Implant Surfaces

In-depth bioinformatic analysis of LncRNA profiles in the blood clots mediated by different nano-scaled implant surfaces was performed using high-throughput sequencing technology, and the potential LncRNA-targeted mRNAs were revealed.

LncRNA expression profiles within the blood clots formed on different surfaces were significantly distinct. Compared with P-Ti, 508 LncRNAs were markedly upregulated and 61 LncRNAs were downregulated within the clots mediated by TNA 15. 250 LncRNAs within the clots mediated by TNA 15 were significantly upregulated when compared with TNA 60, while 28 LncRNAs were downregulated. For TNA 15 *vs* TNA 120 group, 92 LncRNAs were upregulated and 205 LncRNAs were downregulated. Through bioinformatic analysis, pivotal LncRNAs involved in the LncRNA-mRNA regulation were unraveled. LOC103346307, LOC108175175, and LOC108176660 in LncRNA profiles within the blood clots mediated by TNA 15 targeted the majority of upregulated mRNAs in the respective comparison with P-Ti, TNA 60, and TNA 120, while LOC103352121, LOC103348180, and LOC108176465 aimed to the masses of downregulated mRNAs (Fig. 13.4). Bioinformatic identification was performed to export the co-expression networks of LncRNA-mRNA, which manifested the LncRNAs LOC103346307, LOC103352121, LOC108175175, LOC103348180, LOC108176660, and LOC108176465 were the key mediators within the inchoate blood clots on nano-dimensional surfaces.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis illustrated that different surface nano-dimensions can determine the biological functions of LncRNA-targeted mRNAs within the blood clots. The identified key LncRNAs exert crucial roles in maneuvering the transcription of targeted mRNA. Compared with the P-Ti surface, the upregulated LncRNA-targeted mRNAs within the blood clots mediated by the TNA 15 surface were prominently clustered in the pathways associated with cell growth and metabolism and the major pathways included the signaling pathways PI3K-Akt, cAMP, and Jak-STAT and the cellular activities such as focal adhesion, regulation of actin cytoskeleton, and platelet

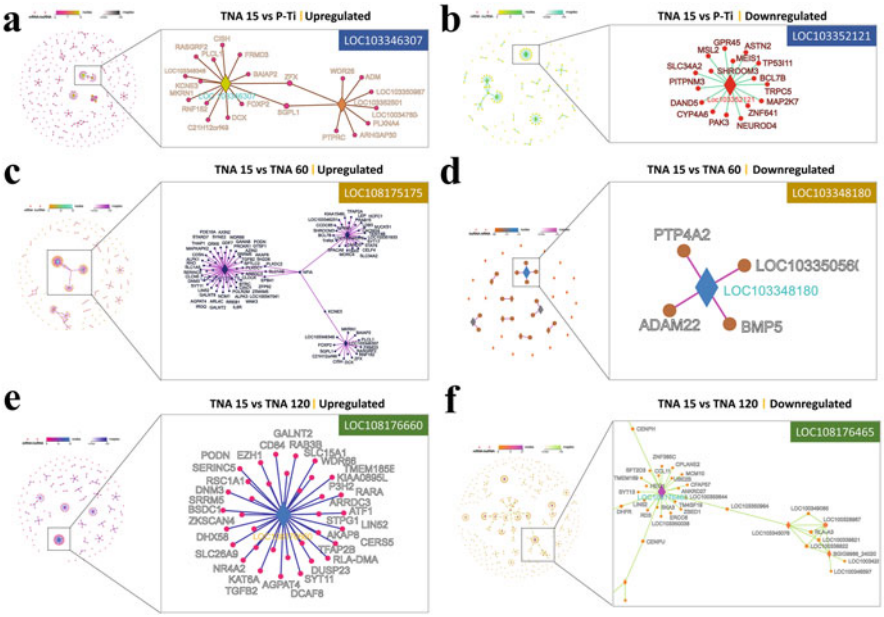


Fig. 13.4 Pivotal LncRNAs identified in different comparison groups (Bai et al. 2021a). (Copyright Multidisciplinary Digital Publishing Institute) (**a**) and (**b**) Pivotal LncRNAs identified in TNA 15 *vs* P-Ti; (**c**) and (**d**) Pivotal LncRNAs identified in TNA 15 *vs* TNA 60; (**e**) and (**f**) Pivotal LncRNAs identified in TNA 15 *vs* TNA 120

activation (Fig. 13.5a). In addition, the targeted mRNAs of downregulated LncRNAs were mainly concentrated in the pathways related to inflammation responses. The pathways with the most significant enrichment were chemokine, TNF, Toll-like receptor, C-type lectin receptor, T cell receptor, and IL-17 signaling pathways (Fig. 13.5b).

As shown in Fig. 13.5c, the targeted mRNAs of upregulated LncRNAs in TNA 15 *vs* TNA 60 were principally focused on the signaling pathways related to cell growth and metabolism (e.g., Wnt, Hippo, and PI3K-Akt signaling pathways). The targeted mRNAs of downregulated LncRNAs were mainly enriched in mTOR, p53, Toll-like receptor, and B cell receptor signaling pathways and the cell behaviors such as apoptosis and Fc gamma R-mediated phagocytosis (Fig. 13.5d). In TNA 15 *vs* TNA 120 comparison group, the enriched signaling pathways of the upregulated LncRNAs targeted mRNAs were also primarily concentrated in cell growth and metabolism (Fig. 13.5e). Wnt and TGF-β signaling pathways and adherens junction were dominant. Additionally, the downregulated LncRNA-targeted mRNAs were observably clustered in the signaling pathways associated with inflammation and the chemokine and T cell receptor signaling pathways, Fc gamma R-mediated phagocytosis, cellular senescence, and inflammatory mediator regulation of TRP were main enrichment items (Fig. 13.5f).

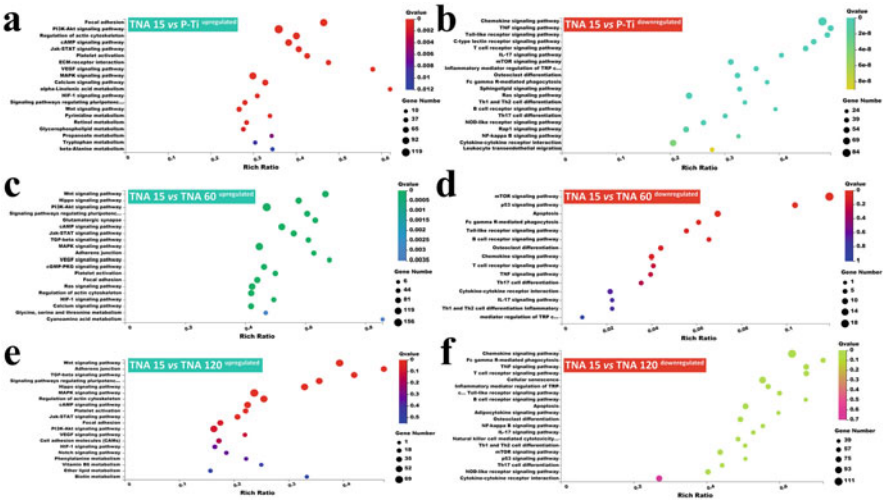


Fig. 13.5 KEGG enrichment items of the LncRNA-targeted mRNAs in different comparison groups (Bai et al. 2021a). (Copyright Multidisciplinary Digital Publishing Institute) (**a**) and (**b**) KEGG enrichment pathways in TNA 15 *vs* P-Ti comparison group; (**c**) and (**d**) KEGG enrichment pathways in TNA 15 *vs* TNA 60 comparison group; (**e**) and (**f**) KEGG enrichment pathways in TNA 15 *vs* TNA 120 comparison group

In comparison with the P-Ti, the functions of the targeted mRNAs of LncRNA profiles mediated by TNAs showed synchronous appearances. Upregulated pathways were synchronized in Wnt, TGF-β, and VEGF signaling pathways, and simultaneously downregulated pathways were concentrated in TNF and NF-κ signaling pathways and osteoclast differentiation. Wnt signals take a pivotal role in the development and differentiation of osteogenic-lineage cells during osseointegration. Meanwhile, the Wnt signaling pathway is a typical pathway for osteogenic differentiation (Baron and Kneissel 2013). In addition, the Wnt signaling pathway is important in regulating bone homeostasis. The signaling pathway activation leads to enhanced bone mass and the inhibition causes attenuated bone mass and strength. TGF-β signaling pathway is another critical pathway that proved to promote bone repair and remodeling and regulate new bone formation (Dole et al. 2017). Silencing of the TGF-β signals likewise can cause a reduction of bone matrix mineralization.

During new bone forming, neovascularization at the bone-implant interface is considered the precondition for sufficient osseointegration. VEGF signal can induce vasculature-network formation by promoting the expression of pro-angiogenesis cytokines (Ferrara et al. 2003). As a downstream target of VEGF, endothelial nitric oxide synthase (eNOS) stimulates the synthesis of vascular nitric oxide (NO), thus contributing to angiogenesis (Ahmad et al. 2006). Wnt, TGF-β, and VEGF signaling pathways take an equally important role in angiogenesis and osteogenesis and exert their effects throughout osseointegration. Macrophages in the bone marrow can undergo osteoclast differentiation under inflammation and stress and further

transform into bone-resorbing polymorphonuclear cells, that is, osteoclasts (Park et al. 2017). Osteoclasts secrete multiple proteases (such as TRAP and CTSK) which can degrade bone matrix (mainly collagen I), thus resulting in bone resorption, impeding the formation of de novo bone, and promoting osteoclastogenesis (Boyle et al. 2003). Osteoclast differentiation is an impediment to osseointegration. TNF is a critical inflammatory cytokine signal of immune responses, contributing to the inflammation and the synthesis and secretion of pro-inflammatory factors (Chu 2013; Yao et al. 2020). Additionally, the TNF signaling pathway exerts essential regulatory functions in vivo *(*Zhao 2017*)*. It directly elevates the number of osteoclast precursors and indirectly promotes osteoclastogenesis by upregulating the transcription of RANK (activator of NF-κ signals) in osteoclast precursors, thus performing a crucial enhanced effect on bone resorption. As a transcription factor family, NF-κ exerts significant signal-regulatory roles in promoting osteoclast functions (Chang et al. 2009). Growing evidence suggests the activated NF-κ signaling pathway impedes osteogenic differentiation and restrains new bone formation, thus not conducive to bone regeneration and repair (Krum et al. 2010). In addition, the NF-κ signal coordinates with other inflammation-regulating factors (such as IL-6, IL-1β, and TNF-α) to augment inflammation development. Suppressing inflammation is beneficial to angio/osteo-genesis and osseointegration (Bai et al. 2018a). Consequently, the downregulated pathways centralized by the LncRNA-targeted mRNAs mediated by TNAs mainly exert effects on inhibiting osteoclast differentiation and inflammation.

The different surface nano-dimensions are capable of regulating LncRNA profiles in the hematoma surrounding the implants. The TNA, especially TNA 15, mediates the initial blood clots to upregulate LncRNAs (LOC103346307, LOC108175175, and LOC108176660) which targeted mRNAs correlated to Wnt, TGF-β, and VEGF signaling pathways, and downregulate LncRNAs (LOC103352121, LOC103348180, and LOC108176465) which targeted mRNAs related to osteoclast differentiation, TNF, and NF-κ signaling pathways. Consequently, the blood clot formed on TNA 15 surface exhibits beneficial promoting effects for angiogenesis and osteogenesis and inhibits bone resorption and inflammation, ultimately facilitating osseointegration. Another study suggested that TNA 15 was highly prospective for ameliorating osseointegration through manipulating a favorable immune microenvironment, which was generated by mediating a thin and compact fibrous network decorated specialized blood clot that could release growth mediators such as TGF-β and PDGF-AB (Bai et al. 2021b). The results of LncRNA profiles in the blood clots demonstrate that TNA 15-mediated clot possesses favorable regulatory effects on osseointegration, and elucidates the underlying mechanism. Perspectives on the regulatory roles of LncRNA in the blood clots deepen the current understanding of osseointegration (Fig. 13.6).

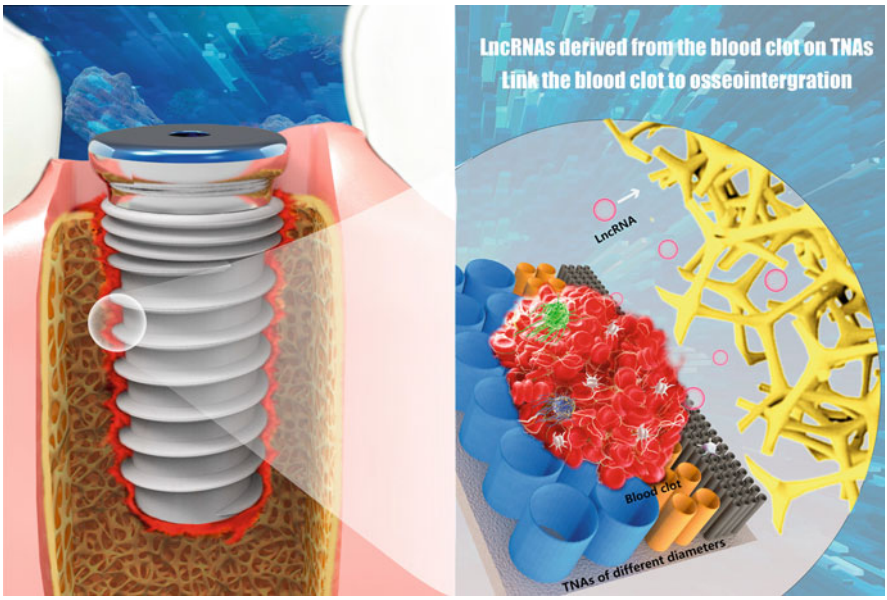


Fig. 13.6 Relationship schematic between the LncRNAs within the blood clots formed on nano-dimensional implant surfaces and osseointegration (Bai et al. 2021a). (Copyright Multidisciplinary Digital Publishing Institute)

13.4 Summary and Perspective

The importance of early blood clots formed on the implant surfaces for osseointegration has received increasing attention. Implant surfaces with nano-dimensional structures can govern the blood coagulation processes yielding appropriate clot structures, features, and degradation properties, which in turn affect the responses of relevant cells and the inchoate stage of osseointegration. The role of blood clots in manipulating immune responses is convincingly demonstrated. However, the pathways by which blood clots regulate the function of osseointegration-related cells are still rarely identified. LncRNAs exert significant regulatory effects on the transcription of protein-coding genes and many biological processes. This chapter introduces the effects of TNAs on clot features, the regulation of LncRNA profile expression derived from the hematoma, and the relationship between the LncRNAs and osseointegration. The current knowledge indicates that the LncRNA profiles within blood clots could be mediated by implant surfaces, which have a decisive function in regulating the inflammation, neovascularization, and new bone formation, thus providing a novel perspective for implant surface modification to promote osseointegration. Expanded efforts shall be made to explore the deeper mechanisms by which blood clots regulate osseointegration and understand the regulatory role of LncRNAs in various stages of osseointegration.

References

Ahmad S, Hewett PW, Wang P, Al-Ani B, Cudmore M, Fujisawa T, Haigh JJ, Le Noble F, Wang L, Mukhopadhyay D, Ahmed A (2006) Direct evidence for endothelial vascular endothelial growth factor receptor-1 function in nitric oxide-mediated angiogenesis. Circ Res 99(7):715–722

Amaral PP, Mattick JS (2008) Noncoding RNA in development. Mamm Genome 19(7–8):454–492

Andrae J, Gallini R, Betsholtz C (2008) Role of platelet-derived growth factors in physiology and medicine. Genes Dev 22(10):1276–1312

Anitua E, Sánchez M, Nurden AT, Nurden P, Orive G, Andía I (2006) New insights into and novel applications for platelet-rich fibrin therapies. Trends Biotechnol 24(5):227–234

Ashe HL, Monks J, Wijgerde M, Fraser P, Proudfoot NJ (1997) Intergenic transcription and transinduction of the human β-globin locus. Genes Dev 11(19):2494–2509

Bai L, Du Z, Du J, Yao W, Zhang J, Weng Z, Liu S, Zhao Y, Liu Y, Zhang X, Huang X, Yao X, Crawford R, Hang R, Huang D, Tang B, Xiao Y (2018a) A multifaceted coating on titanium dictates osteoimmunomodulation and osteo/angio-genesis towards ameliorative osseointegration. Biomaterials 162:154–169

Bai L, Liu Y, Du Z, Weng Z, Yao W, Zhang X, Huang X, Yao X, Crawford R, Hang R, Huang D, Tang B, Xiao Y (2018b) Differential effect of hydroxyapatite nano-particle versus nano-rod decorated titanium micro-surface on osseointegration. Acta Biomater 76:344–358

Bai L, Yang Y, Mendhi J, Du Z, Hao R, Hang R, Yao X, Huang N, Tang B, Xiao Y (2018c) The effects of TiO_2 nanotube arrays with different diameters on macrophage/endothelial cell response and ex vivo hemocompatibility. J Mater Chem B 6(39):6322–6333

Bai L, Chen P, Tang B, Hang R, Xiao Y (2021a) Correlation between LncRNA Profiles in the blood clot formed on nano-scaled implant surfaces and osseointegration. Nanomaterials 11(3):674

Bai L, Zhao Y, Chen P, Zhang X, Huang X, Du Z, Crawford R, Yao X, Tang B, Hang R, Xiao Y (2021b) Targeting early healing phase with titania nanotube arrays on tunable diameters to accelerate bone regeneration and osseointegration. Small 17(4):2006287

Baron R, Kneissel M (2013) WNT signaling in bone homeostasis and disease: from human mutations to treatments. Nat Med 19(2):179–192

Beltran M, Puig I, Peña C, García JM, Alvarez AB, Peña R, Bonilla F, de Herreros AG (2008) A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition. Genes Dev 22(6):756–769

Blair P, Flaumenhaft R (2009) Platelet α-granules: basic biology and clinical correlates. Blood Rev 23(4):177–189

Boyle WJ, Simonet WS, Lacey DL (2003) Osteoclast differentiation and activation. Nature 423(6937):337–342

Browder T, Folkman J, Pirie-Shepherd S (2000) The hemostatic system as a regulator of angiogenesis. J Biol Chem 275(3):1521–1524

Burkhardt MA, Gerber I, Moshfegh C, Lucas MS, Waser J, Emmert MY, Hoerstrup SP, Schlottig F, Vogel V (2017) Clot-entrapped blood cells in synergy with human mesenchymal stem cells create a pro-angiogenic healing response. Biomater Sci 5(10):2009–2023

Chan LW, White NJ, Pun SH (2015) Synthetic strategies for engineering intravenous hemostats. Bioconjug Chem 26(7):1224–1236

Chang J, Wang Z, Tang E, Fan Z, McCauley L, Franceschi R, Guan K, Krebsbach PH, Wang C-Y (2009) Inhibition of osteoblastic bone formation by nuclear factor-κB. Nat Med 15(6):682–689

Chen Z, Klein T, Murray RZ, Crawford R, Chang J, Wu C, Xiao Y (2016) Osteoimmunomodulation for the development of advanced bone biomaterials. Mater Today 19(6):304–321

Chen Z, Bachhuka A, Wei F, Wang X, Liu G, Vasilev K, Xiao Y (2017) Nanotopography-based strategy for the precise manipulation of osteoimmunomodulation in bone regeneration. Nanoscale 9(46):18129–18152

Chu W-M (2013) Tumor necrosis factor. Cancer Lett 328(2):222–225

Davie JE (2003) Understanding peri-implant endosseous healing. J Dent Educ 67(8):932–949

Dole NS, Mazur CM, Acevedo C, Lopez JP, Monteiro DA, Fowler TW, Gludovatz B, Walsh F, Regan JN, Messina S, Evans DS, Lang TF, Zhang B, Ritchie RO, Mohammad KS, Alliston T (2017) Osteocyte-intrinsic TGF-β signaling regulates bone quality through perilacunar/canalicular remodeling. Cell Rep 21(9):2585–2596

Du X, Plow EF, Frelinger AL III, O'Toole TE, Loftus JC, Ginsberg MH (1991) Ligands "activate" integrin αIIbβ3 (platelet GPIIb-IIIa). Cell 65(3):409–416

Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. J Investig Dermatol 127(3):514–525

Feng J, Bi C, Clark BS, Mady R, Shah P, Kohtz JD (2006) The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. Genes Dev 20(11):1470–1484

Ferrara N, Gerber H-P, LeCouter J (2003) The biology of VEGF and its receptor. Nat Med 9(6): 669–676

Guenther MG, Levine SS, Boyer LA, Jaenisch R, Young RA (2007) A chromatin landmark and transcription initiation at most promoters in human cells. Cell 130(1):77–88

Gurtner GC, Werner S, Barrandon Y, Longaker MT (2008) Wound repair and regeneration. Nature 453(7193):314–321

He Y, Vogelstein B, Velculescu VE, Papadopoulos N, Kinzle KW (2008) The antisense transcriptomes of human cells. Science 322(5909):1855–1857

Herter JM, Rossaint J, Zarbock A (2014) Platelets in inflammation and immunity. J Thromb Haemost 12(11):1764–1775

Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, Stadler PF, Hertel J, Hackermüller J, Hofacker IL, Bell I, Cheung E, Drenkow J, Dumais E, Patel S, Helt G, Ganesh M, Ghosh S, Piccolboni A, Sementchenko V, Tammana H, Gingeras TR (2007) RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science 316(5830):1484–1488

Kim E-S, Kim J-J, Park E-J (2010) Angiogenic factor-enriched platelet-rich plasma enhances in vivo bone formation around alloplastic graft material. J Adv Prosthodont 2(1):7–13

Kolar P, Schmidt-Bleek K, Schell H, Gaber T, Toben D, Schmidmaier G, Perka C, Buttgereit F, Duda GN (2010) The early fracture hematoma and its potential role in fracture healing. Tissue Eng Part B Rev 16(4):427–434

Krum SA, Chang J, Miranda-Carboni G, Wang C-Y (2010) Novel functions for NFκB: inhibition of bone formation. Nat Rev Rheumatol 6(10):607–611

Listgarten MA, Lang NP, Schroeder HE, Schroeder A (1991) Periodontal tissues and their counterparts around endosseous implants. Clin Oral Implants Res 2(1):1–19

Marco F, Milena F, Gianluca G, Vittoria O (2005) Peri-implant osteogenesis in health and osteoporosis. Micron 36(7–8):630–644

Martianov I, Ramadass A, Barros AS, Chow N, Akoulitchev A (2007) Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript. Nature 445(7128):666–670

Mercer TR, Dinger ME, Mattick JS (2009) Long non-coding RNAs: insights into functions. Nat Rev Genet 10(3):155–159

Miron RJ, Bosshardt DD (2016) OsteoMacs: key players around bone biomaterials. Biomaterials 82:1–19

Nagy JA, Benjamin L, Zeng H, Dvorak AM, Dvorak HF (2008) Vascular permeability, vascular hyperpermeability and angiogenesis. Angiogenesis 11(2):109–119

Nurden AT, Nurden P, Sanchez M, Andia I, Anitua E (2008) Platelets and wound healing. Front Biosci 13(9):3532–3548

Oryan A, Alidadi S, Moshiri A (2013) Current concerns regarding healing of bone defects. Hard Tissue 2(2):1–12

Park JH, Lee NK, Lee SY (2017) Current understanding of RANK signaling in osteoclast differentiation and maturation. Mol Cells 40(10):706–713

Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E, Chang HY (2007) Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell 129(7):1311–1323

Roy SC, Paulose M, Grimes CA (2007) The effect of TiO_2 nanotubes in the enhancement of blood clotting for the control of hemorrhage. Biomaterials 28(31):4667–4672

Shahneh F, Grill A, Klein M, Frauhammer F, Bopp T, Schäfer K, Raker VK, Becker C (2021) Specialized regulatory T cells control venous blood clot resolution through SPARC. Blood 137(11):1517–1526

Sivaraman B, Latour RA (2011) Delineating the roles of the GPIIb/IIIa and GP-Ib-IX-V platelet receptors in mediating platelet adhesion to adsorbed fibrinogen and albumin. Biomaterials 32(23):5365–5370

Statello L, Guo C-J, Chen L-L, Huarte M (2020) Gene regulation by long non-coding RNAs and its biological functions. Nat Rev Mol Cell Biol 22(2):96–118

Wang KC, Chang HY (2011) Molecular mechanisms of long noncoding RNAs. Mol Cell 43(6): 904–914

Wang X, Arai S, Song X, Reichart D, Du K, Pascual G, Tempst P, Rosenfeld MG, Glass CK, Kurokawa R (2008) Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. Nature 454(7200):126–130

Wang Y, Zhang Y, Miron RJ (2016) Health, maintenance, and recovery of soft tissues around implants. Clin Implant Dent Relat Res 18(3):618–634

Wapinski O, Chang HY (2011) Long noncoding RNAs and human disease. Trends Cell Biol 21(6): 354–361

Willingham AT, Orth AP, Batalov S, Peters EC, Wen BG, Aza-Blanc P, Hogenesch JB, Schultz PG (2005) A strategy for probing the function of noncoding RNAs finds a repressor of NFAT. Science 309(5740):1570–1573

Witham J, Ouboussad L, Lefevre PF (2013) A NF-κB-dependent dual promoter-enhancer initiates the lipopolysaccharide-mediated transcriptional activation of the chicken lysozyme in macrophages. PLoS One 8(3):e59389

Yang J, Zhou Y, Wei F, Xiao Y (2016) Blood clot formed on rough titanium surface induces early cell recruitment. Clin Oral Implants Res 27(8):1031–1038

Yao Y, Liu Q, Adrianto I, Wu X, Glassbrook J, Khalasawi N, Yin C, Yi Q, Dong Z, Geissmann F, Zhou L, Mi Q-S (2020) Histone deacetylase 3 controls lung alveolar macrophage development and homeostasis. Nat Commun 11(1):1–15

Zarb GA, Koka S (2012) Osseointegration: promise and platitudes. Int J Prosthodont 25(1):11

Zhao B (2017) TNF and bone remodeling. Curr Osteoporos Rep 15(3):126–134

Current Approaches in Vertical Bone Augmentation and Large Bone Deficiencies in the Orofacial Region

14

Cedryck Vaquette, Saso Ivanovski, and Martin Batstone

14.1 Introduction

Bone regeneration is required following tooth extraction, surgical resection or trauma. While there exits a plethora of bone reconstruction techniques, they all possess advantages and disadvantages. The last few decades have seen a shift from the utilisation of autologous bone graft towards either synthetic biomaterials or allogenic and/or xenogeneic biomaterials. This chapter addresses two of the main issues encountered in density and maxillofacial reconstruction, namely vertical bone augmentation and the reconstruction of large-volume bone deficiencies.

C. Vaquette (✉)
The University of Queensland, School of Dentistry, Centre for Oral Regeneration, Reconstruction and Rehabilitation (COR3), Herston, QLD, Australia

Herston Biofabrication Institute, Metro North Hospital and Health Service, Brisbane, QLD, Australia
e-mail: c.vaquette@uq.edu.au

S. Ivanovski
The University of Queensland, School of Dentistry, Centre for Oral Regeneration, Reconstruction and Rehabilitation (COR3), Herston, QLD, Australia

M. Batstone
Herston Biofabrication Institute, Metro North Hospital and Health Service, Brisbane, QLD, Australia

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_14

14.2 Vertical Bone Augmentation

14.2.1 Clinical Problem

Bone resorption is naturally occurring in the oral–facial area following tooth extraction, surgical resection and trauma leading to the destruction of the bony contours of the face. Consequently, this poses significant functional and aesthetic issues and prevents the placement of restorative prosthetic devices such as dental implants. Due to the limited amount of remaining host bone, regenerative bone therapies are required in order to re-establish the lost bone volume and height for enabling dental placement necessary for the restauration of the masticatory function of the jaw. Therefore, vertical bone augmentation is the formation of bone outside the extraskeletal envelop and represents significant challenges in the oral–facial region. In this application, bone is growing in a vertical manner and its growth is not supported by pre-existing bony walls as it is the case in the regeneration of multi-walled bone deficiencies. Moreover, the long-term maintenance of the regenerated bone is also problematic due to the unpredictable resorption of the newly formed bone triggered by the surgical re-entry performed for the purpose of placement a dental implant.

14.2.2 Current Surgical Approaches and Limitations

The current surgical solutions encompass a variety of invasive techniques ranging from autologous bone grafting, distraction osteotomy and guided regeneration. The following section describes the major approaches currently utilised in the clinical setting for the purpose of vertical bone augmentation.

The one of the other commonly utilised techniques is the so-called Guided Bone Regeneration which relies on the utilisation of an occlusive membrane for ensuring space maintenance (allowing bone ingrowth) and selective cell repopulation for preventing the infiltration of undesirable non-bone forming cell types (fibroblasts and epithelial cells). The membrane can be of non-degradable (such of PTFE) or bioresorbable biomaterials and can be utilised with or without bone grafting biomaterials such as autologous bone blocks or bone chips, synthetic or anorganic bone grafting particulate. Non-degradable membranes have been commonly used in the past due to their higher stiffness when compared to resorbable membrane resulting in enhanced space maintenance properties required for vertical bone formation. However, they are also associated with increased exposure due to soft tissue dehiscence which is obviously detrimental for bone formation. In addition, unpredictable bone resorption following surgical re-+entry for the removal of the protective membrane and dental implant placement is common and can be a significant issue.

Bone block grafting in combination with an occlusive membrane is considered as one of the gold standard techniques for the reconstruction of atrophied bony structures in the craniofacial area (Von Arx and Buser 2006; Neovius and Engstrand

2010). This method requires the harvesting of an autologous block of bone generally from the chin, the retromolar area or extra-orally in the hip region. While bone block grafting possesses several significant advantages such as high osteogenic potential, excellent initial space maintenance, it also requires the harvesting of the bone from a donor site, potentially resulting in morbidity and pain. In addition, the long space maintenance of the block is generally limited, and bone resorption is frequent. The utilisation of bone chips provides certain advantages such as the capacity to better shape the defect, high osteogenic potential and also results in the presence of porosity beneficial for blood clot formation and subsequent vascularisation which is essential for osteogenesis to occur. Obviously, bone chips also require a second surgical site for harvesting which can potentially result in adverse reactions and the volume of bone harvested is also limited. Several other solutions have been developed utilising biomaterials from either xenogeneic or synthetic sources. Xenogeneic bone grafting biomaterials require specific preparation and processing in order to ensure their immunogenicity potential and is completely removed prior to implantation. This drastically reduces the osteogenic potential of these biomaterials when compared to autograft. Synthetic biomaterials are produced under a highly controlled laboratory setting and as such their chemical composition, dissolution rate and surface properties could be adjusted depending on the targeted application. The currently commercially available synthetic bone grafting biomaterials are generally based on various phases of calcium phosphate ceramic. Most common synthetic calcium phosphate grafting materials are hydroxyapatite, dicalcium and tricalcium phosphate or a mixture of these phases. The calcium/phosphate ratio broadly determines the rate of dissolution which has a direct impact of bone formation and subsequent stability.

While this solution can promote vertical bone formation, the utilisation of particulate bone grafting materials can represent a challenge in the clinical setting due to their poor handling properties. In addition, the lack of control over the particle packing in the defect can significantly reduce the overall porosity of the elevated volume and hence, can prevent rapid vascularisation and subsequent bone formation.

Other surgical techniques have been also developed in order to augment bone in a vertical manner. Such is the case of distraction osteogenesis, which relies on the gradual separation of the resident bone to encourage healing a new bone formation. In this strategy, the bone segment is separated from the host bone. An intraoral distractor is then utilised in order to increase regularly the gap between the resident bone and the separated segment. While this promotes bone healing, it is highly invasive, painful and is associated with high rates of complication (Rocchietta et al. 2008) ranging from infection, failure of the intraoral device and premature consolidation (Kende et al. 2021).

In an attempt to enable long-term space maintenance, a surgical approach known as the tent pole grafting technique has been developed. This involves the placement of titanium screws protruding over the resident bone with the interstitial volume filled with bone grafting materials and the whole area covered using a resorbable membrane. This technique resulted in significant vertical bone formation but required complex surgical intervention for managing soft tissue healing and enabled

tension free wound closure to prevent downstream wound dehiscence and graft exposure (Le et al. 2010).

Urban et al. (2015) described a novel combination of hard and soft tissue grafting to correct severely atrophic anterior maxillas in six patients without the unfavourable translocation of the mucogingival line or loss of the vestibule and keratinised mucosa that often results from broad release of the buccal mucosa to achieve tension-free closure in vertical augmentation procedures. Vertical bone augmentation was achieved using titanium-reinforced polytetrafluoroethylene (PTFE) membranes with a combination of 1:1 autogenous bone and anorganic bovine bone-derived mineral (Bio-Oss, Geistlich Pharma). After this initial phase of vertical augmentation, implants were placed with secondary bone grafting before a third and fourth phase of treatment involving very advanced soft tissue modifications. The mean vertical bone increase was 5.83 mm, with adequate bone height achieved in all six cases to allow for implant placement. Although the results of this study are impressive, the total treatment time was nearly 18 months and involved four complex surgeries before the implants reached the restorative phase.

Given the technique sensitivity and/or relatively high complication rate associated with the above techniques, a significant body of research has been investigating alternative methods to improve the predictability of vertical bone dimension and facilitate dental implant placement. The field of bone tissue engineering (BTE) is currently investigating many clinical applications in the spinal, appendicular and craniofacial regions of the skeleton (Oryan et al. 2014; Stevens 2008). Within the craniofacial complex, bioengineered bone has shown potential for periodontal regeneration, sinus augmentation, socket preservation, both lateral and vertical bone augmentation (Scheller et al. 2009; Tevlin et al. 2014; Ward et al. 2010; Xiao 2014).

Within the context of bone tissue engineering utilising a template biomaterial, or scaffold, for enabling bone ingrowth, an ideal construct should possess the following properties: it should enable long-term space maintenance, be highly porous in order to encourage neo-vascularisation, bioactive to favour bone formation, should slowly degrade over time to ensure multiple cycles of bone turn over, resulting in a stable mature bone before the complete degradation of the biomaterial.

In these aspects, the utilisation of regenerative medicine strategies combined with additive manufacturing technologies may be able to circumvent several limitations encountered in the conventional surgical protocols for vertical augmentation.

14.2.3 Additive Manufacturing Strategies for Vertical Bone Augmentation

Several approaches using additive manufacturing technologies have been utilised both in developmental research and in the clinical setting. These various strategies can be divided in three main streams using either bioceramic, polymer or metals.

14.2.3.1 Additively Manufactured Bioceramic Scaffolds

Three-dimensional (3D)-printed bioceramic scaffolds for the bone regeneration can be manufactured using powder printing or using a direct writing approach. One of the earliest research endeavours using additively manufactured bioceramic scaffold for orofacial bone regeneration relied on a modified 3D-printing powder technology. In this strategy, a mixture of α/β tricalcium phosphate (TCP) particles were chemically reacted by spraying a solution of phosphoric acid. This resulted in the binding of the particles while modifying the bioceramic composition which was composed post-printing of mainly brushite, some unreacted α/β tricalcium phosphate and a third phase in small quantity; monetite (dicalcium phosphate anhydrate). This technology enabled the manufacturing of anatomically relevant scaffolds for bone regeneration in the craniofacial area. A proof-of-concept study was performed using a 3D-printed human skull model to demonstrate the capacity of this technology to manufacture anatomical patient-matched scaffold. In this approach, the bony defects were imaged using micro-computed tomography and further processed to design and manufacture an implant with the precise shape and dimension of the defect. These patient matched scaffolds were placed in the defects of a human 3D-printed skull model and some manual smoothing was required in order to ensure prefect fit. These scaffolds were further tested in a lapine animal model to assess their performance towards vertical bone augmentation when compared to an onlay autologous bone graft (Tamimi et al. 2009). To this end, a 9 mm diameter, 2 mm thick 3D-printed scaffold monetite scaffold was placed over the calvarial bone of rabbit. Similarly, an autologous block bone graft with the same dimension was also implanted for comparison purposes. The 3D-printed scaffold and the block bone graft were secured using a conventional self-drilling osteosynthesis screw demonstrating the potential for clinical translation of the 3D-printed scaffold. Eight weeks post-implantation, both the 3D-printed scaffold and the autologous bone graft displayed excellent integration with the surrounding tissue, even though resorption, mediated by enhanced osteoclastic activity, was observed in the bone graft. Evidence of degradation of the 3D-printed monetite scaffold was also found and bone formation within this synthetic construct was constrained to the direct vicinity of the resident bone bed. Consequently, there was no significant difference in bone height between the 3D-printed scaffold and the autologous bone graft. The limited osteogenesis performance of the 3D-printed scaffolds can be attributed to the low porosity of the scaffold and the lack of interconnectivity of the pore network which impeded rapid tissue and cellular infiltration, and consequently vascularisation and bone formation. A subsequent study investigated the impact of initial height of the 3D-printed scaffold on extraskeletal bone formation, and this confirmed that the lack of pore interconnectivity and the low porosity of the scaffold prevented osteogenesis (Torres et al. 2011) as shown in Fig. 14.1a–g. Scaffolds with increased porosity were further developed by introducing several channels within the constructs (Tamimi et al. 2014) and their bone formation performance was also assessed in the lapine extraskeletal bone formation model previously used in the aforementioned studies. While this represented an improvement in the amount of bone formed within the scaffold, osteogenesis was still limited, and only observed near the resident bone bed

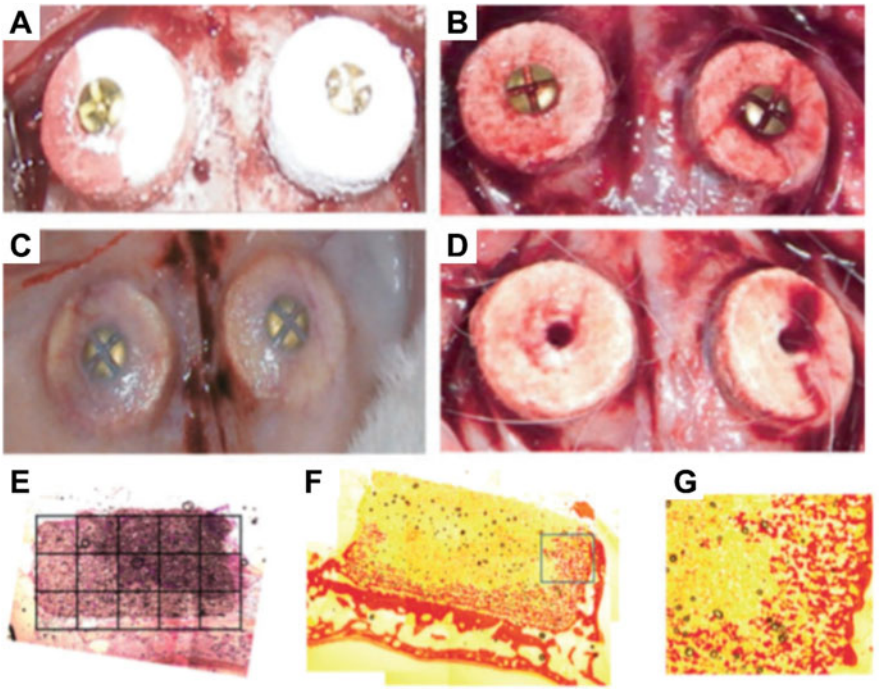


Fig. 14.1 Vertical bone augmentation regenerative outcome in a rabbit extraskeletal model of a bioceramic monetite scaffold manufactured using powder 3D-printing. (**a**) Morphology of the scaffold once implanted and secured with a fixation screw, (**b**) blood infiltration within the scaffold shortly after implantation. (**c**) Morphology of the scaffold after 8 weeks in vivo. (**d**) Removal of the fixation screws at 8 weeks post-implantation, (**e–g**) Tissue morphology observed by histology (picro-sirius staining) demonstrating limited bone formation mostly located in the direct vicinity of the resident bone bed. Reproduced with permission from Torres et al. (2011)

or in the proximity of the channels. Consequently, these studies clearly demonstrated that this particular bioceramic powder 3D-printing method is not a suitable technology for manufacturing 3D scaffold for achieving extensive and homogenous bone formation despite the high bioactivity of the bioceramic. Again, this was due to the inherent characteristic of the fabrication technique resulting in the formation of low porosity scaffolds with small pore with only limited interconnectivity, which presented a barrier for bone formation. This exemplified the necessity of developing structure with macroscopic pores sizes, fully interconnected and with high porosity in order to facilitate rapid tissue infiltration, vascularisation which is crucial for subsequent bone formation.

A direct printing approach, as opposed to the fusion of bioceramic particles, may provide more flexibility in the organisation of the pore resulting in increased pore sizes along with enhanced porosity and interconnectivity proven to be beneficial to bone formation. This approach was developing by Carrel et al. using an extrusion based 3D-printing technology for the manufacturing of highly porous scaffolds with

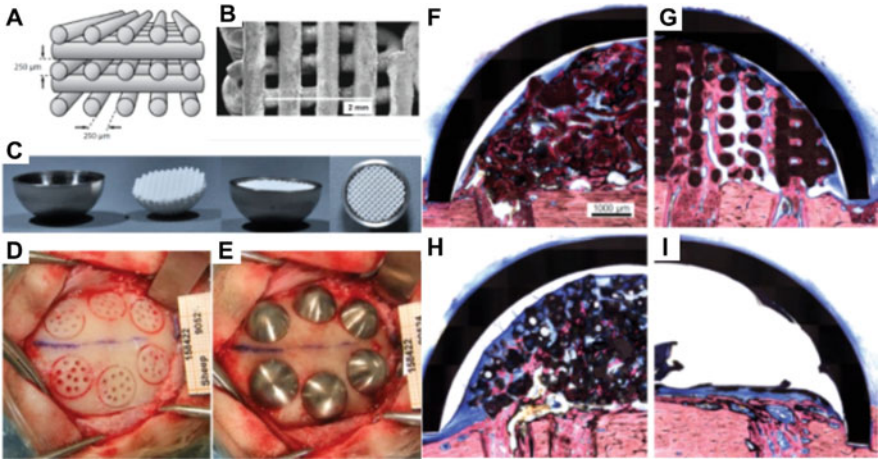


Fig. 14.2 Bioceramic scaffold manufacturing via direct extrusion printing in an extraskeletal model in sheep. (**a**) Schematic representation of internal organisation of the 3D-printed scaffold called Osteoflux, (**b**) SEM images of the scaffold, (**c**) system utilised for proceeding to the implantation of the scaffold involving a titanium dome according to the principles of guided bone regeneration. (**d**) Surgical preparation of the implantation sites by the creation of circular grooves and transcortical perforation. (**f**) Scaffold implantation under the domes. (**f–i**) Regenerative assessment of the various groups demonstrating high level of bone formation in the 3D-printed scaffold (**f**) Bio-Oss, (**g**) 3D-printed Osteoflux, (**h**) Ceros, (**i**) empty dome (blood clot). Reproduced with permission from Carrel et al. (2016)

fully interconnected and macroscopic pores (Carrel et al. 2016) as shown in Fig. 14.2a, b. In this strategy, a mixture of calcium deficient hydroxyapatite and α-TCP was utilised for extruding 400 μm diameter struts which could be deposited in a highly organised and layer-by-layer manner to form 250 μm interconnected pore. The performance towards extraskeletal bone regeneration of this newly developed bioceramic 3D-printed scaffold, referred as to osteoflux (OF), was compared to two commercially available particulate bone grafting biomaterials namely, Bio-Oss (BO) and Ceros (CO) with macroscopic particle sizes (Bio-Oss: 0.25–1 mm and Ceros: 0.5–0.7 mm). This was performed using an ovine extraskeletal bone regeneration model which consisted in the creation of transcortical holes placed within a circular groove used to secure a titanium dome hosting either the 3D-printed scaffold or the particulate bone grafting biomaterials (Fig. 14.2c–e). This experiment demonstrated that the 3D-printed scaffold significantly outperformed the particulate bone grafting products at the early timepoint of 8 weeks post-implantation when bone fill and maximal bone height were assessed (Fig. 14.2f–i). This was hypothesised to be caused by the increased porosity and interconnected porous network of the 3D-printed scaffold as opposed to the highly tortuous pore network created by the compaction of the bone grafting particles. As a consequence, vascularisation occurred very rapidly in the 3D-printed scaffold which resulted in early bone formation, while this was delayed in the particulate bone grafting groups. Interestingly, similar levels of bone formation were detected in the three groups

(3D-printed OF, BO and CO) at the 16-week timepoint indicating that the 3D-printed bioceramic scaffold favoured earlier bone formation but was not conducive to long-term enhanced osteogenesis outcomes.

The performance of the 3D-printed scaffold was further assessed in a more clinically relevant model using a dog model. To this end, an edentulous area was surgically created in the dog's mandible by the extraction of premolars and molars in order to obtain a shallow bony defect into which a 10 mm length, 10 mm width and 5 mm height 3D-printed scaffold was inserted as an onlay graft, it was then covered using a collagen membrane to mirror conventional clinical protocols. The scaffold was fixated using Teflon loops placed in two transcortical tunnels. This suggests that standard biomechanical fixation using titanium screws was challenging and possibly not possible for the 3D-printed scaffold, perhaps due to its brittleness inherent to any bioceramics. While the study reported regenerative outcome on only one animal, uniformly distributed newly formed extraskeletal bone was extensively present in the scaffold, with a bone fill around 30% of the elevated volume and bone height reaching up to 5 mm. Despite the limitation of this study in terms of animal number and method of implantation, it established the proof of concept that a bioceramic 3D-printed scaffold can be utilised for vertical bone augmentation. In addition, these series of studies by Carrel et al. clearly demonstrated the superiority of the direct bioceramic printing technology when compared to the bioceramic powder 3D-printing. This was attributed to the scaffold bioactivity and the enhanced pore size and pore interconnection which better supported early vascularisation and subsequently bone formation in the vertical direction.

The bioactivity of the bioceramic 3D-printed scaffold can be further enhanced by the addition of osteogenic clues, such as bone morphogenetic Protein-2 (BMP-2). Moussa et al. developed a strategy whereby the bioceramic 3D-printed scaffold was primed with 100 μg of BMP-2 and subsequently implanted in the aforementioned ovine extraskeletal bone regeneration model for 8 and 16 weeks (Moussa et al. 2015). This resulted in high level of bone formation at both 8 and 16 weeks, filling the entire volume and height of the scaffold/dome. Interestingly, the presence of the BMP-2 also induced an acceleration of the bioceramic degradation. Indeed, only traces of the scaffold were observed at this late timepoint, possibly indicating that the osteogenic cue may reduce the physical space maintenance capacity of the bioceramic scaffold.

Despite these early pre-clinical positive outcomes, the utilisation of bioceramic 3D-printed constructs remains still challenging due to the potentially technically complex manufacturing process (which seems to have been well overcome in the case of Carrel's studies but still requires an excellent technical know-how) and more importantly due to the limitations in satisfactorily fixating the scaffold using standard clinical practices, that is, titanium screws. In turns, this can significantly impede clinical translation and other biomaterials with enhanced flexibility have been explored for the purpose of vertical bone formation.

14.2.3.2 Additively Manufactured Polymeric Constructs

As an alternative manufacturing approach, the 3D-printing of thermoplastic polymers such as the most commonly used aliphatic polyesters (polylactic acid [PLA], polycaprolactone [PCL] etc.) enables the facilitated fabrication of scaffolds with a highly organised internal architecture, high porosity and pore interconnectivity creating a physical environment favourable for the establishment of new vascularisation and subsequent bone formation. While the degree of technical complexity seen in printing bioceramic is decreased, polymeric 3D-printed medical devices have not been yet widely translated to the clinic. Indeed, only a few reports have described the clinical performances of such devices towards bone regeneration in the craniofacial region (socket preservation (Goh et al. 2015), periodontal regeneration (Rasperini et al. 2015)).

As early as 2013, a first report was published which utilised a 3D-printed β-TCP/PCL scaffold implanted in the dog's mandible for 8 weeks. In this approach the bioactivity of the polymer 3D-printed scaffold was enhanced by the addition of bone marrow mesenchymal stem cells and this resulted in an increase in bone regeneration compared to the non-cellularised scaffold. Indeed, a 50% bone fill was observed in the cellularised scaffold, while only 20% bone fill was present in the control group. The limited bone regeneration capacity of the scaffold without cells highlights the necessity of increasing the construct bioactivity; indeed polymer scaffolds are, by nature, bioinert and bio-functionalisation strategies are required for overcoming this limitation. An interesting feature of this polymeric scaffold was that it was biomechanically secured using several titanium screws, indicating the suitability of such structures to use clinical standard fixation practices.

A more recent report from our group reported the utilisation of a biphasic scaffold for vertical bone augmentation in a lapine extraskeletal model. The scaffold was developed in mimicry to the native tissue composed of a dense cortical plate and a more porous cancellous bone. Therefore, the scaffold consisted of two compartments, a mechanically robust 3D-printed porous shell into which a highly porous melt electrowritten scaffold was inserted, mimicking the physical features of the cancellous bone (Fig. 14.3a). The specific structure of the 3D-printed shell ensured long-term space maintenance required for enabling bone formation in the vertical direction, while the highly porous melt electrowritten scaffold facilitated rapid cell and tissue infiltration, and vascularisation though its macroscopic and fully interconnected porous network, required for subsequent osteogenesis. The exterior 3D-printed compartment was 60% porous and made of 400 μm diameter PCL struts forming 1 mm pores, while the inner melt electrospun scaffold was 90% porous and composed of 10 μm fibres forming 250 μm pores. As the biphasic scaffold was manufactured using PCL, it was further biofunctionalised using BMP-2 encapsulated in a heparinised gelatine/acid hyaluronic hydrogel and implanted as an onlay graft over the calvarial bone of rabbit for 8 weeks. Upon surgical implantation, an occlusive dome in PLA was placed over the scaffold in accordance with the principles of guided bone regeneration whereby a cell occlusive barrier is utilised to prevent the infiltration of undesirable cells (such as fibroblast skin cells in this particular case or gingival fibroblasts and epithelial cells in clinical cases) as depicted

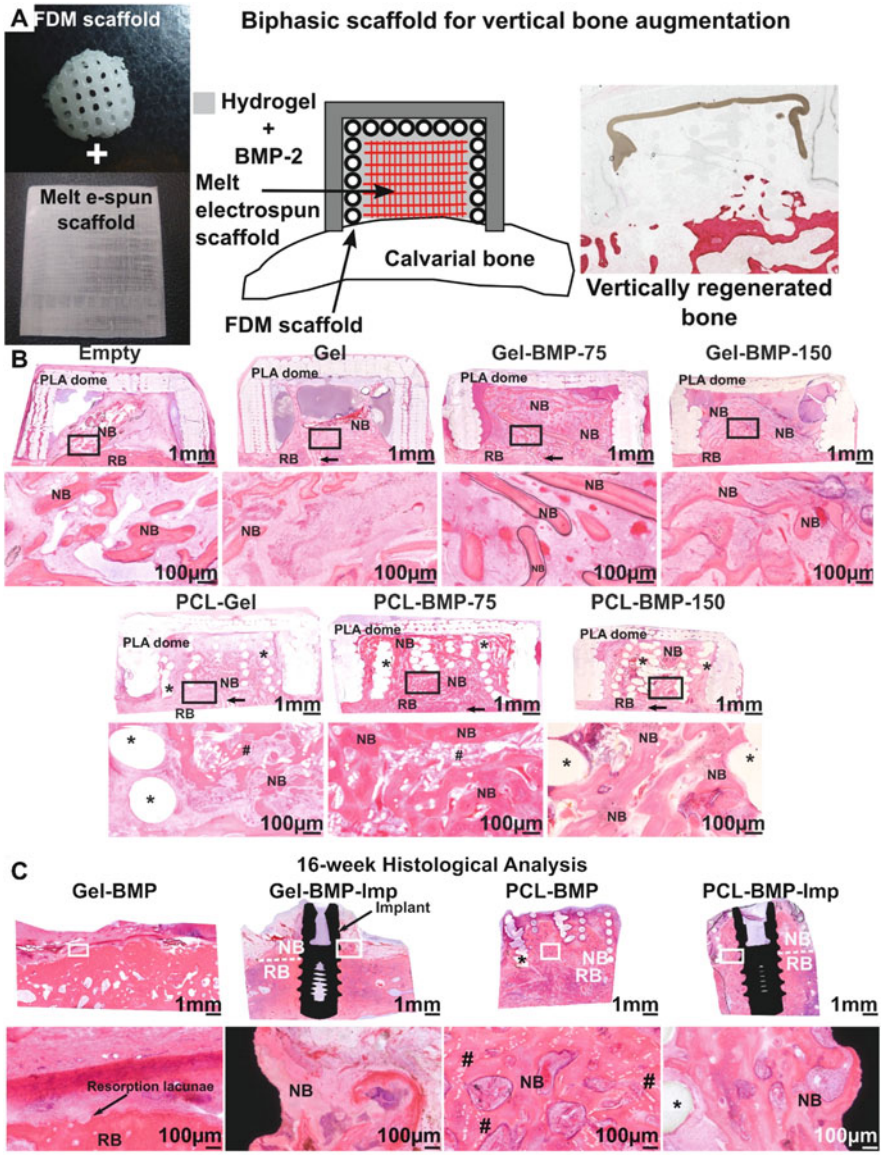


Fig. 14.3 Polymeric 3D-printed scaffold for vertical bone augmentation. (**a**) 3D-printed biphasic scaffold for vertical bone augmentation made of polycaprolactone infiltrated with a hydrogel containing BMP-2 in a lapine model. Reproduced with permission from Kumar et al. (2018). (**b**) Assessment of the performance of the biphasic scaffold fabricated by utilising two different additive manufacturing technologies (3D printing for the outer shell providing long-term space maintenance and melt electrospinning writing for the internal compartment with high porosity, thus favouring vascularisation and bone formation), Vertical bone formation in an ovine extraskeletal bone regeneration model demonstrating the necessity of BMP-2 incorporation to achieve significant bone formation. (**c**) Second stage of the study assessing the maintenance of previously regenerated bone upon surgical re-entry and dental implant placement. This demonstrated the prevention of

in Fig. 14.3a. The biphasic scaffold exerted a high level of biomechanical stability and created space maintenance required for osteogenesis to occur. However, bone regeneration was limited to the direct vicinity of the resident bone bed in all groups with or with scaffold or BMP-2 (Fig. 14.3a). This was attributed to the surgical model which lacked transcortical perforations, even though a circular groove was created (in order to better secure the position of the scaffold and PLA dome). The presence of these perforations in other studies was demonstrated to play a crucial role in enabling early bleeding in the elevated volume. Consequently, their absence impeded blood clot formation and the initiation of the healing cascade leading to vascularisation of the augmented volume and subsequent bone formation. While the limited amount of bone observed in the augmented volume was more an indication of poor defect preparation, the proof of concept using a biphasic polymeric scaffold was vertical bone augmentation was established.

The bone vertical bone regeneration capacity of the biphasic scaffold was further assessed using the ovine extraskeletal model initially developed by Carrel et al. This follow up study also utilised BMP-2 which was encapsulated in a heparinised gelatine hyaluronic acid hydrogel and several doses of the osteogenic cue were investigated (Vaquette et al. 2021). In a first phase, the regenerative performance of the biphasic scaffold with 75 or 150 μg of BMP-2 was compared to those of the hydrogel (without the biphasic scaffold) with identical doses placed in a PLA dome for space maintenance purposes. This demonstrated that the presence of the biphasic scaffold improved bone formation (Fig. 14.3b), and this was attributed to the enhanced retention capacity of the polymeric 3D-printing scaffold as opposed to when the hydrogel was implanted alone. Interestingly, there were no significant differences in the amount of bone formed between the two doses of BMP-2, indicating that osteogenesis is triggered when a threshold in the BMP-2 dose is attained and that no further improvements are seen when higher doses are utilised. This finding further confirmed previous studies indirectly comparing various BMP-2 for a given defect volume albeit for orthopaedic applications (Cipitria et al. 2013; Reichert et al. 2012). A second stage in this study investigated the maintenance of the regenerated bone following surgical re-entry and dental implant placement. To this end, two groups were implanted; the hydrogel loaded with BMP-2 and the biphasic scaffold loaded with the hydrogel containing the BMP-2. Initial bone regeneration was allowed for 8 weeks and surgical re-entry was performed for the removal of the occlusive PLA dome and the placement of commercially available titanium implant. A further 8 weeks of implant osseointegration was allowed prior to retrieving the specimens. This demonstrated that the hydrogel only samples displayed full bone resorption down to the initial resident bone bed whereas the biphasic scaffold specimens enabled the retention of the previously elevated bone (Fig. 14.3c). Therefore, the presence of the biphasic scaffold resulted in the

Fig. 14.3 (continued) bone resorption when the biphasic scaffold was present. Reproduced with permission from Vaquette et al. (2021)

reproducible maintenance of the augmented bone tissue and prevented its early resorption. Interestingly, the osseointegration of the dental implant in the bone formed within the biphasic scaffold was very high (around 80%). While this study only investigated the early maintenance of elevated bone, a prolonged in vivo implantation could provide interesting insight into bone maintenance when the biphasic scaffold is partially or fully degraded. Indeed, PCL requires several years to degrade (typically 3–5 years) which could enable several cycles of bone remodelling, hence creating a mature and potentially long-term stable bone.

More recently, Zhang et al. investigated the performance of a PLGA/HA/β-TCP mixture in dioxane which was subsequently 3D-printed on a cooled collector. The scaffold featured a pre-tapped hole used for accommodating a dental implant. The scaffold and dental implant were simultaneously implanted in a lapine extraskeletal model. This demonstrated that the presence of the inorganic particles (HA/β-TCP) was essential for osteogenesis to occur in the extraskeletally created space and a 20% bone fill was achieved. While his study established a proof of concept in using a 3D-printed scaffold simultaneously to a dental implant, the amount and height of elevated bone were still limited and mostly located near the resident bone. In addition, the dental implant osseointegration was minimal and seemed to be prevented by the presence in very close proximity of the 3D-printed struts.

These previous research endeavours clearly suggest that the biofunctionalisation of 3D-printed polymeric scaffolds is a necessity for inducing extensive bone formation in the elevated space and that a highly porous inner compartment is also required for enabling high osseointegration.

The present section focussed on the regeneration of the small to medium size bone deficiencies in the mandibular region and other techniques and technologies are required when the volume of bone to regenerate is above a few cubic centimetres. The following section describes the most recent advancement in the regeneration of large bone deficiencies.

14.3 Large Bone Volume Deficiencies: Orofacial Regenerative Medicine

14.3.1 Current Approaches

Large volume defects in the craniofacial region are most commonly created during the ablation of head and neck malignancy though trauma, developmental anomalies and iatrogenic injury remain important causes. Their reconstruction is complicated by the complex anatomy of the head and neck region. Defects occasionally involve one tissue type (e.g. reconstruction of the ascending ramus for odontogenic tumours is primarily a bone issue), but this is a rare occurrence and frequently multiple tissue types are involved. An example would be maxillary resection for malignancy which would include mucosa, bone, dentition and sensory nerves.

Current reconstructive techniques are variable depending on the exact defect and a commonly used method of stratification is the reconstructive ladder. Primary

closure and healing by secondary intention are reserved for the repair of defects primarily in skin or mucosa and of limited extent.

Grafts which include autografts, allografts and to a limited extent, xenografts (skin, mucosa, bone,) are suitable reconstructive techniques for larger defects but all suffer the limitation of absence of vascular supply thus rely on several key factors for success. Firstly, a healthy vascularised non infected tissue bed without communication with the external environment is required to provide vascularity to the grafted bed. This excludes large wounds, contaminated or infected defects or patients with tissue compromise such as that caused by radiotherapy. Secondly immobility is required to allow nutrient supply and the ingrowth of blood vessels usually over approximately 1 week for mucosal or skin grafts and longer for bone grafts. Finally, there is a critical volume issue beyond which tissue necrosis occurs before vascular ingrowth can provide blood supply to the grafted tissue. This limits the thickness of soft tissue grafts and means that bone grafts often provide a scaffold rather than viable cells.

Local and distant rotational flaps to the head and neck overcome the issue of blood supply and allow the transfer of larger volumes of soft tissue as they have blood supply via a nutrient vessel (e.g. the long buccal artery and vein in a buccinator flap or superficial temporal artery and vein in a scalp flap). Unfortunately though, local and regional rotational flaps in the head and neck are limited to soft tissue only, with no real ability to transfer bone of any volume. In addition, the donor site defect is often visible externally, or causes functional deficits. Their current role in head and neck reconstruction is limited to smaller soft tissue defects where colour match of the skin is important.

Free tissue transfer is the mainstay in the reconstruction of large defects of the head and neck, particularly those which comprise multiple tissue types (Batstone 2018). These are autografts harvested from the same patient whereby bone, skin, fat, muscle and nerve can be taken in combination with a defined vascular supply. They are disconnected from their donor site, transferred and adapted to the defect with microvascular anastomosis of the supplying artery and draining vein to vessels in the head and neck. This technique overcomes the requirements of the recipient bed and allows successful reconstruction of large, irradiated and even infected defects. In addition, the donor site is transferred 'out' of the head and neck to a less anatomically and aesthetically critical area.

This technique has been performed for decades and with improvements in equipment, patient selection and surgical techniques success rates approach 100% (Van Genechten and Batstone 2016). Recent technologic advances have improved the accuracy of reconstruction, particularly of bone, with the use of 3D printing, and virtual surgical planning. Computed tomographic data of the tumour or recipient site can be imported, digitally adjusted and planned allowing the production of cutting guides, and patient-specific implants (plates and screws) to more accurately reconstruct the target organ.

Virtual surgical planning has allowed more accurate templating and adaptation of grafted tissue to the defect; however, they all require adaptation of the inherently

dissimilar tissues. Completely accurate reconstruction of bone remains elusive, as does the incorporation of functional muscle and nerve tissue.

Dental rehabilitation frequently relies on the use of prostheses, and osseointegrated dental implants which are reliable and successful but there remains significant room for improvement particularly with regard to the soft tissue environment (Khadembaschi et al. 2021).

Donor site morbidity is a feature of all autograft harvest. That donor site morbidity is variable depending on the tissue harvested and the volume required. For example, a soft tissue skin and fat containing 'anterolateral thigh free flap' have a lower donor site morbidity than a skin, fat, muscle and bone containing 'composite scapula free flap'.

14.3.2 Additive Manufacturing and Other Experimental Techniques

Various regenerative approaches have been undertaken which primarily focus on the bony structures of the craniofacial skeleton particularly the mandible and cranium (Kumar et al. 2020). The most utilised approaches incorporate customised or 3D-printed scaffolds of various materials broadly categorised into ceramics (Hydroxyapatite, beta-tri calcium phosphate etc.) and polymers (polycaprolactone, polylactic acid, polylactic-*co*-glycolic acid). The absolute requirements are of biocompatibility though the ideal material would be—osteoinductive, osteoconductive, resorbable, able to be 3D printed or otherwise customised, and of sufficient strength to maintain stability until replaced by bone. Some materials possess some of these characteristics but suffice to say the ideal material does not currently exist. To generate bone growth of any quantum, most include the addition of bioactive components. These overcome the absence of osteoinductive or osteogenic potential and include autologous bone chips, patient derived stem cells, platelet-rich plasma or fibrin and human bone morphogenetic protein 2. Although some results are encouraging, they are all limited by the need to gain vascular supply from surrounding tissue. This has proven to be the ultimate limiting factor as oxygen and cellular nutrition can only diffuse over a certain distance and it has not yet proven possible to fabricate large segments with an axial supplying vessel. This can be compared with the current technique of free tissue transfer which utilises known axial supplying vessels to harvest bone, muscle, skin, fat and nerves all in combination. This limits the applications to those patients with soft tissue coverage, and ideal host conditions (e.g. prior radiotherapy or infection are contraindications). Even with these ideal circumstances, success rates are in the largest series 65% which is significantly lower than contemporaneous techniques such as free flap reconstruction (Stoor et al. 2017). Attempts to overcome the problems of exact tissue adaptation and vascular supply have included prefabricated or prelaminated free flaps. They include the growth of a bone free flap in the same patient in an area of lower donor site morbidity prior to transfer (Urban et al. 2015; Warnke et al. 2006). They still consist of vascularised autografts but are partially prefabricated in the patient with an attempt to either improve the contour of the transplanted tissue or minimise the donor site morbidity

but maintain the advantages of multiple tissue types in an independently vascularised construct. They partially overcome the limitations of contour mismatch as the contour can be controlled by 3D printing the scaffold and maintain a transferrable axial blood supply courtesy of the donor site. They still have of course the disadvantage of some degree of donor site morbidity. In addition, the bone growth often takes some time which limits their applicability to secondary reconstruction cases rather than primary cancer or defect management. Procedural complexity and reliability have also prevented any widespread use.

14.3.3 Future Directions

The management of large volume defects in the craniofacial region is challenging by virtue of the anatomical complexity of the region, and the multiple tissue types that are frequently incorporated into each defect. The potential exposure to the external environment, potential need for radiotherapy in malignancy cases and the aesthetic demands of the area add additional challenges. Ideally tissue engineering would allow the manufacture of a personalised reconstruction with multiple potential tissue types in the same construct. Fabrication time is important for some clinical scenarios—critical, for example, in cancer ablative surgery or major craniofacial trauma, less critical for secondary reconstruction of developmental anomalies.

This could feasibly be achieved with a 3D printed scaffold (either printed, and seeded with cells, or bioprinted) that is grown ex vivo and then transferred to the patient. The challenges in this approach centre around maintaining vascularity and nutrition as the size of the construct exceeds that which can be achieved with diffusion of nutrients and oxygen alone. In addition, far greater understanding of the complexity of the growth factor cascade and local mechanical environmental features such as scaffold design and material choice will be required to understand how cell differentiation is driven to a final result. Extra corporeal flap perfusion allows the nutrient supply of transplanted tissue with ex vivo blood on a bypass machine (Wolff et al. 2016) and experience from this technique may help to facilitate transfer especially into hostile recipient sites.

An alternative approach is the use of xenografts whereby the construct is fabricated in a host animal prior to transfer to the recipient. This would overcome the problem of donor site morbidity and to a certain extent the issues surrounding operative complexity. Duration of 'fabrication' may continue to be an issue for primary reconstructive scenarios. Xenografting is currently widely used for tissue replacement in areas such as cardiac valves, cartilage and artificial dermis. The main barrier to this approach is the antigenicity of xenografted tissue which relies on treatment of the tissue to remove antigens. These treatments preclude viability in transplanted tissue. Approaches to transplant viable xenografts (or xenotransplants) include modification of the organ or construct (antibody masking, transgenic modification, gene knockout) or modification of the host (immunosuppression, bone marrow chimerism, microchimerism). Additionally, modification of the construct

would be required to achieve an anatomical match for the recipient. Ethical and societal barriers may prove to be impossible to overcome for this approach.

References

Batstone MD (2018) Reconstruction of major defects of the jaws. Aust Dent J 63:S108–S113

Carrel J-P, Wiskott A, Moussa M, Rieder P, Scherrer S, Durual S (2016) A 3D printed TCP/HA structure as a new osteoconductive scaffold for vertical bone augmentation. Clin Oral Implants Res 27:55–62

Cipitria A, Reichert JC, Epari DR, Saifzadeh S, Berner A, Schell H, Mehta M, Schuetz MA, Duda GN, Hutmacher DW (2013) Polycaprolactone scaffold and reduced rhBMP-7 dose for the regeneration of critical-sized defects in sheep tibiae. Biomaterials 34(38):9960–9968

Goh BT, Teh LY, Tan DBP, Zhang Z, Teoh SH (2015) Novel 3D polycaprolactone scaffold for ridge preservation–a pilot randomised controlled clinical trial. Clin Oral Implants Res 26(3): 271–277

Kende PP, Sarda AS, Desai H, Landge J, Nimma V, Varte V (2021) Complications of distraction osteogenesis: narrative review. Oral Surg 14(4):412–422

Khadembaschi D, Russell P, Beech N, Batstone MD (2021) Osseointegrated implant survival, success and prosthodontic outcomes in composite free flaps: a 10-year retrospective cohort study. Clin Oral Implants Res 32:1251–1261

Kumar SPT, Hashimi S, Saifzadeh S, Ivanovski S, Vaquette C (2018) Additively manufactured biphasic construct loaded with BMP-2 for vertical bone regeneration: a pilot study in rabbit. Mater Sci Eng C 92:554–564

Kumar VV, Rometsch E, Thor A, Wolvius E, Hurtado-Chong A (2020) Segmental mandibular reconstruction using tissue engineering strategies: a systematic review of individual patient data. Craniomaxillofac Trauma Reconstr 13:267–284

Le B, Rohrer MD, Prassad HS (2010) Screw "tent-pole" grafting technique for reconstruction of large vertical alveolar ridge defects using human mineralized allograft for implant site preparation. J Oral Maxillofac Surg 68(2):428–435

Moussa M, Carrel JP, Scherrer S, Cattani-Lorente M, Wiskott A, Durual S (2015) Medium-term function of a 3D printed TCP/HA structure as a new osteoconductive scaffold for vertical bone augmentation: a simulation by BMP-2 activation. Materials 8(5):2174–2190

Neovius E, Engstrand T (2010) Craniofacial reconstruction with bone and biomaterials: review over the last 11 years. J Plast Reconstr Aesthet Surg 63(10):1615–1623

Oryan A, Alidadi S, Moshiri A, Maffulli N (2014) Bone regenerative medicine: classic options, novel strategies, and future directions. J Orthop Surg Res 9(1):1

Rasperini G, Pilipchuk S, Flanagan C, Park C, Pagni G, Hollister S, Giannobile W (2015) 3D-printed bioresorbable scaffold for periodontal repair. J Dent Res 94(9 Suppl):153S–157S

Reichert JC, Cipitria A, Epari DR, Saifzadeh S, Krishnakanth P, Berner A, Woodruff MA, Schell H, Mehta M, Schuetz MA (2012) A tissue engineering solution for segmental defect regeneration in load-bearing long bones. Sci Transl Med 4(141):141ra93

Rocchietta I, Fontana F, Simion M (2008) Clinical outcomes of vertical bone augmentation to enable dental implant placement: a systematic review. J Clin Periodontol 35(s8):203–215

Scheller E, Krebsbach P, Kohn D (2009) Tissue engineering: state of the art in oral rehabilitation. J Oral Rehabil 36(5):368–389

Stevens MM (2008) Biomaterials for bone tissue engineering. Mater Today 11(5):18–25

Stoor P, Suomalainen A, Mesimaki K, Kontio R (2017) Rapid prototyped patient specific guiding implants in critical mandibular reconstruction. J Craniomaxillofac Surg 45:63–70

Tamimi F, Torres J, Gbureck U, Lopez-Cabarcos E, Bassett DC, Alkhraisat MH, Barralet JE (2009) Craniofacial vertical bone augmentation: a comparison between 3D printed monolithic monetite blocks and autologous onlay grafts in the rabbit. Biomaterials 30(31):6318–6326

Tamimi F, Torres J, Al-Abedalla K, Lopez-Cabarcos E, Alkhraisat MH, Bassett DC, Gbureck U, Barralet JE (2014) Osseointegration of dental implants in 3D-printed synthetic onlay grafts customized according to bone metabolic activity in recipient site. Biomaterials 35(21): 5436–5445

Tevlin R, McArdle A, Atashroo D, Walmsley G, Senarath-Yapa K, Zielins E, Paik K, Longaker M, Wan D (2014) Biomaterials for craniofacial bone engineering. J Dent Res 93(12):1187–1195

Torres J, Tamimi F, Alkhraisat MH, Prados-Frutos JC, Rastikerdar E, Gbureck U, Barralet JE, López-Cabarcos E (2011) Vertical bone augmentation with 3D-synthetic monetite blocks in the rabbit calvaria. J Clin Periodontol 38(12):1147–1153

Urban I, Monje A, Wang H (2015) Vertical ridge augmentation and soft tissue reconstruction of the anterior atrophic maxillae: a case series. Int J Periodontics Restorative Dent 35:613–623

Van Genechten ML, Batstone MD (2016) The relative survival of composite free flaps in head and neck reconstruction. Int J Oral Maxillofac Surg 45:163–166

Vaquette C, Mitchell J, Fernandez-Medina T, Kumar S, Ivanovski S (2021) Resorbable additively manufactured scaffold imparts dimensional stability to extraskeletally regenerated bone. Biomaterials 269:120671

Von Arx T, Buser D (2006) Horizontal ridge augmentation using autogenous block grafts and the guided bone regeneration technique with collagen membranes: a clinical study with 42 patients. Clin Oral Implants Res 17(4):359–366

Ward B, Brown S, Krebsbach P (2010) Bioengineering strategies for regeneration of craniofacial bone: a review of emerging technologies. Oral Dis 16(8):709–716

Warnke PH, Wiltfang J, Springer I, Acil Y, Bolte H, Kosmahl M, Russo PA, Sherry E, Lützen U, Wolfart S, Terheyden H (2006) Man as living bioreactor: fate of an exogenously prepared customized tissue-engineered mandible. Biomaterials 27:3163–3167

Wolff KD, Mücke T, von Bomhard A, Ritschl LM, Schneider J, Humbs M, Fichter AM (2016) Free flap transplantation using an extracorporeal perfusion device: first three cases. J Craniomaxillofac Surg 44:148–154

Xiao Y (2014) Bone tissue engineering for dentistry and orthopaedics. Biomed Res Int 2014: 241067

In-Vitro and In-Vivo Tracking of Cell-Biomaterial Interaction to Monitor the Process of Bone Regeneration

15

Anwesha Barik and Moumita Das Kirtania

15.1 Introduction

Skeletal structure, major part of which is composed of bone, provides the mechanical support, aids in haematopoiesis, acts as the primary storage of mineral as well as enhances mobility coordinating with muscles in vertebrate animals including human (Terranova et al. 2016). This multifunctional tissue is highly sensitive towards wide variety of bio-chemical and bio-mechanical stimuli due to which preservation of structural integrity often needs well distribution of stress/strain caused by mechanical loading and weight-bearing physical activities (Bailey and McCulloch 1990). Having the natural capability for regeneration and repair, small wounds in the skeletal structure often do not need any clinical management. Large segmentation non-union bone fractures, in contrast, can only be healed either by grafting artificial bone substitutes or placing titanium, zirconia or other bio-inert metallic implants as the 'gold standard' of such therapy (Yang et al. 2013; Montazerolghaem et al. 2016). In spite of increasing demand of biomaterials worldwide, many patients suffer from unavailability of such technologies which eventually force them to choose the cost-effective option such as limb amputation requiring long-term hospitalization indirectly causing a negative impact on the socio-economic status (Wang et al. 2014).

Bone, having a multi-scale structure, is primarily divided in two parts among which cortical bone creates the dense, non-porous external surface of the bone and comprises of about 99 and 90% of total available calcium and phosphate of the human body. In contrast to that cancellous bone is distributed throughout the core of the bone with interspersed haematopoietic stem cells, adipose tissues, blood vessels and spanning lamellar trabeculae. Although the trabecular or cancellous bone

A. Barik (✉) · M. D. Kirtania
Department of Pharmaceutical Technology, School of Medical Sciences, Adamas University, Barasat, West Bengal, India
e-mail: anwesha1.barik@adamasuniversity.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_15

comprises about 20% of the total weight of the bone, effective surface area is much higher compared to cortical bone possibly owing to its superior porosity (McKittrick et al. 2010). Difference in age, genetic diversity, inherited unique biological features, defect geometry all together influence in variation of structure and composition of bone thereby requiring different requirements to be fulfilled for effective bone regeneration. In lieu of this, biomaterials guide the progenitor cells to fill the bone defects by restoring the structure, function of bone and type of cells present in the native bone. Ideal biomaterial should not only mimic the biological and mechanical performance of the bone but also should meet the requirements of being non-toxic, in-vivo biodegradable, bioactive. Moreover, excessive immunogenic reaction against implantation of such foreign bodies should not cause hindrance to apposition of new bone, matrix mineralization. Tunable properties in implants/scaffolds such as nano- or micro-structuring help to alter cellular morphology as required for cellular attachment and migration on the non-porous implants. The multi-scale porous scaffolds whereas provide sufficient space and microenvironment requisite for tissue in-growth through pores while maintaining regular supply of oxygen, nutrient through blood vessels, nerve conduction and deposition of minerals (Fedorovich et al. 2011). Due to patient-specific difference in site and structure of native bone and bone defects, interior and exterior structure of the implants should be customized to fit in to the defect site to render early primary stability (Saiz et al. 2013). This approach has been found to encourage cellular interaction resulting in successful artificial-to-biological translation (Hench 2015; Knight and Hankenson 2013). This necessitates the tracking of the in-vitro and in-vivo biological response at host-implant interface to ensure complete integration of tissue in and surrounding the biomaterial without causing incompatibility as summarized in the following sections of this article.

15.2 Significance of Tracking Bone-Biomaterial Interaction

The bone regeneration and reconstruction were initially started with implantation of autologous bonegrafts collected from ribs, calvarium, iliac crest from patient's own physiological system (Frohlich et al. 2008; Zomorodian and Baghaban 2012; Romagnoli and Brandi 2014). The use of autografts was still limited by restricted access as well as chronic pain at the site of tissue collection and potential risk of infection at the donor site despite some positive aspects including low immunogenicity (Romagnoli and Brandi 2014). The autografts and allografts moreover may only be considered as osteogenic to initiate osteogenesis and neo-vascularization provided that the host-graft interface is sufficiently stable and macroporous with optimal presence of pre-osteogenic cells at host bed (Delloye et al. 2007). Often the terminology 'osteogenic' creates confusion with other two related terms 'osteoinductive' and 'osteoconductive' both of which bears separate meaning. Cells can migrate from the host to osteoconductive scaffolds to be proliferated and differentiated further in to matured osteoblasts leading to ingrowth of bone whereas the scaffolds only can induce in-vivo osteogenecity when preserved with

osteoinductive factors such as transforming growth factor-beta (TGF-β) type 1 or 2 capable to differentiate mesenchymal stem cells, i.e., osteoprogenitor cells to mature osteoblasts (Urist 1965). With expanding clinical requirement, the research paradigm on bone reconstructing scaffolds has immensely shifted towards more 'bioactive' materials with capability to integrate with surrounding bones which will be eventually resorbed and replaced by body's own tissue with minimal dissimilarity at a time-dependant manner (Stevens 2008). Despite several proofs of similarities of inorganic or organic biomaterials in providing structural support, concerns exist predominantly over the insufficient toughness and mechanical stability provided by both the polymeric and metallic scaffolds especially for load-bearing applications (Kokubo 1995). Inorganic biomaterials such as tricalcium phosphate, bioactive glass have the ability to precipitate naturally mimicking hydroxyapatite (HA) in-vitro and in-vivo in presence of biological fluid. These are found to stimulate complex osteogenic gene transduction network, although they are unable to provide adequate mechanical strength due to the brittle nature especially of the bioactive silica glass even after enforcing crystalline HA in to their molecular structure (Kokubo 1995; Tsigkou et al. 2007; Jell and Stevens 2006).The rate of resorption can, however, be manipulated by simultaneously tailoring the surface chemistry, topographical features and controlling the in-vitro and in-vivo release of ions from the scaffolds in to bone microenvironment. Unlike those materials, the natural (collagen etc.) and synthetic polymers (polycaprolactone, copolymers of polylactic acid, and polyglycolic acid) can be utilized in fabrication of three-dimensional (3D) scaffolds with controllable porosity, topology and cross-linking density employing several conventional and advanced layer-by-layer manufacturing methods including computed tomography (CT) scan-guided computer-aided 3D printing (Hollister 2005). Majority of the biomaterials are being developed to mimic the natural architecture of bone which is composed of compact central cortical region with recapping osteon surrounded by spongy shock-absorbable trabecular bone with interspersed hydroxyapatite crystals and collagen fibres thus making it strong, stiff and mechanically stable (Shao et al. 2022). Poor mechanical properties thereby elicited by the polymers somehow can be improved by incorporating inorganic materials to mimic Young's modulus similar to native bone without compromising interwoven network formation by the pores while maintaining optimum degradation rate, bioactivity and viscoelastic properties (Filippi et al. 2020). Literature review also revealed improved osteogenic potential of surface modified biomaterials compared to that of unmodified materials as mentioned by Kazimierczak and Przekora (Kazimierczak and Przekora 2020). Apart from inorganic coatings, plasma modifications, hydrothermal and laser sintering methods, alteration of surface chemistry by incorporation of combinations of few among fibroblast growth factor (FGF), TGF-β, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), bone morphogenetic protein (BMP) type 2 or 4 in to scaffold are reported to induce superior osteogenic gene expression simultaneously accelerating angiogenesis and osseointegration at the implantation site (Przekora 2019). This approach probably provides cues directly to stimulate

innate biological response and chemotactic cell behaviour aiding cell adhesion, proliferation and differentiation (Alam et al. 2007).

All of these above-mentioned materials and their composites have tried to create a niche cellular microenvironment to meet the necessary features applicable for bone repair since long but all the approaches have not been able to achieve that huge diversity. Moreover, lack of clarity in elucidating detailed molecular mechanism involving cell-ECM interaction, cell-cell interaction and cell-scaffold interaction make it difficult to explore the novel theoretical and practical directions to achieve successful biological translation (Gao et al. 2017). A few experimental demonstrations can be obtained from literature in this regard to identify some of the associated pathways involved in osteogenic differentiation of osteoprogenitor stem cells. Among all, TGF-β/BMP pathway gets activated through binding of TGF β-1 to the respective receptor followed by TGF β-2 binding, autophosphorylation and recruitment of SMAD proteins to the receptors. This leads to differentiation of undifferentiated stem cells to mature osteoblasts by inducing chemotaxis of the stem cells to the extracellular matrix (ECM) and the ending of the process is marked with deposition of mineral as well as collagen synthesis in the matrix (Gao et al. 2017). Similarly the role of Wnt/β-catenin signalling pathway, Hedgehog signalling pathway, Notch signalling pathway, MAPK signalling pathway in activating early and late stages of osteogenic differentiation in response to several stimuli also has been explained in various in-vitro and in-vivo studies (Engin et al. 2008; Delpiano and Acker 1985; Shi et al. 2015; Wang et al. 2016; Yuan et al. 2016; Hu et al. 2013). Variation in the physical parameters such as porosity irrespective of being micro or nanosized has been found to significantly promote protein adsorption, focal adhesion, cell viability and upregulate osteogenic gene expression accentuated by the large effective surface area and porous volume. This necessitates the detailed study of cell-biomaterial interaction apart from conventional tracking of biomarkers (Gao et al. 2017). However, the major concern lies in understanding the complex bone regeneration modulated by a combination of growth factors, inflammatory modulators, that is, cytokines released in the diversely populated bone microenvironment. This further necessitates understanding the effect of administered active biomolecules or drugs in the bone remodelling process either positively or negatively. Since the introduction of several molecules sometimes may destroy the natural bone healing phenomenon by disrupting homeostasis between growth factors/cytokines.

15.3 In-Vitro Evaluation Methods

Mediated by ligand-integrin receptor binding, the bone tissue engineering scaffolds strongly influence the adhesion and migration of surrounding cell niche consisting of adult stem cells, pluripotent stem cells, umbilical cord blood mesenchymal stem cells, embryonic stem cells leading to in-growth of tissues through the porous 3D architecture of the scaffolds. The adipocytes, in addition to those cells, result in the formation of microvascular networks albeit it takes days even weeks to transform in

to fully functionalized vascularized bone tissues (Kang et al. 2015). Vascular smooth muscle cells or pericytes whereas are required for structural stabilization of newly developed endothelialized blood vessels in the bone (Neff et al. 2011; Bergers and Song 2005). Cell-biomaterial interaction directly modulates these cytoskeletal activity, matrix modelling and cellular contraction requisite for fracture healing (Friedl et al. 1998; Yannas et al. 1989). Modification of scaffolds by alteration in the concentration of adhesive ligands or polymers (natural or synthetic) has been found to significantly influence the strength of cell-scaffold interaction while causing differential integrin binding according to the availability of receptors (Murphy et al. 2013). This phenomenon was explained by a previously published literature where comparatively less compact and mechanically stable gelatin methacrylate scaffold (fabricated with 5% gelatin) with low compressive modulus exhibited superior osteogenecity compared to those fabricated with high concentration of gelatin (Celikkin et al. 2018). Therefore, the optimization of cellular interaction at cell-scaffold interface is necessary utilizing the standard procedure to evaluate stimulation of cellular adhesion, migration, proliferation and differentiation of osteoprogenitor cells towards maturity. All the established in-vitro assays are summarized in Fig. 15.1.

15.3.1 Cytotoxicity and Cell Metabolic Activity

According to the guidelines framed by International Standard ISO 10993-5:2009, direct as well as indirect contact approaches are employed to evaluate 'biocompatibility' of biomaterials with an aim to protect human from potential biological risk arising from medical devices. In-vitro cytotoxicity therefore inspects the impact of the developed material towards targeted cells cultured and maintained in appropriate conditions. Direct contact approach involves culturing of cells seeded on scaffolds or implants followed by fluorescence microscopy-based observation of live cells tagged with Calcein (excitation/emission at the wavelength of 495/515 nm), a specific fluorescent dye for living cells and dead cells tagged with propidium iodide (excitation/emission at the wavelength of 560/720 nm), another specific dye for dead cells, although the evaluation is seemingly qualitative (Lagonegro et al. 2017). Quantitative measurement from the same experiment can be done by measuring the total area covered by the cells normalized to that covered by the cells cultured on negative control, that is, without any biomaterial. The pattern of cell growth although can depend on the type of substrate as it has been commonly found that cells are grown in clusters when cultured on flat surfaces while giving a confusing estimation about the cellular coverage measured by the fluorescence assay (Lagonegro et al. 2017). Literature also showed that an optimum gap of 50–70 nm between integrin binding motifs can aid in formation of focal adhesion and cellular attachment which can be inhibited once the spacing exceeds 100 nm usually observed with nano-structured biomaterials eventually leading to cell death (Cavalcanti-Adam et al. 2006; Bershadsky et al. 2006). Indirect contact test on the other side analyses the effect of release of cytotoxic agents from the material of interest. Briefly, the extracts

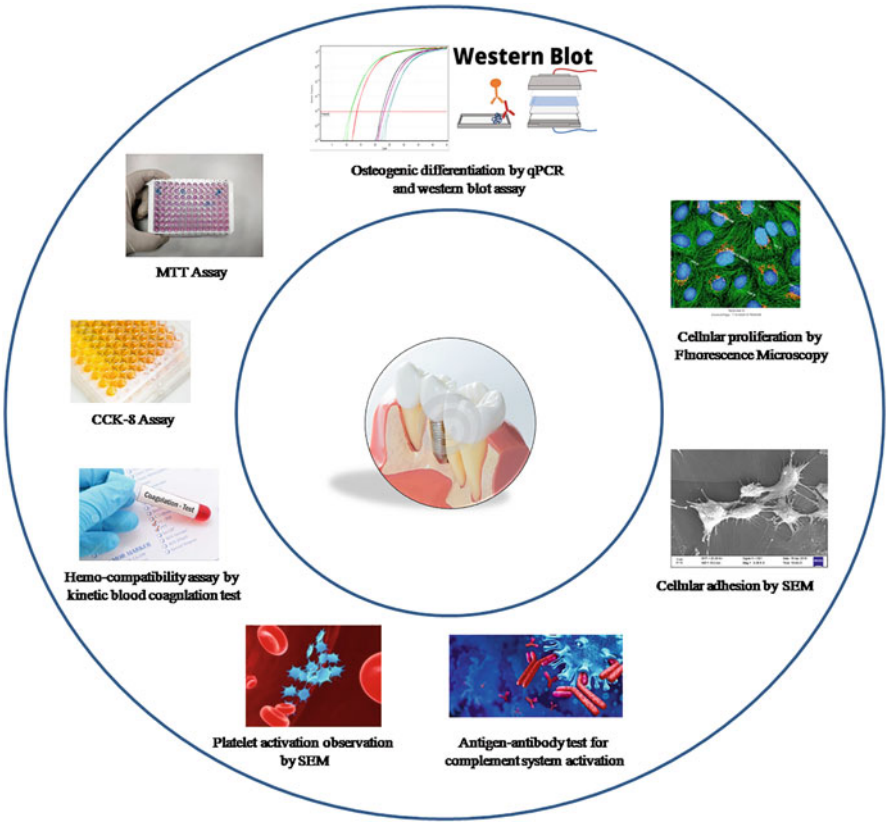


Fig. 15.1 Summarized representation of in-vitro assays to evaluate cell-biomaterial interaction

are prepared by immersing the materials in pristine culture medium to develop the conditioned medium. That conditioned medium is added then to the fresh culture medium at increasing concentration followed by culturing of targeted cells for a short duration, that is, 24 h. Cell viability is further determined following the standard protocol of MTT colorimetric assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye). The outcome may be further validated by several independent methods including chemiluminescence while quantifying the intracellular ATP present in metabolically active cells. Cell counting kit-8 also may be used in this regard where the incorporating reagent WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) gets bioreduced in exposure to cellular dehydrogenase secreted from the metabolically active cells. This in turn produces formazan product, on solubilization of which in aqueous cell culture media gives orange colour and therefore can be detected by spectrophotometry (Tong et al. 2016).

One of the major drawbacks associated with the indirect test is lack of clarity in revealing the reason of cytotoxic effects caused especially by calcium phosphate-based biomaterials. These apparently lead to reduced cell viability owing not only to the release of ions due to spontaneous dissolution of calcium phosphate but also introduction of ionic reactivity resulting in drastic changes in the ionic composition of culture medium turning it to be an unfavourable environment (Klimek et al. 2016; Przekora et al. 2014). The commercially defined cytotoxicity evaluation of ceramics especially can show misleading results as evident from the long- and short-term ion interaction assay while altering the concentration of divalent cations (Ca++ and Mg2++) and phosphate ions in pristine cell culture medium (Przekora et al. 2014). This assay depicted that abnormally high concentration of calcium in the culture media released from the ceramics might lead to in-vitro cytotoxicity on human foetal osteoblast cells (hFOB) although the ion reactivity of calcium-lacking hydroxyapatite scaffold was strongly affected by the composition of medium and duration of exposure. Alongside, the increased ion-adsorption especially with scaffolds fabricated from unsintered hydroxyapatite lead to critically low concentration of divalent cations resulting in inhibition of cellular metabolism, activation of integrin receptors, cell adhesion as well as viability (Malafaya and Reis 2009). The increased concentration of phosphate ions, on the contrary, showed less toxicity of hFOB cell line as evident from the results of MTT assay (Przekora et al. 2014). Other limitations include incompatibility of silicon oxycarbide nanowires with the chemical reagents of MTT assay which has been found to adversely affect the outcome as the assay is based on the principle of redox reactions (Lagonegro et al. 2017). In lieu of this, covering of cell monolayer by agar mixed culture medium following incubation with scaffolds/implants has been found to be more reliable in this aspect. Cells cultured in this manner are usually inspected to track changes in cell morphology, cell lysis and instances of probable detachment, loss of membrane integrity and vacuolization by means of microscope. This approach also has been executed by the same group (Lagonegro et al. 2017).

15.3.2 Hemocompatibility and Immunogenic Response

Biomaterials including implants or scaffolds meant for use in bone tissue engineering must possess hemocompatibility as a series of complex physiological reactivity happens while differing the degree of adjustment at blood-biomaterial interface. The risk of potential deleterious effect ensuing out of incompatibility may extend up to implant failure along with other severe consequences. Adsorption of circulating platelets and plasma proteins on the surface of substrates leads to activation of blood coagulation cascade mediated by leukocyte adhesion, in-vivo adhesion and activation of platelets resulting in blood coagulation (Liu et al. 2014a). Cellular matrix is formed provisionally on colonization of cells leading to the conversion of prothrombin to thrombin. It leads to formation of dense fibrin network of clots surrounding the implants, although the phenomenon is more prominent in cardiovascular implants, stents. After coagulation persists, reverse mechanisms are

initiated to dissolve the thrombus where zymogen, plasminogen is converted to plasmin and results in breaking of fibrin network to form fibrin degradation products (FDP) (Zorio et al. 2008; Liu et al. 2014b).

Primary evaluation of hemocompatibility of scaffolds involves detection of FDPs on incubation of targeted cell seeded scaffolds in freshly collected whole human blood compared to the FDPs formed by incubation of whole human blood without scaffolds (Weber et al. 2021). Briefly, after incubation of about 90 min, blood is transferred into a fresh container coated with anticoagulant and subjected for the measurement of blood cell numbers to find out significant deviation in platelets, erythrocytes and leukocytes. The analysis of complement activation markers also can be performed to evaluate the potential of the biomaterial in triggering inflammatory response leading to activation of polymorphonuclear (PMN) leukocytes. The level of PMN elastase and proteolytic enzyme released from the leukocytes in the exudates blood needs to be measured therefore. Activation of coagulation cascade can be tracked by measuring the level of thrombin-antithrombin III (TAT) complex which is formed by counter activity of fibrin by coagulation inhibitor antithrombin III. The same can also be evaluated indirectly by performing kinetic blood coagulation test. The formation of blood clot can be replicated in-vitro by adding drops of whole human blood on surface of implants and is incubated at 37°C for about 5 min. Recalcification then may be done by uniformly mixing calcium chloride in warm blood followed by dilution with distilled water to separate the supernatant comprising of lysate-free red-blood corpuscles (RBCs). The absorbance of the supernatant is measured by means of UV-Vis spectroscopy at about 540 nm (Lagonegro et al. 2017). The outcome may be corroborated by quantification of p-selectin by specific immunosorbent assay. Release of p-selectin has been usually found to be significantly high with response to oxide containing biomaterials (silicon oxide, titanium oxide) compared to the control polypropylene tissue culture substrate (Lagonegro et al. 2017). P-selectin is considered to be an important biomarker expressed on platelet membranes while indicating platelet aggregation mediated by fibrinogen binding on being released from alpha-granules (Body 1996; Shattil et al. 1987). Increased surface area available due to micro-roughening often results in increased plasma protein deposition resulting in high platelet activity, although it should also be noted that the micro-structured metallic implants with features of <3 μm may not provide enough area for adhering platelets having size of 3 μm at resting condition (Park et al. 2001). Accelerated protein adsorption seems to be the primary reason behind high index of blood coagulation on those structures compared to machined implants.

Complement system activation as a result of interaction of blood with foreign material takes place and gets terminated by formation of SC5b-9 complex thus indicating its suitability to be the important marker, whereas platelet activation can be detected by the presence of β-TG in blood which also gets released from alpha granules in the interacting platelets.

In addition to these above-mentioned biochemical assays, blood-biomaterial interaction can be examined by Scanning Electron Microscopy (SEM) imaging where the biomaterials are subjected to interact with platelet rich plasma for a brief

duration. The samples then are to be fixed with 4% w/v paraformaldehyde solution following dehydration with serially increasing concentration of ethanol. The shapes of the platelets indicative of the activation might be determined from the three-dimensional images acquired from SEM of the gold-sputtered plasma-soaked implants or scaffolds and the adhering platelets might be quantified by lactate dehydrogenase assay as well (Lagonegro et al. 2017; Park et al. 2001). Apparently, the platelets appear to be star-shaped at early activation phase, whereas the fully activated platelets are found to be octopus-shaped. Additionally, the platelets also can be observed for the pseudopod formation, aggregation, and spreading on the implant surface. The appearance of the dense fibrin matrix surrounding implants can also be observed by SEM imaging. The aliquot of platelet rich plasma also can be subjected to flow-cytometry.

15.3.3 Cellular Adhesion and Proliferation

Accomplishment of cellular adhesion which is divided in three consecutive major steps, that is, cellular attachment, cell spreading and focal adhesion, is observed to be crucial to produce cellular mass sufficient for cellular proliferation and further differentiation thus necessitating the determination of cellular adhesion. The adherence of the anchorage-dependant cells, that is, osteoblasts, pre-osteoblasts, mesenchymal stem cells on biomaterial substrates, is therefore necessary. In-vitro immersing or in-vivo implantation of the bone scaffolds instantaneously induces adsorption of several tissue and plasma proteins at the surface of the substrates. The plasma proteins (albumin etc.) and adhesive glycoproteins (fibronectin, vitronectin, vinculin, laminin etc.) undergo conformational change thereby significantly affecting the strength of adsorption and further cell adhesion (Salakhutdinov et al. 2008; Burmeister et al. 1996). Experimental evidence demonstrated that the hydrophobic implants induce greater deformation of adsorbed proteins resulting in unfolding compared to that observed on the surface of hydroxyl group-functionalized hydrophilic implants (Keselowsky et al. 2003). The more hydrophilic group the implant surface contains, the more protein gets adsorbed on the surface (Chang and Wang 2011). Implants and scaffolds pre-coated with those adhesive proteins and immunoglobulins even sometimes with adhesion-specific peptide sequence RGD (arginine-glycine-aspartic acid) tend to be recognized more by the recruiting target cells present in the vicinity of the implant (LeBaron and Athanasiou 2000; Yamada 1991). These biological cues specifically attach to the integrin binding domain in the cell membrane of osteoblasts or osteoprogenitor cells thus mediating the cell adhesion to a great extent. Cellular attachment followed by interaction of actin cytoskeleton to the proteins present in ECM results in formation of subcellular structures including focal adhesion plaques (FAP). This phenomenon gets further accentuated when the surface of the implant is nano-structured while fabricating ridges, pores, channels, fibres as the integrin binding sequences are also nano-sized (Ercan and Webster 2014; Le et al. 2013).

Biological activity of the implants and scaffolds is usually evaluated visually by microscopical observation. After incubation for desired duration, the cell-scaffold constructs are fixed with 2.5% of glutaraldehyde solution followed by dehydration with increasing concentration of ethanol solutions. The dried samples afterwards are observed under SEM to characterize cell distribution, cell-scaffold interaction, intracellular connection and morphology (Lagonegro et al. 2017; Lee et al. 2006). SEM observation, although being the extensively used evaluation method, qualitative fluorescence analysis also can be performed on the cell-scaffold constructs to observe cytoskeleton and focal adhesion of the cells growing on the surface. On washing with phosphate-buffered saline (PBS), the non-specific binding sites of the samples are blocked by bovine serum albumin and then staining was done using primary antibody of any of the before-mentioned cytoskeletal proteins labelled with fluorescent dye tagged secondary antibody. Further staining of actin cytoskeleton and nucleus also is performed using other dyes phalloidin and DAPI (4',6-diamidino-2-phenylindole) respectively prior to observing under fluorescence microscopy (Lagonegro et al. 2017). Similar to the cytotoxicity evaluation, samples can be evaluated quantitatively by MTT assay or CCK-8 method as well (Zhang et al. 2018).

15.3.4 Osteogenic Differentiation

On protein adsorption on the surface of substrates and subsequent cellular adhesion, osteoconductive implants aid in cell proliferation of partially differentiated osteogenic lineage and ECM formation. Osteoinductive implants or scaffolds including stem cell-incorporated or osteogenic growth factor-loaded systems have been found to recruit osteoprogenitor cells leading towards differentiation (Albrektsson and Johansson 2001; El-Ghannam 2005; Xu et al. 2017). This phenomenon is a complex dynamic metabolism-controlled process comprising of three consequent phases. While proliferating, the osteoblasts start to synthesize primarily collagen I protein and other ECM proteins including osteopontin, osteocalcin, bone sialoproteins. At initial stage, bone alkaline phosphatase (bALP) activity seems to be low accompanied with low ability to produce osteopontin which increases exponentially afterwards leading to synthesis and maturation of ECM. The bALP activity reaches the maximum point at this stage when cells stop proliferating as well as matrix mineralization is also observed to be low. Increased level of osteopontin, osteocalcin and collagen type I at this second phase seems to crosslink with osteoid to form the template for deposition of calcium phosphate crystals moving towards the third and last stage of matrix mineralization. The end of osteogenic differentiation is marked with sharp decline in bALP activity, high serum level of osteocalcin and osteopontin, both of which are calcium binding proteins along with formation of mineralized stable bone matrix (Polo-Corrales et al. 2014). This process, however, summarizes the characteristic markers at the different phases. Starting from the initial stages of osteogenic differentiation, that is, last stage of cellular proliferation to ECM formation, can be characterized by increasing activity of bALP by means of q-PCR

(polymerase chain reaction), enzyme-linked immunosorbent assay (ELISA), Western-blot assay, immunofluorescence-based staining. The late stage of osteogenic differentiation whereas can be evaluated by studying relative gene expression of relevant osteogenic markers (osteocalcin, osteopontin, collagen I, bone sialoprotein) and levels of corresponding proteins in bone. Alizarin Red S assay is also a reliable method for qualitative and quantitative evaluation of matrix mineralization.

15.4 In-Vivo Evaluation Methods

Biological response to the metallic, polymeric and composite materials used for fabrication of bone tissue engineering implants and scaffolds largely relies on the size, shape, surface modification, surgical site and method of implantation as well as the duration of implantation therapy. Similar to the in-vitro cellular activity, in-vivo biocompatibility and osteogenecity have been found to be correlated with the acute and chronic inflammatory responses happened right after the surgical implantation of scaffolds. In lieu of this, the interaction of a plethora of immunogenic and non-immunogenic cells including macrophages, fibroblasts, osteoblasts, osteoclasts, multinucleated giant cells surrounding implants is needed to be evaluated. Knowledge about in-vivo activation and communication of cells with materials otherwise would be incomplete if gross morphological, immunohistochemical and histomorphometry analyses were not performed at all.

15.4.1 Inflammatory Response

Naturally obtained polymers such as collagen-based biomaterials display physiological reactivity determined by mononuclear cells and sometimes pathological reaction while inducing multinucleated giant cells after subcutaneous implantation in small animal model (Ghanaati 2012; Ghanaati et al. 2011a). These materials are usually observed to be well-integrated with the surrounding subcutaneous host connective tissues with an evident mild peripheral vascularization pattern (Ghanaati 2012). Ghanaati et al. showed that the presence of stable collagen membrane allowed the penetration of anti-inflammatory mononuclear cells inside the core resulting in tissue in-growth over time while the polytetrafluoroethylene (PTFE) membrane as negative control prevented penetration of mononuclear cells rather inducing the formation of pro-inflammatory multinucleated giant cells at both of the surfaces. Thus, the negative control membranes posed as an effective functional barrier inhibiting tissue in-growth. Even the silk-fibroin membrane, although having superior biocompatibility, has been found to delay the penetration of both the pro-inflammatory multinucleated giant cells and anti-inflammatory mononucleated cells resulting in lagging events including membrane breakdown followed by neotissue formation and transmembrane vascularization. The outcome of this above-mentioned study, however, contradicts other findings where significant decrease in thickness was observed after

in-vivo implantation of non-cross-linked collagen membrane over time with instance of transmembrane vascularization on breaking down of the membrane (Rothamel et al. 2005; Schwarz et al. 2006). Among the bone substitutes, subcutaneous placement of deproteinized bovine bone grafts sintered at low temperature in mice model experimentally showed a temporary immune response in terms of localization of multinucleated giant cells at early phase (Barbeck et al. 2014). The response was remarkably decreased with in-vivo time-based degradation of small granules present in the graft. Synthetic biomaterials including bioactive glass also showed similar pathological immune response, although at a comparatively milder level (Ravarian et al. 2013). This emphasizes the advantage of pre-processing of bone engineering scaffolds or grafts especially fabricated with hydroxyapatite, silk fibroin, beta-tricalcium phosphate either with human blood or sometimes culturing of human osteoblasts with or without endothelial cells before implantation (Barbeck et al. 2015a, 2016; Ghanaati et al. 2011b). All of these have been found to attribute to significant induction of multinucleated giant cells with markedly increased vascularization. Pre-seeding scaffolds only with osteoblasts even have been found to release several soluble factors including pro-angiogenic stimuli enough to stimulate host endothelial cells to adhere, proliferate and form neovessels (Ghanaati et al. 2011b).

As it has been previously mentioned, the inflammatory and in-vivo biodegradation patterns are usually determined on subcutaneous implantation of the synthetic as well as natural bone graft substitutes. The resorption rates of these materials are directly related with induction of multinucleated giant cells and transmembrane vascularization even up to the central region which is integral to the process of guided bone regeneration, therefore highlighting the importance of such evaluation. Briefly the experimental animals are treated with the samples implanted in a pre-formed subcutaneous pocket at the subscapular region (Barbeck et al. 2015b; Ghanaati et al. 2010). After desired duration, the animals are subjected to euthanasia and explanted samples are fixed with 4% paraformaldehyde solution followed by decalcification and dehydration with increasing concentration of ethanol. Histomorphometrical analysis is performed thereafter with histological sections prepared from paraffin-embedded scaffold implantation bed. The presence of multinucleated giant cells with surface expression of tartrate-resistant acid phosphatase (TRAP) as 'look-alike' osteoclasts typical to the induced phagocytotic cells is detected. The cells, however, lack some of the other quintessential characteristics of osteoclasts such as ruffled border (Barbeck et al. 2015b). In-vivo implantation of similar grafts in human also showed the presence of those multinucleated giant cells; however, they lack the osteoclast activity seeming to influence bone regeneration (Al-Maawi et al. 2017). The phenomenon happens possibly owing to the fact that resident multinucleated giant cells in host tissue, being induced through integrin β 3, behave similarly to the osteoclasts, while biomaterial-adherent ones, being positive for integrin β 2, act as interleukin-4 mediated immunogenic foreign body giant cells only (Barbeck et al. 2017; McNally and Anderson 2002).

Trindade et al. evaluated host-biomaterial relation in lieu of immune system activation on implantation of titanium implants in osteotomy defect created at distal femur in rabbit model compared with sham-operated rats (Trindade et al. 2018).

After euthanasia, implants from half of the animals were retrieved to collect the bone for evaluating relative expression of genes responsible for induction of neutrophils, T and B lymphocytes, activation of complement system, macrophage activity. Bone samples with embedded implants were collected from rest of the animal for performing histological analysis for the expression of relevant components in innate immune system. Those components included bone resorption markers: receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), tartrate resistant acid phosphatase (TRAP) and cathepsin K (CathK). Both the studies resulted in evident innate immunity building through up-regulation of neutrophil activity as a marker of acute inflammation, increased macrophage fusion, thereby inhibiting neutrophil apoptosis as a marker of chronic inflammation surrounding titanium implants. The uprising balance towards reparative M2 phenotype of macrophages apparently shifted the paradigm towards anti-inflammatory activity and wound healing while decreasing the level of inflammatory M1 phenotype over time. This outcome corroborated with the previous hypothesis (Gensel and Zhang 2015).

15.4.2 Biomechanics

In-vivo osseointegration surrounding implants largely depends on achievement of the early implant stability. The stability further relies on several factors including surgical technique adapted, quality and quantity of the residual host bone before implantation, compatibility of implant-bone interface as well as microscopic and macroscopic surface features of the implant (Büchter et al. 2006). All these contributing factors effectively delay the total time needed for the completion of prosthetic reconstruction. Surgical trauma and incapability of attachment of adjacent tissue directly on the implant surface often result in fibrous tissue formation rather than direct apposition of newly formed bone in close agreement with the host tissue. Early achievement of mechanical stability thereby biological and functional restoration, that is, osseointegration, may be ensured by reducing the possibility of micromotion by decreasing the gap at bone-implant interface (Szmukler-Moncler et al. 2000). Micro-movement at a minimum keeping the range of 500–3000 μ strain moreover can be achieved by improved geometry of the implants especially for the metallic ones (Buser et al. 1991; Joos et al. 2000). Comparative study among implants with different geometries such as cylindrical, threaded cylindrical, cylindrical with steps, threaded cylindrical with steps and double-disk experimentally depicted that the threaded implants distributed the strain homogeneously on the surrounding tissue leading to physiological and non-physiological transmission of mechanical strength (Joos et al. 2000). This appears to be necessary for long-term implant stability.

Biomechanical evaluation is usually performed via measuring the removal torque and resonance frequency measurement (RFM). After in-vivo implantation of implants in targeted bone (tibia, femur, maxilla and mandible) of the small animal, vibrational frequency of the implant with tissue is measured from intact euthanized

animals to evaluate implant stability right after the implantation as well as after euthanasia. High frequency thereby is associated with increased stiffness at bone-implant interface and higher peri-implant bone density (Meredith et al. 1996; Pagliani et al. 2013). Less lateral displacement also has been found with more mechanically stable implants which can be further reflected as improved osseointegration. In lieu of this, multiple probes need to be placed perpendicular to the transducer (/sensor) at various regions surrounding the implant.

Loosening of the implantable screws is often a matter of concern as frictional force acting at contact region of both the biological and implant surfaces significantly affect the preload values of the implants (Lang et al. 2003). The more force or torque is applied during tightening of the screws, the less frictional coefficient it will have in future. This can be directly correlated to the increased resistance to micro-motion and friction resulting in inhibition of loosening of implants. This feature largely relies on the structure, threading, texture and degree of lubrication of the implants (Burguete et al. 1994; Wang et al. 2009). Nano-structuring of titanium implants has been found to accelerate bone growth and osteoblast response establishing high shear strength after 4 week of implant placement. The micro-roughened implants although ensured tissue in-growth through the micropores resulting similarly high interfacial bonding but after around 12 weeks of implantation (Xia et al. 2012). Counter-clockwise rotation is usually applied at a fixed degree of rotation per second on the retrieved samples and the torque rotation curve is recorded to measure maximum torque on the curve at which the implant eventually gets removed from the newly grown tissue. The interfacial stiffness also may be calculated from the same curve according to standard procedure (Büchter et al. 2006).

15.4.3 Histomorphometry and Immunohistochemistry to Evaluate Osteoblast and Osteoclast Response

In-vitro immunohistochemical evaluation of the growth of osteoblasts, formation of blood capillaries along with the expression of relevant osteogenic proteins such as osteocalcin, osteoprotegerin, vascular endothelial growth factor should be ideally validated from the similar study performed on the explanted implants after in-vivo implantation. After decalcification and paraffin-embedding, the samples are cut in thin histological sections followed by observation under optical microscope. Suitable dyes (such as haematoxylin, eosin, toluidine blue) are used for staining of the cells or tissues along with counterstaining. The samples are usually observed for formation of osteoblasts, osteocytes and its morphology, presence of macrophage and multinucleated giant cells, reconstructed vasculature, newly formed bone with lamellar structure, ratio of cortical bone to trabecular bone in the peri-implant zone (Bahraminasab et al. 2021). At different time points, the morphology of different cells gets altered depending on the texture of the implant. Osteocytes, the terminally differentiated form of osteoblasts, may be considered as a key player both in new bone formation as well as in bone remodelling mediated by receptor activator of

nuclear factor-κB ligand (RANKL), osteoprotegerin, receptor activator of nuclear factor-κB (RANK). This necessitates visualization of lacuna-canalicular structure formed by osteocytes residing inside lacunae for alteration of size, morphology, alignment, connectivity with other proximal and distant osteocytes, accessibility to blood vessels (Shah et al. 2016; Bohner et al. 2017). Direct bone-to-implant contact may also be observed by staining the interfacial thin tissue layer with Alizarin S and Brilliant-Cresyl-blue (Büchter et al. 2006). These lamellar structures also may be observed under fluorescence microscope without labelling (Büchter et al. 2006). Moreover, the significant difference (if any) in the bone-to-implant contact ratio at different time points (e.g. 7 days, 14 days, 28 days, 56 days) can be calculated via quantitative histomorphometric analysis. The calculated parameters are bone-to-implant contact (%BIC) and bone area fraction occupancy (BAFO) where %BIC defines percentage of implant surface directly in contact with anchored bone over the length of implant and BAFO indicates total area of mineralized matrix deposited by bone between the pitches, valleys of threads over the unit microscopic field (Folkman et al. 2020). Several studies although did not find any influence of different surgical techniques and drilling dimensions on those parameters. Preparation of bone before surgery, insertion torque rather apparently influenced the parameters as high insertion torque and no preparation of bone (i.e. cortical perforation) caused micro-fractures, generation of high temperature, necrosis of host tissues due to compression leading to failure in osseointegration (Cha et al. 2015; Stocchero et al. 2019).

15.4.4 In-Vivo Imaging for Bone Tissue Engineering

Several non-destructive real-time imaging techniques are available to track the progress of peri-implant bone healing and even the accuracy of the surgical placement of the implants. These techniques include X-ray computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound (US), optical imaging (OI), single photon emission computed tomography (SPECT) and positron emission tomography (PET) which provide anatomical and sometimes metabolic information during and post-implantation (Fragogeorgi et al. 2019).

Nuclear imaging such as SPECT and PET detects the emitted photons from the isotope combined chemically and biologically active substances using ionizing radiation. Region of interest can be identified and measured with high sensitivity through SPECT, although the uptake of radiotracer agents in metallic hardware (screws, plates, rods) can overlap the region of interest in the reconstructed 3D images of the pre-clinical/clinical models (Lima et al. 2020). This overlapping is usually represented using the term 'shining metal artifact' which apparently indicates significantly increased projected region statistics (Duncan and Ingold 2018). If ignored, this phenomenon might be misinterpreted as having loosening or infection around implants due to which high radiotracer activity is observed in reality (Römer et al. 2005; Murer et al. 2020). These evaluation techniques, however, are limited by the brief biological half-lives of the radiotracer elements or radiopharmaceuticals as

the suitability of using such compounds for long-term tracking the progress of slow physiological process (i.e. bone regeneration, osseointegration of implants) cannot be justified (Fragogeorgi et al. 2019). The poor resolution in these methods sometimes renders it inconclusive in terms of identification of the source of new bone formation; confusion occurs whether the bone formation is happening at the peri-implant region or in the host tissue (Wong and Piert 2013). In contrast to that, PET imaging provides quantitative measurement of radiotracer uptake in bone and relative osteoblast activity through compartmental analysis integrated with PET platform (Cheng et al. 2013).

Micro-CT imaging utilizes X-ray to perform scans of the experimental animal or object from multiple angles followed by identification and measurement of region of interest subjected to 3D reconstruction of composite images. This imaging can be performed non-invasively for the small animal models under anaesthesia to track quality and quantity of newly formed bone tissues surrounding implants and also to the tissue embedded implants retrieved from the euthanized animals at different time points of post-implantation. The morphological information may be obtained macroscopically from the objects treated with contrast agents as well as microscopic features including structure of osteoblasts, vasculature, lamellar structure, trabecular connectivity all can be identified via advanced synchrotron-radiation enabled micro-CT (Fragogeorgi et al. 2019). With an objective of whole-body imaging, majority of the pre-clinical study prefers to fuse the information obtained from nuclear imaging to the anatomical information obtained from micro-CT based 3D reconstructed images. This owes to the fact that micro-CT provides improved spatial resolution especially in dense tissues (Beckmann and Maier 2011). A few drawbacks are noted for long-term micro-CT imaging such as detrimental effect of long-exposed radiation, risk of having inconclusive data as the poor integration at bone-implant interface or unclear delineation of host bone tissue to scaffold/implant engineered bone can be as complicated as an artifact (Ventura et al. 2014). Figure 15.2 describes a protocol which can be adapted for micro-CT imaging of implants retrieved from euthanized small animal models to track the progress of bone healing.

MRI selectively measures the movement of protons present exclusively in water containing tissues. On application of magnetic field, the protons spin and get displaced from their original position thereby creating a magnetization vector (Ventura et al. 2014). This is the reason behind the fact that MRI exclusively produces high-contrast images for water-abundant soft tissues compared to the low water containing hard tissues such as bone. Around 20% volume of the cortical bone is composed of water specifically present in haversian canals and canalicular network, whereas some of the rest volume is strongly bound to the collagenous matrix and crystals of hydroxyapatite (Timmins and Wall 1977; Elliott and Robinson 1957). This necessitates employing indirect measurement of peri-implant bone apposition based on gradual disappearance of MRI signals (Washburn et al. 2004). In contrast to that interaction of high frequency sound wave with hard tissues can be measured via US imaging systems depending on the attenuation, absorption and reflection of the sound. Irrespective of the hardness, tissues bear a natural feature to act as an impedance barrier to acoustic waves depending on speed of the sound wave

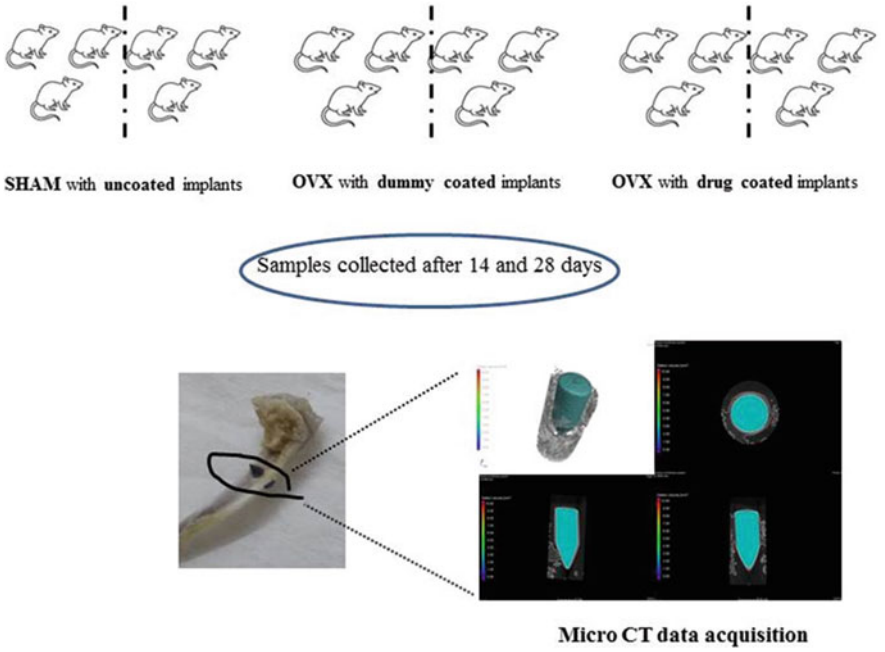


Fig. 15.2 Representation of an experimental protocol for micro-CT imaging for evaluation of in-vivo peri-implant bone growth

and density of the tissue. Several pre-clinical and clinical scenarios may hereby be noted with diverse application of US imaging ranging from monitoring drug treatment, gene-therapy to bone healing engineered by scaffolds/implants (Bez et al. 2017; Hériveaux et al. 2019). Some other highly sensitive imaging modalities include fluorescence imaging (FLI), bioluminescence imaging (BLI) and fluorescence molecular tomography (FMT) which enable photon detection but with poor penetrability (Ventura et al. 2014).

15.5 In-Silico Evaluation Methods

Early achievement of primary stability leading to faster osseointegration can be made possible by fabricating customized patient specific implants. It was conceptualized from the lack of flexibilities in customizing implant length, diameter, surface geometry, threading parameters for a particular fracture (Chen et al. 2012). Customized root-analogue dental implants, since its inception, have been an attractive approach to provide the scope of minimally invasive implantation without requiring traumatic surgical procedures such as sinus lift, drilling, bone augmentation (Pirker and Kocher 2008). It is therefore necessary to perform the in-silico finite

element analysis (FEA) to predict the relationship of biomechanical behaviour of the implants namely magnitude of stress/strain surrounding implants with peri-implant bone growth (Dos Santos et al. 2017). Several studies compared the overall stress distribution pattern and resulting stress on neighbouring bone using general threaded implants with press-fit design accompanied with same standard abutment with that caused by customized route-analogue implant. Summarized results indicated towards more uniform stress distribution, comparatively low alveolar bone stress, improved primary stability and reduced micro-movement with targeted press-fit dental implants (Anssari Moin et al. 2016; Chen et al. 2017). The process initiates the virtual design and development of implant models with requisite geometrical features which are to be virtually placed into simplified bone model composed of equal proportion of cortical and trabecular bone (Dantas et al. 2020). The models are then imported in to the working interface of COMSOLMultiphysics software to develop finite element models. Previously fed with the information regarding mechanical properties of several metals and non-metals acquired from the published literature, external loads are virtually applied from opposite directions (axial and oblique) on the models provided all the bone-bone and bone-metal interface are perfectly bonded. The fixed constraint is applied then in all the possible degrees of freedom at the interface of cutting section and also at the bottom of trabecular and cortical bone. Quantitative analysis finally reveals the effect of applied loading pressure on shear strain on cortical bone. Displacement of the placed implant along with tooth from the socket also can be calculated by means of standard mathematical formula. Therefore, it is absolutely necessary to identify the zone of bone resorption as the major loss of implant stability happens due to improper loading and poor distribution of stress (Dantas et al. 2020). Lorkowski et al. also performed FEA to virtually assess the severity of femoral strain caused by multi-hole plate stabilator accompanied with cerclage wire loops especially in osteoporotic bone suffering from peri-prosthetic fractures compared to that caused in non-osteoporotic healthy bone (Lorkowski et al. 2021). The more screws or wire loops it needs to stabilize the fixture, the more strain it is found to cause leading to reduced bone elasticity, implant destabilization, repeated micro-fractures especially evident in the osteoporotic bone (Lorkowski et al. 2014). X-ray based imaging, being one of the routine examinations for the treatment of peri-prosthetic fractures, is apparently the best source to approximate minute details about altered morphology of bones especially for the elderly patients required for the 3D modelling of the patient-specific bone structure. This seems to be convenient to understand the severity of osteoporosis or illustrate ongoing changes while reducing inconvenient sufferings caused by traumatic revision surgeries, implant exchanges or surgical interventions of repeated fractures (Lorkowski et al. 2021; Lee et al. 2018).

15.6 Future Directions

The advanced technology of biomaterials and cellular biology together has a significant role in driving stem cell fate by mimicking the sequence of physiological events involved in cellular adhesion, proliferation, differentiation, even the production, clearance and interaction of osteoprogenitors at the surface or through pores of the novel biomaterials. Cell-biomaterial interaction, however, can be controlled either by co-culturing cell-sheets on scaffolds prior to implantation and some other approaches include intelligent cell seeding on surface, functional coating on implant surfaces as well. Cell morphology seeded to be an important parameter significantly effecting the cell migration and proliferation on the biomaterial surface. Frost et al. explored the ideal morphology while culturing the porcine dermal fibroblasts on different prototypes of implant surface (Frost et al. 2021). Elongated cellular morphology, being quantified with cell aspect ratio (CAR), was found to be accelerating the cellular proliferation where high CAR value was indicative of rounded cell morphology favouring cell migration. They also outlined the future potential of this work in performing high throughput screening of the prospective surface prototypes before going for in-vitro cell-based assays. Biomaterial, screened by this methodology and designed accordingly might produce better response by generating dynamic cell morphology depending on the sequence of bio-molecular events necessary for tissue regeneration. A novel bio-engineering approach involving microfluidics was applied in a recent research where the clustering pattern and migratory response of retinal progenitor cells were evaluated in presence of some of the widely used extracellular substrates such as concanavalin A, laminin, poly-L-lysine and it was found that the migration of the cluster of the cells was subjected to exogenous chemotactic signalling of fibroblast growth factor (Pena et al. 2019). Although these above-mentioned novel approaches were not applied in optimizing implant/scaffold-based bone regenerative therapies, they may have a pivotal role in investigating potential of the bio-substrates to obtain optimum cell-biomaterial interaction, if utilized. Prior screening of such implants may help to control peri-implant microenvironment experimentally to avoid delayed osseointegration, risk of fibrous encapsulation and loosening of implants. All the evaluation strategies, discussed in this article, however, are not limited only in conventional in-vitro (stem cells etc.) and in-vivo models (small animals specifically rodents) but Vimalraj et al. also highlighted the preference towards zebrafish models as an excellent platform to study host bone-implant interaction (Vimalraj et al. 2021). Apart from several known advantages such as cost-effective, ability for whole-body imaging for optical transparency, genetic adaptability and short life cycle, several inductive signals generated during bone morphogenesis in zebrafish have also the potential to influence bone formation in mammals similar to conventional in-vivo models. The flexibility to study any kind of fabricated scaffold such as hydrogel, film, porous freeze-dried scaffold, electrospun network in such versatile in-vivo platform certainly will provide the edge over others to give insight into biocompatibility, bioactivity of novel materials under investigation.

Conflict of Interest Statement The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

Alam S, Ueki K, Marukawa K, Ohara T, Hase T, Takazakura D et al (2007) Expression of bone morphogenetic protein 2 and fibroblast growth factor 2 during bone regeneration using different implant materials as an onlay bone graft in rabbit mandibles. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 103:16–26

Albrektsson T, Johansson C (2001) Osteoinduction, osteoconduction and osseointegration. Eur Spine J 10:S96–S101

Al-Maawi S, Orlowska A, Sader R, James Kirkpatrick C, Ghanaati S (2017) In vivo cellular reactions to different biomaterials—physiological and pathological aspects and their consequences. Semin Immunol 29:49–61

Anssari Moin D, Hassan B, Wismeijer D (2016) A patient specific biomechanical analysis of custom root analogue implant designs on alveolar bone stress: a finite element study. Int J Dent 2016:8242535

Bahraminasab M, Arab S, Safari M, Talebi A, Kavakebian F, Doostmohammadi N (2021) In vivo performance of Al2O3-Ti bone implants in the rat femur. J Orthop Surg Res 16:79

Bailey DA, McCulloch RG (1990) Bone tissue and physical activity. Can J Sport Sci 15:229–239

Barbeck M, Udeabor S, Lorenz J, Kubesch A, Choukroun J, Sader R et al (2014) Induction of multinucleated giant cells in response to small sized bovine bone substitute (Bio-Oss^{TM}) results in an enhanced early implantation bed vascularization. Ann Maxillofac Surg 4:150–157

Barbeck M, Najman S, Stojanović S, Mitić Ž, Živković JM, Choukroun J et al (2015a) Addition of blood to a phycogenic bone substitute leads to increased *in vivo* vascularization. Biomed Mater 10:055007

Barbeck M, Dard M, Kokkinopoulou M, Markl J, Booms P, Sader R et al (2015b) Small-sized granules of biphasic bone substitutes support fast implant bed vascularization. Biomatter 5: e1056943

Barbeck M, Unger RE, Booms P, Dohle E, Sader RA, Kirkpatrick CJ et al (2016) Monocyte preseeding leads to an increased implant bed vascularization of biphasic calcium phosphate bone substitutes via vessel maturation. J Biomed Mater Res A 104:2928–2935

Barbeck M, Booms P, Unger R, Hoffmann V, Sader R, Kirkpatrick CJ et al (2017) Multinucleated giant cells in the implant bed of bone substitutes are foreign body giant cells—new insights into the material-mediated healing process. J Biomed Mater Res A 105:1105–1111

Beckmann N, Maier P (2011) Noninvasive small rodent imaging: significance for the 3R principles. In: Kiessling F, Pichler BJ (eds) Small animal imaging: basics and practical guide. Springer, Berlin, Heidelberg, pp 47–57

Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol 7:452–464

Bershadsky A, Kozlov M, Geiger B (2006) Adhesion-mediated mechanosensitivity: a time to experiment, and a time to theorize. Curr Opin Cell Biol 18:472–481

Bez M, Sheyn D, Tawackoli W (2017) In situ bone tissue engineering via ultrasound-mediated gene delivery to endogenous progenitor cells in mini-pigs. Sci Transl Med 9:eaal3128

Body SC (1996) Platelet activation and interactions with the microvasculature. J Cardiovasc Pharmacol 27(Suppl 1):S13–S25

Bohner M, Baroud G, Bernstein A, Doebelin N, Galea L, Hesse B et al (2017) Characterization and distribution of mechanically competent mineralized tissue in micropores of β-tricalcium phosphate bone substitutes. Mater Today 20:106–115

Büchter A, Joos U, Wiesmann H-P, Seper L, Meyer U (2006) Biological and biomechanical evaluation of interface reaction at conical screw-type implants. Head Face Med 2:5

Burguete RL, Johns RB, King T, Patterson EA (1994) Tightening characteristics for screwed joints in osseointegrated dental implants. J Prosthet Dent 71:592–599

Burmeister JS, Vrany J, Reichert WM, Truskey GA (1996) Effect of fibronectin amount and conformation on the strength of endothelial cell adhesion to HEMA/EMA copolymers. J Biomed Mater Res 30:13–22

Buser D, Schenk RK, Steinemann S, Fiorellini JP, Fox CH, Stich H (1991) Influence of surface characteristics on bone integration of titanium implants. A histomorphometric study in miniature pigs. J Biomed Mater Res 25:889–902

Cavalcanti-Adam EA, Micoulet A, Blümmel J, Auernheimer J, Kessler H, Spatz JP (2006) Lateral spacing of integrin ligands influences cell spreading and focal adhesion assembly. Eur J Cell Biol 85:219–224

Celikkin N, Mastrogiacomo S, Jaroszewicz J, Walboomers XF, Swieszkowski W (2018) Gelatin methacrylate scaffold for bone tissue engineering: the influence of polymer concentration. J Biomed Mater Res A 106:201–209

Cha JY, Pereira MD, Smith AA, Houschyar KS, Yin X, Mouraret S et al (2015) Multiscale analyses of the bone-implant interface. J Dent Res 94:482–490

Chang H-I, Wang Y (2011) Cell responses to surface and architecture of tissue engineering scaffolds. Regenerative medicine and tissue engineering-cells and biomaterials. InTechOpen

Chen X, Xie L, Du R, Deng F (2012) Design and fabrication of custom-made dental implants. J Mech Sci Technol 26:1993–1998

Chen J, Zhang Z, Chen X, Zhang X (2017) Influence of custom-made implant designs on the biomechanical performance for the case of immediate post-extraction placement in the maxillary esthetic zone: a finite element analysis. Comput Methods Biomech Biomed Engin 20:636–644

Cheng C, Alt V, Dimitrakopoulou-Strauss A, Pan L, Thormann U, Schnettler R et al (2013) Evaluation of new bone formation in normal and osteoporotic rats with a 3-mm femur defect: functional assessment with dynamic PET-CT (dPET-CT) using 2-deoxy-2-[(18)F]fluoro-D-glucose ((18)F-FDG) and (18)F-fluoride. Mol Imaging Biol 15:336–344

Dantas TA, Carneiro Neto JP, Alves JL, Vaz PCS, Silva FS (2020) In silico evaluation of the stress fields on the cortical bone surrounding dental implants: comparing root-analogue and screwed implants. J Mech Behav Biomed Mater 104:103667

Delloye C, Cornu O, Druez V, Barbier O (2007) Bone allografts. J Bone Joint Surg 89-B:574–580

Delpiano MA, Acker H (1985) Extracellular pH changes in the superfused cat carotid body during hypoxia and hypercapnia. Brain Res 342:273–280

Dos Santos Marsico V, Lehmann RB, de Assis Claro CA, Amaral M, Vitti RP, Neves ACC et al (2017) Three-dimensional finite element analysis of occlusal splint and implant connection on stress distribution in implant-supported fixed dental prosthesis and peri-implantal bone. Mater Sci Eng C Mater Biol Appl 80:141–148

Duncan I, Ingold N (2018) The clinical value of xSPECT/CT Bone versus SPECT/CT. A prospective comparison of 200 scans. Eur J Hybrid Imaging 2:1–12

El-Ghannam A (2005) Bone reconstruction: from bioceramics to tissue engineering. Expert Rev Med Devices 2:87–101

Elliott SR, Robinson RA (1957) The water content of bone. I. The mass of water, inorganic crystals, organic matrix, and CO2 space components in a unit volume of the dog bone. J Bone Joint Surg Am 39-A:167–188

Engin F, Yao Z, Yang T, Zhou G, Bertin T, Jiang MM et al (2008) Dimorphic effects of Notch signaling in bone homeostasis. Nat Med 14:299–305

Ercan B, Webster T (2014) Cell response to nanoscale features and its implications in tissue regeneration: an orthopedic perspective. In: Nanotechnology and regenerative engineering. p 145–190

Fedorovich NE, Alblas J, Hennink WE, Öner FC, Dhert WJA (2011) Organ printing: the future of bone regeneration? Trends Biotechnol 29:601–606

Filippi M, Born G, Chaaban M, Scherberich A (2020) Natural polymeric scaffolds in bone regeneration. Front Bioeng Biotechnol 8:474

Folkman M, Becker A, Meinster I, Masri M, Ormianer Z (2020) Comparison of bone-to-implant contact and bone volume around implants placed with or without site preparation: a histomorphometric study in rabbits. Sci Rep 10:12446

Fragogeorgi EA, Rouchota M, Georgiou M, Velez M, Bouziotis P, Loudos G (2019) In vivo imaging techniques for bone tissue engineering. J Tissue Eng 10:2041731419854586

Friedl P, Zänker KS, Bröcker EB (1998) Cell migration strategies in 3-D extracellular matrix: differences in morphology, cell matrix interactions, and integrin function. Microsc Res Tech 43: 369–378

Frohlich M, Grayson WL, Wan LQ, Marolt D, Drobnic M, Vunjak-Novakovic G (2008) Tissue engineered bone grafts: biological requirements, tissue culture and clinical relevance. Curr Stem Cell Res Ther 3:254–264

Frost OG, Owji N, Thorogate R, Kyriakidis C, Sawadkar P, Mordan N et al (2021) Cell morphology as a design parameter in the bioengineering of cell–biomaterial surface interactions. Biomater Sci 9:8032–8050

Gao C, Peng S, Feng P, Shuai C (2017) Bone biomaterials and interactions with stem cells. Bone Res 5:17059

Gensel J, Zhang B (2015) Macrophage activation and its role in repair and pathology after spinal cord injury. Brain Res 22:1–11

Ghanaati S (2012) Non-cross-linked porcine-based collagen I–III membranes do not require high vascularization rates for their integration within the implantation bed: a paradigm shift. Acta Biomater 8:3061–3072

Ghanaati S, Barbeck M, Orth C, Willershausen I, Thimm BW, Hoffmann C et al (2010) Influence of β-tricalcium phosphate granule size and morphology on tissue reaction in vivo. Acta Biomater 6:4476–4487

Ghanaati S, Schlee M, Webber MJ, Willershausen I, Barbeck M, Balic E et al (2011a) Evaluation of the tissue reaction to a new bilayered collagen matrix *in vivo* and its translation to the clinic. Biomed Mater 6:015010

Ghanaati S, Unger RE, Webber MJ, Barbeck M, Orth C, Kirkpatrick JA et al (2011b) Scaffold vascularization in vivo driven by primary human osteoblasts in concert with host inflammatory cells. Biomaterials 32:8150–8160

Hench LL (2015) The future of bioactive ceramics. J Mater Sci Mater Med 26:86

Hériveaux Y, Nguyen V-H, Brailovski V, Gorny C, Haïat G (2019) Reflection of an ultrasonic wave on the bone−implant interface: effect of the roughness parameters. J Acoust Soc Am 145:3370–3381

Hollister SJ (2005) Porous scaffold design for tissue engineering. Nat Mater 4:518–524

Hu N, Jiang D, Huang E, Liu X, Li R, Liang X et al (2013) BMP9-regulated angiogenic signaling plays an important role in the osteogenic differentiation of mesenchymal progenitor cells. J Cell Sci 126:532–541

Jell G, Stevens MM (2006) Gene activation by bioactive glasses. J Mater Sci Mater Med 17:997–1002

Joos U, Vollmer D, Kleinheinz J (2000) [Effect of implant geometry on strain distribution in peri-implant bone]. Mund Kiefer Gesichtschir 4:143–147

Kang Y, Mochizuki N, Khademhosseini A, Fukuda J, Yang Y (2015) Engineering a vascularized collagen-β-tricalcium phosphate graft using an electrochemical approach. Acta Biomater 11: 449–458

Kazimierczak P, Przekora A (2020) Osteoconductive and osteoinductive surface modifications of biomaterials for bone regeneration: a concise review. Coatings 10:971

Keselowsky BG, Collard DM, García AJ (2003) Surface chemistry modulates fibronectin conformation and directs integrin binding and specificity to control cell adhesion. J Biomed Mater Res A 66:247–259

Klimek K, Belcarz A, Pazik R, Sobierajska P, Han T, Wiglusz RJ et al (2016) "False" cytotoxicity of ions-adsorbing hydroxyapatite—corrected method of cytotoxicity evaluation for ceramics of high specific surface area. Mater Sci Eng C 65:70–79

Knight MN, Hankenson KD (2013) Mesenchymal stem cells in bone regeneration. Adv Wound Care (New Rochelle) 2:306–316

Kokubo T (1995) Bioactivity of bioactive filler-resin cement: proceedings of the 8th International Symposium on Ceramics in Medicine. Bioceramics 8:213–217

Lagonegro P, Rossi F, Galli C, Smerieri A, Alinovi R, Pinelli S et al (2017) A cytotoxicity study of silicon oxycarbide nanowires as cell scaffold for biomedical applications. Mater Sci Eng C 73: 465–471

Lang LA, Kang B, Wang RF, Lang BR (2003) Finite element analysis to determine implant preload. J Prosthet Dent 90:539–546

Le X, Poinern GEJ, Ali N, Berry CM, Fawcett D (2013) Engineering a biocompatible scaffold with either micrometre or nanometre scale surface topography for promoting protein adsorption and cellular response. Int J Biomater 2013:782549

LeBaron RG, Athanasiou KA (2000) Extracellular matrix cell adhesion peptides: functional applications in orthopedic materials. Tissue Eng 6:85–103

Lee SJ, Lim GJ, Lee J-W, Atala A, Yoo JJ (2006) In vitro evaluation of a poly(lactide-co-glycolide)–collagen composite scaffold for bone regeneration. Biomaterials 27:3466–3472

Lee J-M, Kim T-S, Kim T-H (2018) Treatment of periprosthetic femoral fractures following hip arthroplasty. Hip Pelvis 30:78–85

Lima TVM, Bhure U, Pérez Lago MS, Thali Y, Matijasevic S, Roos J et al (2020) Impact of metal implants on xSPECT/CT Bone reconstruction: the "shining metal artefact". Eur J Hybrid Imaging 4:18

Liu X, Yuan L, Li D, Tang Z, Wang Y, Chen G et al (2014a) Blood compatible materials: state of the art. J Mater Chem B 2:5718–5738

Liu Y, Cai D, Yang J, Wang Y, Zhang X, Yin S (2014b) In vitro hemocompatibility evaluation of poly (4-hydroxybutyrate) scaffold. Int J Clin Exp Med 7:1233–1243

Lorkowski J, Mrzygłód MM, Kotela A, Kotela I (2014) Application of rapid computer modeling in the analysis of the stabilization method in intraoperative femoral bone shaft fracture during revision hip arthroplasty—a case report. Pol Orthop Traumatol 79:138–144

Lorkowski J, Wilk R, Pokorski M (2021) In silico evaluation of treatment of periprosthetic fractures in elderly patients after hip arthroplasty. In: Pokorski M (ed) Medical and biomedical updates. Springer International Publishing, Cham, pp 115–123

Malafaya PB, Reis RL (2009) Bilayered chitosan-based scaffolds for osteochondral tissue engineering: influence of hydroxyapatite on in vitro cytotoxicity and dynamic bioactivity studies in a specific double-chamber bioreactor. Acta Biomater 5:644–660

McKittrick J, Chen PY, Tombolato L, Novitskaya EE, Trim MW, Hirata GA et al (2010) Energy absorbent natural materials and bioinspired design strategies: a review. Mater Sci Eng C 30: 331–342

McNally AK, Anderson JM (2002) Beta1 and beta2 integrins mediate adhesion during macrophage fusion and multinucleated foreign body giant cell formation. Am J Pathol 160:621–630

Meredith N, Alleyne D, Cawley P (1996) Quantitative determination of the stability of the implant-tissue interface using resonance frequency analysis. Clin Oral Implants Res 7:261–267

Montazerolghaem M, Rasmusson A, Melhus H, Engqvist H, Karlsson OM (2016) Simvastatin-doped pre-mixed calcium phosphate cement inhibits osteoclast differentiation and resorption. J Mater Sci Mater Med 27:83

Murer AM, Hirschmann MT, Amsler F, Rasch H, Huegli RW (2020) Bone SPECT/CT has excellent sensitivity and specificity for diagnosis of loosening and patellofemoral problems after total knee arthroplasty. Knee Surg Sports Traumatol Arthrosc 28:1029–1035

Murphy CM, O'Brien FJ, Little DG, Schindeler A (2013) Cell-scaffold interactions in the bone tissue engineering triad. Eur Cell Mater 26:120–132

Neff LP, Tillman BW, Yazdani SK, Machingal MA, Yoo JJ, Soker S et al (2011) Vascular smooth muscle enhances functionality of tissue-engineered blood vessels in vivo. J Vasc Surg 53:426–434

Pagliani L, Sennerby L, Petersson A, Verrocchi D, Volpe S, Andersson P (2013) The relationship between resonance frequency analysis (RFA) and lateral displacement of dental implants: an in vitro study. J Oral Rehabil 40:221–227

Park JY, Gemmell CH, Davies JE (2001) Platelet interactions with titanium: modulation of platelet activity by surface topography. Biomaterials 22:2671–2682

Pena CD, Zhang S, Majeska R, Venkatesh T, Vazquez M (2019) Invertebrate retinal progenitors as regenerative models in a microfluidic system. Cell 8:1301

Pirker W, Kocher A (2008) Immediate, non-submerged, root-analogue zirconia implant in single tooth replacement. Int J Oral Maxillofac Surg 37:293–295

Polo-Corrales L, Latorre-Esteves M, Ramirez-Vick JE (2014) Scaffold design for bone regeneration. J Nanosci Nanotechnol 14:15–56

Przekora A (2019) The summary of the most important cell-biomaterial interactions that need to be considered during in vitro biocompatibility testing of bone scaffolds for tissue engineering applications. Mater Sci Eng C 97:1036–1051

Przekora A, Czechowska J, Pijocha D, Ślósarczyk A, Ginalska G (2014) Do novel cement-type biomaterials reveal ion reactivity that affects cell viability in vitro? Open Life Sci 9:277–289

Ravarian R, Zhong X, Barbeck M, Ghanaati S, Kirkpatrick CJ, Murphy CM et al (2013) Nanoscale chemical interaction enhances the physical properties of bioglass composites. ACS Nano 7: 8469–8483

Romagnoli C, Brandi ML (2014) Adipose mesenchymal stem cells in the field of bone tissue engineering. World J Stem Cells 6:144

Römer W, Kuwert T, Olk A, Hennig F, Bautz W (2005) Assessment of aseptic loosening of the acetabular component in a total hip replacement with 99m Tc-DPD-SPECT/spiral-CT hybrid imaging. Nuklearmedizin 44:N58–N60

Rothamel D, Schwarz F, Sager M, Herten M, Sculean A, Becker J (2005) Biodegradation of differently cross-linked collagen membranes: an experimental study in the rat. Clin Oral Implants Res 16:369–378

Saiz E, Zimmermann EA, Lee JS, Wegst UG, Tomsia AP (2013) Perspectives on the role of nanotechnology in bone tissue engineering. Dent Mater 29:103–115

Salakhutdinov I, VandeVord P, Palyvoda O, Matthew H, Handa H, Mao G et al (2008) Fibronectin adsorption to nanopatterned silicon surfaces. Biomedical Optics: Optical Society of America, p BSuE11

Schwarz F, Rothamel D, Herten M, Sager M, Becker J (2006) Angiogenesis pattern of native and cross-linked collagen membranes: an immunohistochemical study in the rat. Clin Oral Implants Res 17:403–409

Shah FA, Stenlund P, Martinelli A, Thomsen P, Palmquist A (2016) Direct communication between osteocytes and acid-etched titanium implants with a sub-micron topography. J Mater Sci Mater Med 27:1–9

Shao R, Dong Y, Zhang S, Wu X, Huang X, Sun B et al (2022) State of the art of bone biomaterials and their interactions with stem cells: current state and future directions. Biotechnol J 17: e2100074

Shattil SJ, Cunningham M, Hoxie JA (1987) Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood 70:307–315

Shi Y, Chen J, Karner CM, Long F (2015) Hedgehog signaling activates a positive feedback mechanism involving insulin-like growth factors to induce osteoblast differentiation. Proc Natl Acad Sci 112:4678–4683

Stevens MM (2008) Biomaterials for bone tissue engineering. Mater Today 11:18–25

Stocchero M, Jinno Y, Toia M, Ahmad M, Papia E, Yamaguchi S et al (2019) Intraosseous temperature change during installation of dental implants with two different surfaces and different drilling protocols: an in vivo study in sheep. J Clin Med 8:1198

Szmukler-Moncler S, Piattelli A, Favero GA, Dubruille JH (2000) Considerations preliminary to the application of early and immediate loading protocols in dental implantology. Clin Oral Implants Res 11:12–25

Terranova L, Mallet R, Perrot R, Chappard D (2016) Polystyrene scaffolds based on microfibers as a bone substitute; development and in vitro study. Acta Biomater 29:380–388

Timmins PA, Wall JC (1977) Bone water. Calc Tissue Res 23:1–5

Tong S, Xu D-P, Liu Z-M, Du Y, Wang X-K (2016) Synthesis of and in vitro and in vivo evaluation of a novel TGF-1-SF-CS three-dimensional scaffold for bone tissue engineering. Int J Mol Med 38:367–380

Trindade R, Albrektsson T, Galli S, Prgomet Z, Tengvall P, Wennerberg A (2018) Osseointegration and foreign body reaction: titanium implants activate the immune system and suppress bone resorption during the first 4 weeks after implantation. Clin Implant Dent Relat Res 20:82–91

Tsigkou O, Hench LL, Boccaccini AR, Polak JM, Stevens MM (2007) Enhanced differentiation and mineralization of human fetal osteoblasts on PDLLA containing Bioglass composite films in the absence of osteogenic supplements. J Biomed Mater Res A 80:837–851

Urist MR (1965) Bone: formation by autoinduction. Science 150:893–899

Ventura M, Boerman OC, de Korte C, Rijpkema M, Heerschap A, Oosterwijk E et al (2014) Preclinical imaging in bone tissue engineering. Tissue Eng Part B Rev 20:578–595

Vimalraj S, Yuvashree R, Hariprabu G, Subramanian R, Murali P, Veeraiyan DN et al (2021) Zebrafish as a potential biomaterial testing platform for bone tissue engineering application: a special note on chitosan based bioactive materials. Int J Biol Macromol 175:379–395

Wang RF, Kang B, Lang LA, Razzoog ME (2009) The dynamic natures of implant loading. J Prosthet Dent 101:359–371

Wang C, Wang S, Li K, Ju Y, Li J, Zhang Y et al (2014) Preparation of laponite bioceramics for potential bone tissue engineering applications. PLoS One 9:e99585

Wang Q, Chen B, Cao M, Sun J, Wu H, Zhao P et al (2016) Response of MAPK pathway to iron oxide nanoparticles in vitro treatment promotes osteogenic differentiation of hBMSCs. Biomaterials 86:11–20

Washburn NR, Weir M, Anderson P, Potter K (2004) Bone formation in polymeric scaffolds evaluated by proton magnetic resonance microscopy and X-ray microtomography. J Biomed Mater Res A 69:738–747

Weber M, Umrath F, Steinle H, Schmitt LF, Yu LT, Schlensak C et al (2021) Influence of human jaw periosteal cells seeded β-tricalcium phosphate scaffolds on blood coagulation. Int J Mol Sci 22:1–15

Wong KK, Piert M (2013) Dynamic bone imaging with 99mTc-labeled diphosphonates and 18F-NaF: mechanisms and applications. J Nucl Med 54:590–599

Xia L, Feng B, Wang P, Ding S, Liu Z, Zhou J et al (2012) In vitro and in vivo studies of surface-structured implants for bone formation. Int J Nanomedicine 7:4873–4881

Xu HH, Wang P, Wang L, Bao C, Chen Q, Weir MD et al (2017) Calcium phosphate cements for bone engineering and their biological properties. Bone Res 5:1–19

Yamada KM (1991) Adhesive recognition sequences. J Biol Chem 266:12809–12812

Yang F, Wang J, Hou J, Guo H, Liu C (2013) Bone regeneration using cell-mediated responsive degradable PEG-based scaffolds incorporating with rhBMP-2. Biomaterials 34:1514–1528

Yannas IV, Lee E, Orgill DP, Skrabut EM, Murphy GF (1989) Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin. Proc Natl Acad Sci U S A 86:933–937

Yuan Z, Li Q, Luo S, Liu Z, Luo D, Zhang B et al (2016) PPARγ and Wnt signaling in adipogenic and osteogenic differentiation of mesenchymal stem cells. Curr Stem Cell Res Ther 11:216–225

Zhang Y, Li R, Wu W, Ya Q, Tang X, Ye W et al (2018) Adhesion and proliferation of osteoblast-like cells on porous polyetherimide scaffolds. Biomed Res Int 2018:1491028

Zomorodian E, Baghaban Eslaminejad M (2012) Mesenchymal stem cells as a potent cell source for bone regeneration. Stem Cells Int 2012:980353

Zorio E, Gilabert-Estellés J, España F, Ramón LA, Cosín R, Estellés A (2008) Fibrinolysis: the key to new pathogenetic mechanisms. Curr Med Chem 15:923–929

The Prospects of RNAs and Common Significant Pathways in Cancer Therapy and Regenerative Medicine

16

Manaswini Gattupalli, Parry Dey, Shanmugam Poovizhi, Riya Ben Patel, Debasish Mishra, and Satarupa Banerjee

16.1 Introduction

Human cells normally expand and multiply via cell division to generate new cells as needed and in cancer, some cells in the body grow out of control and spread to other parts of the body. Cells die as they become old or injured, and new cells replace them. Cancerous tumours can affect adjacent tissues and spread to other parts of the body, resulting in the formation of new tumours by a process called metastasis. Regeneration is a natural mechanism by which injured or damaged tissues and organs are replaced through cell proliferation into a normal tissue. Tissue regeneration, similar to cancer, has certain hallmarks, which are restricted to lineages and are activated only during damage (Goldman and Poss 2020). Repair of an organ is the restoration of interrupted continuity of injured tissue mass and formation of scar tissue, while regeneration refers to the restoration of interrupted continuity of the damaged tissue mass with original anatomical characters and original tissue function, yet no scar formation (Mason and Dunnill 2008). Many signalling pathways play a major role in the process as well. In this chapter, the role of different signalling pathways has been discussed, which modulate both the process of carcinogenesis and regeneration. Role of different signalling pathways in cancer has been depicted in Fig. 16.1.

Manaswini Gattupalli and Parry Dey contributed equally to this work.

M. Gattupalli · P. Dey · S. Poovizhi · R. B. Patel · D. Mishra (✉) · S. Banerjee (✉)
School of Bio Sciences and Technology (SBST), Vellore Institute of Technology (VIT), Vellore, Tamil Nadu, India
e-mail: debasish@vit.ac.in; satarupa.banerjee@vit.ac.in

N. Chakravorty, P. C. Shukla (eds.), *Regenerative Medicine*,
https://doi.org/10.1007/978-981-19-6008-6_16

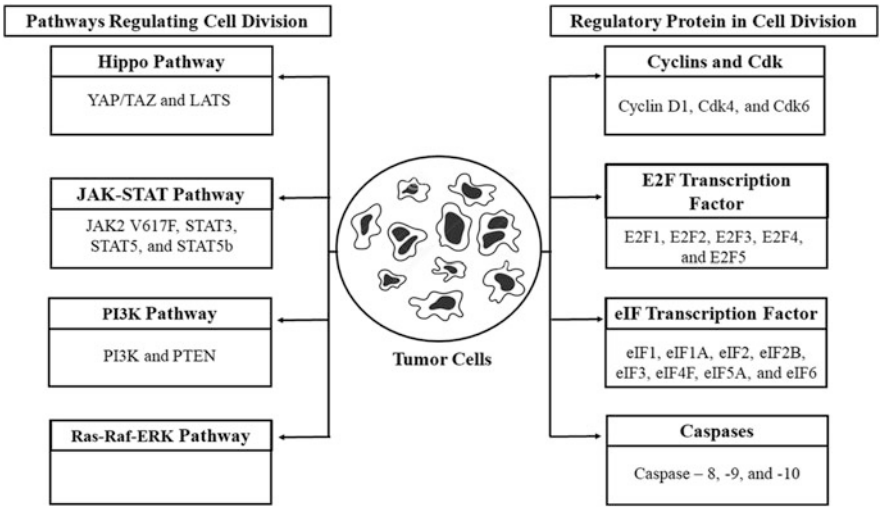


Fig. 16.1 Regulation of cancer by various signalling pathways

16.1.1 Similarities and Differences Between Cancer and Regeneration

The similarities and differences between cancer and regeneration have been shown in Table 16.1 (Charni et al. 2017; Boilly et al. 2017; Oviedo and Beane 2009; Smetana et al. 2013).

16.1.2 Significant Pathways of Regeneration and Cancer

Early signs of regeneration include:

1. A layer of epithelium covers the injured region, as one of the initial responses.
2. There is an immediate triggering of reactive oxygen species (ROS) in the injured area for wound detection.
3. The site of injury shows inflammation because of macrophages and T cells, which further direct the site of injury into a site for regeneration.
4. There is production of hydrogen peroxide.
5. Targets of ROS include vital signalling pathways like JNK, WNT and FGF. These pathways are essential for cell regeneration.
6. Mitogens are released by dying cells, by MAPK/CREB pathway, to cause the activation of tissue regeneration in the healthy cells of the injured site (Chen and Poss 2017).

There are a few hallmarks of regeneration of heart revascularization

Table 16.1 Similarities and differences between cancer and regeneration

Similarities—cancer and regeneration	Differences—cancer and regeneration
Inflammation, construction and remodelling of tissues are common in both cancer and regeneration	Tumorigenesis has surpassed one/many of the regulations and is unrestrained, while regeneration is a strictly controlled process
Various cell signalling pathways like WNT, PI3K and MEK, are commonly seen in both cancer and regeneration	Dysregulation in any one of the factors like genes/regulatory proteins/pathways can lead to cancer
Cell proliferation, cell differentiation, cell migration are commonly triggered by the same regulatory processes	Cell proliferation and cell migration, when uncontrolled, lead to cancer. Inflammation can become a pre-condition
Triggering cancer and the rate of regeneration are influenced by telomeres length and the extent of the activity of telomerase	Regeneration of organs in humans is limited and is commonly incomplete because of the formation of scar tissue, while any tissue can turn cancerous due to impairment
Blastema provides stem cells, which contributes to the regeneration of damaged tissue and also contributes to the growth of cancer cells	Cancers can be formed from improper regeneration or loss of regeneration or loss of a tissue morphogenesis
Regulation of microenvironment, such as extra-cellular matrix or cytokines, contributes to regeneration and as well as metastasis	Common pathways are strictly controlled in regeneration, while common pathways are usually unrestrained in cancer
Suppression of tumours can also lead to suppression of regeneration	End result of cancer is death, but end result of regeneration is life

1. Modulating the developmental signalling pathways
2. Directing the cell cycle regulators
3. Reprogramming resident stromal cells into required specific tissue
4. Stem cell or progenitor cell leads to paracrine effect, but this is an indirect central hallmark (Bertero and Murry 2018).

In cancer, a few pathways associated with cell cycle regulation, JNK, WNT, FGF signalling, the mTOR pathway, PI3K, JAK-STAT, Hippo signalling, modulation by miRNAs also have significant contribution and have been detailed accordingly in this study.

16.2 Cell Cycle and Checkpoints

'Antipathic pleiotropy' is a key idea in evolutionary theories of aging, which states that aging itself is not an evolutionary selected programme, but rather the result of selection for organismal fitness during development and reproductive phases (Kirkwood and Melov 2011). The aging of stem cells and progenitor cells is also influenced by checkpoint genes. Under genotoxic stress, checkpoint genes play a major role in suppressing cancer, but they also limit the ability of stem cells and progenitor cells to maintain and repair tissues under accumulated DNA damage and during physiological aging (Chambers et al. 2007; Krishnamurthy et al. 2006; Siegl-

Cachedenier et al. 2007). According to the results of checkpoint enhancement, the maintenance of physiologic control of checkpoint genes has a bearing on lifespan and cancer effects. Checkpoint activation cannot delay aging nor protect against cancer in conditions of high DNA damage accumulation, such as those found in telomere-dysfunctional mice. Enhanced expression of physiologically regulated checkpoint genes has been shown to reduce cancer formation and extend lifespan in mice with low levels of DNA damage. There may be a way to prevent the clonal expansion of mutated or transformed stem cells through increased expression of physiologically regulated checkpoints. Depletion of damaged stem cells during mouse models of low DNA damage inhibits cancer development and delays aging since abnormal stem cells are removed, and the remaining undamaged stem cells compensate for this depletion. Despite the enhanced clearance of damaged stem cells in mouse models of severe telomere dysfunction, tissue maintenance is unaffected since the functioning of undamaged stem cells diminishes and cannot compensate for the loss of damaged stem cells.

Cell cycle in eukaryotes includes the G0, G1, S, G2 and M phases. They are necessary for the division of the cell and are regulated by three checkpoints called G1/S checkpoint or start checkpoint, G2/M checkpoint and SAC (spindle assembly checkpoint). These checkpoints are critical for the completion of the cell cycle. Along with checkpoints, there are a few proteins like cyclins- and cyclin-dependent kinases (CDKs) that are also a part of the cell cycle regulation. During an injury or damage to the organ or tissue, cell division is triggered and the site of damage is repaired or regenerated. Regeneration of an organ or tissue involves cell differentiation along with cell proliferation. Some cells also might need reprogramming, meaning differentiated cells revert to pluripotent cells and then they divide it into any type of cell. Any aberration in cell cycle or checkpoints could lead to cancer.

16.3 Regulatory Proteins in Cell Division

16.3.1 Cyclins and CDK

The cyclin-dependent kinases (CDKs) are a large group of enzymes with a serine/threonine-specific catalytic core that becomes active on interaction with a cyclin regulatory protein, which modulates substrate specificity and kinase activity (Lim and Kaldis 2013; Loyer and Trembley 2020). In mammals, 30 cyclins and 20 CDKs have been identified so far (Loyer and Trembley 2020; Chotiner et al. 2019). These CDKs have been given a name from CDK1 to CDK20, and CLKs have been named from CDKL1 to CDKL5. Furthermore, cyclins are divided into three groups which include canonical, non-canonical and atypical cyclins. The canonical group contains A, B, D and E sub-families which play a significant part in cell cycle regulation, whereas the non-canonical group includes C, H, K, L, Q and T sub-families which are required in controlling transcription and pre-RNA processing. The third group consists of recently identified cyclins which include G, I, J, O, P and Y (Loyer and Trembley 2020). Studies have shown that

cyclin and CDKs (cyclin-dependent kinase) complexes are critical cell cycle regulators (Chotiner et al. 2019; Deshpande et al. 2005). In general, CDKs act as a promoter for the progression of the cell cycle, while cyclins regulate the transition between cell cycle stages. Therefore, CDK inhibitors (CKIs) serve as a barrier to cell cycle progression in adverse situations and hence regulate CDK–cyclin complex activity (Lim and Kaldis 2013).

16.3.1.1 Cyclins and CDK in Cancer

Deregulated cell cycle control is one of the hallmarks of cancer. Generally, non-cancer cells proliferate according to tissue growth requirements, while the proliferation of cancerous cells occurs uncontrollably which in turn contribute to tumour formation (Deshpande et al. 2005). The deregulation of the CDK–cyclin complex is among the leading causes of uncontrolled cell growth. Cyclins have been reported to have pivotal function in the pathogenesis of cancer (Stamatakos et al. 2010). The formation of tumour is usually linked with genetic or epigenetic alterations in key cell cycle molecules such as cyclins which regulate the activity of CDKs (Malumbres 2007).

Furthermore, cyclin D has been found to act as a carcinogen with a pathogenetic involvement in a variety of malignancies (Stamatakos et al. 2010). Among three types of cyclin D, cyclin D1 reported to be overexpressed in various types of malignancies (Fu et al. 2004). This cyclin D1 binds to *cdk4* or *cdk6* and using different mechanisms such as translocation of a chromosome, amplification of the gene, normal intercellular trafficking disruption, and proteolysis leads to an increase in tumour formation (Stamatakos et al. 2010). In addition to this, dysregulation of cyclin E has shown to be linked with various cancers such as breast, ovarian, colorectal and bladder (Geisen and Möröy 2002; Stamatakos et al. 2010). The exact method by which cyclin E is dysregulated is unknown; however, a few studies have indicated that gene amplification is involved, which results in G1 shortening, decreased cell size and the elimination of serum need for proliferation (Stamatakos et al. 2010).

CDKs are a well-validated target for cancer therapies because of their function in regulating important checkpoints of the cell cycle and transcription. In recent years, continuous attempts have been made to the development of CDK inhibitors (Whittaker et al. 2017). Due to the absence of an exact mechanism of action and relevant biomarkers, poor CDK isoform leads to failed attempts to target CDKs (Whittaker et al. 2017; Canavese et al. 2012). Furthermore, most of the CDKs inhibitors (non-selective CDKs inhibitors) have been found to lack selectivity for cancerous cells and found to be ineffective in most cancers (Whittaker et al. 2017). Despite these, researchers are trying to target CDKs to regenerate the controlled cell division potential via finding a novel molecule with more favourable pharmacokinetics, developing selective CDKs inhibitors, and using CDKIs in combination with conventional cytotoxic (Canavese et al. 2012; Whittaker et al. 2017). For instance, palbociclib is a selective inhibitor of CDK4/6 that has been certified as a therapeutics, and a combination of CDK4/6 inhibitors and hormone therapy is currently being tested for breast cancer (Lin et al. 2018; Whittaker et al. 2017). Moreover, certain

medications with the perfect balance of CDK selectivity, such as the second-generation, orally accessible inhibitor of CDK 2, 5 and 9 CYC065 developed from seliciclib, have been proven to be beneficial against cancer (Whittaker et al. 2017). Additionally, even though the significance of CDK2 in carcinogenesis is questionable, targeted *cdk2* inhibition may provide therapeutic advantage against some malignancies (Tadesse et al. 2018). Therefore, selective inhibitors alone and/or in combination can be used to target CDKs, but yet more research studies are required in this area as there are emerging hurdles such as drug resistance (Whittaker et al. 2017).

16.3.1.2 Cyclins and CDKs in Regeneration

In humans many (types of) cells remain quiescent in the gap phase between cell cycles, until they receive cell proliferation signals. The cells enter the G1 phase. This phase is linked with various cell controlling pathways such as quiescence, senescence, responses to stress and differentiation. During the S phase, a number of signals are received and DNA is replicated. The entry of a cell into the S phase is also termed as a restriction point since cells cannot decide to return to the original stage after this phase. Usually, differentiated cells enter the G0 phase after the G1 phase and perform their specialized functions. These cells must receive signals for their re-entry into the cell cycle and to undergo cell proliferation.

In mammals, such as humans, entry of cells into the restriction point is highly controlled by the retinoblastoma suppressor (RB) pathway.

Cell cycle is strictly controlled by cyclins and CDKs. Various cyclins and CDKs are expressed to regulate each phase of the cell cycle. During the G1 phase, cyclin D-*cdk4/6* is expressed, during the G1-S phase cyclin E-*cdk 2* is expressed, while in the S-G2 phase, cyclin A-*cdk2/1* is expressed, and during the mitotic phase, cyclin B-*cdk 1* is expressed.

The three cyclin-dependent kinases, *cdk4, cdk6 and cdk2*, phosphorylate pRB proteins at various sites. These CDKs signal the RB pathway and control G1 mechanism. In a normal cell, *CDK2*, *CDK4* and *CDK6* are relatively constant on their expression which allows the cell to perform various functions like quiescent, aging, terminal differentiation. Thus, cell proliferation is triggered, and cell cycle is regulated. Aberration in this signalling leads to development of most cancers. These CDKs can be regulated via balancing mRNA stability or translational control.

The most common pathway, mitogen activated protein kinase (MAPK) pathway, activates cyclin D transcription. When optimal expression of cyclins and CDKs is present, it leads to cell proliferation however, when there is overexpression of cyclin D with mitogenic growth factors in the G1 phase, it leads to tumorigenesis. This is because cyclin D triggers proto-oncogene transcription factors. Repression of cyclin D is essential for cell cycle exit, and any dysregulation in this terminal differentiation could lead to reactivation into the S phase of cell cycle (Duronio and Xiong 2013).

16.3.2 E2F

E2F is a class of transcription factors in mammals consisting of eight genes. Based on in vitro research, these genes encode ten proteins that are split into an activator of transcription (E2F1 to E2F3A) and repressor of transcription (E2F3B to E2F8) (Xie et al. 2021; Hollern et al. 2014; Xanthoulis and Tiniakos 2013). E2Fs are essential for various cellular functions such as controlling cell cycle, DNA damage response and apoptosis (Xie et al. 2021; Xanthoulis and Tiniakos 2013). Furthermore, the level of E2F1, E2F2 and E2F3A (activator protein) is found to be increased in the G1-S phase, whereas the high level of E2F7 and E2F8 (atypical repressor) is found in the late S phase and the level of E2F3B, E2F4, E2F5 and E2F6 (canonical repressor) is found to remain same throughout the cell cycle. Moreover, the expression and activity of E2Fs are firmly regulated at multiple levels, including regulation via post-transcriptional, pocket protein, subcellular localization and post-translational mechanisms (Kent and Leone 2019).

16.3.2.1 E2F Transcription Factor in Cancer

E2F transcription factors have been found to make a significant contribution to metastasis regulation (Hollern et al. 2014). High levels of E2F have been linked to a poor prognosis in malignancies of several types, including liver and pancreatic tumours (Kent and Leone 2019). Studies have shown that E2Fs have a dual function; based on the tissue, it may either promote or repress cancer (Xanthoulis and Tiniakos 2013). For instance, a study has shown that knockdown of E2F8 in transformed cells affects the target gene expression, cell survival and the tumour growth in xenograft models. In contrast to this, another study reported the tumour suppressor role of E2F8 using knockout mice, but further analysis is needed to confirm this controversy (Kent and Leone 2019). Furthermore, E2F1 is by far the most researched component of the E2F family in terms of cancers, along with the investigation of a few other genes. Elevated levels of E2F1 and E2F3 expression have been found to be linked with a worse prognosis in non-small cell lung cancer patients. In breast tumours, enhanced level of E2F1 or E2F4 is related to a worse prognosis, but increased E2F5 expression is linked to certain histological subtypes. The level of E2F1-5, E2F7 and E2F8 expression has been found to increase in cases of ovarian cancer. Additionally, higher levels of E2F4 and E2F7 have been connected to improved overall and disease-free survival, respectively, whereas E2F8 expression has been linked to worse overall survival. In prostate tumours, the expression of E2F2 and E2F3 increases whereas that of E2F1 is absent. The expression of E2F3 has been enhanced in bladder urothelial carcinomas, whereas E2F1 is dependent on invasion. Glioblastoma, thyroid cancer, lymph nodes, as well as small cell lung carcinoma metastases from malignant melanoma, have been shown to harbour higher E2F1 expression (Xanthoulis and Tiniakos 2013).

16.3.2.2 E2F Transcription Factor in Regeneration

It is one of the key regulators of cell proliferation. This is in turn controlled by intracellular and extracellular signals. It plays a very important role in maintaining

the inhibition of the cell cycle at the G0/G1 phase in embryonic as well as adult stem cells, during regeneration and cancer. As G1 cells are directed to enter the S phase or return to the G0 phase, the regulation of E2F is essential for the cells to enter into the S phase. E2F members bind to promoters of genes that regulate transcription of the G0/G1 phase. Cyclin-CDK activity is upregulated with the increase in the dissociation of repressive E2F complexes and releasing activator E2F complexes which leads to upregulation of genes with HATs. This family of transcription factors lacks nuclear localization signals, and, therefore, they depend on the RB family of proteins to arrest the cell cycle. On the whole, E2F behaves as a cell activator, while E2F4-5 are those repressors which prevent cell proliferation. During regeneration, expression of E2F4 is optimal and normal; thus, it prevents cell proliferation, while the aberration of E2F4 leads to cancer, pointing E2F4 as an onco-gene. Also, because of aberration in E2F4, cells also escape apoptosis (Hsu and Sage 2016).

16.3.3 eIF

The translation route in eukaryotes is facilitated by a group of enzymes known as eIF (eukaryotic translation initiation) factors (Efiok and Safer 2000). In eukaryotic cells, messenger RNA (mRNA) translation is essential for gene expression and occurs at the initiation stage, which is mostly controlled by eukaryotic initiation factors (eIFs). eIFs are required for mRNA translation and so serve as major targets for various signalling pathways that control gene expression (Hao et al. 2020). eIF-2 is a prominent initiation factor among eIF-1, 1A, 2, 3 and 5 because it triggers a rate-limiting initiation step of translation, that is, it stimulates the interaction of initiator met-tRNA to the 40S ribosomal subunit (Watanabe et al. 2010; Efiok and Safer 2000). As a result, most cells' overall translation rate is determined by eIF-2. Furthermore, the α subunit of eIF-2 is considered as a target for post-translational alterations which in turn contributes to protein synthesis regulation in accordance to cell cycle arrest, division, viral infection and metabolic abnormalities (Efiok and Safer 2000). Moreover, other eIFs are also involved in the initiation process of eukaryotic translation but do not have a prominent role. For instance, eIF4F is responsible for recruiting m7G-capped mRNA to the PIC (43 S pre-IC) (Watanabe et al. 2010).

16.3.3.1 eIF in Cancer

The typical characteristic of carcinogenesis is deregulated mRNA expression (Hao et al. 2020). The abnormal translational pathway promotes tumour development and cellular changes. This pathway is sensitive to cellular conditions such as nutrition availability, energy or stress, production of the ribosome and gene expressions in non-cancerous cells, but it is hyper-activated and pro-oncogenic in malignant cells (Ali et al. 2017). Furthermore, investigations conducted over the last two decades have revealed that a number of eukaryotic initiation factors (increased expression levels of eIF4A, eIF4E and eIF4G, alone with low expression of 4E-BPs or phosphorylation of eIF2) have been reported in various types of malignancies (Ali et al.

2017; Hao et al. 2020). These in turn result in the selective translation of mRNA encoding proteins involved in carcinogenesis, metastasis, or drug resistance, making eIFs a prospective therapeutic target for a variety of malignancies (Hao et al. 2020). There are a number of eIFs which are misregulated in human cancer. For instance, elF1, eIF1A, eIF2, eIF5A, eIF6, eIF2B, eIF3 and eIF4F are found to be dysregulated in most of the malignancies (Hao et al. 2020; Ali et al. 2017).

16.3.3.2 eIF Transcription Factor in Regeneration

Eukaryotic initiation factor is another important regulator during the cell cycle, cell proliferation and apoptosis. It binds to the 5′ terminal 7-methyl GTP cap of DNA transcript and affects the rate of translation. This factor is crucial for the cell cycle to progress and when overexpressed, it becomes oncogenic. In quiescent cells, eIF-4E binds to eIF-4F to act as translational suppressor (Wilkinson and Millar 2000). Under normal conditions, eIF-4E binds to eIF-4G, a translation initiation complex. When it is activated by mitogenic stimulators, it becomes phosphorylated, and it interferes with many pathways such as Ras, PI3K-AKT. In optimal conditions eIF-4E is responsible for angiogenesis, an important factor for cell proliferation (De Benedetti and Graff 2004).

16.3.4 Caspases

Caspases are cysteine-aspartic proteases which are present in an inactive form in the cells and are majorly involved in controlling apoptosis and inflammation (Shalini et al. 2015; Yadav et al. 2021). It is split into two groups—group I are inflammatory caspases which consist of caspase-1, -4, -5, -11 and -12, and group II are apoptotic caspases (Olsson and Zhivotovsky 2011; Yadav et al. 2021). Based on various structures and functions, apoptotic caspases are further separated into: (1) caspase initiators (caspase-2, -8, -9 and -10) and (2) caspase effectors (caspase-3, -6 and -7) (Yadav et al. 2021; Hounsell and Fan 2021). These enzymes are synthesized as inactive zymogens that gain catalytic activity after signalling events that encourage their aggregation into dimers or macromolecular complexes (McIlwain et al. 2013).

16.3.4.1 Caspases in Cancer

Apoptosis evasion is thought to be one of the characteristics of human malignancies. Caspases are responsible for cell death, and various upstream regulatory factors that control their proteolytic activity have been categorized as suppressor of tumour or oncogenic (Olsson and Zhivotovsky 2011). Caspases are implicated in a variety of human diseases, including cancer and inflammatory disorders, and considerable attempts to better understand how these enzymes work and might be managed are underway (McIlwain et al. 2013). Deregulation of caspase results in abnormal apoptosis which eventually leads to decreased apoptosis, irregular growth of cancer cells, and carcinogenesis (Farghadani and Naidu 2021). The dysregulation of caspase can occur via different mechanisms which include inhibition by apoptotic protein, dimerization blockage of initiator caspases etc. (Boice and Bouchier-Hayes

2020). The deficiency of initiator apoptosis has been linked to the formation and progression of cancer. Majorly, altered expression of caspase-8, -9 and -10 is known to be involved in the progress of carcinogenesis (Farghadani and Naidu 2021). In addition to this, studies have reported the dysregulation of single or multiple caspases which contributes to tumour cell growth and development. For instance, in stage II colorectal cancer, caspase-9 is found to be downregulated, whereas, in choriocarcinoma, caspase-8 and -10 are found to be downregulated (Wong 2011).

16.3.4.2 Caspases in Regeneration

Apoptosis is one of the most crucial steps for cell cycle regulation, executed by caspases. Caspases are triggered when they receive signals such as DNA damage, viral infection, loss of cell adhesion and other cellular insults (Bergmann and Steller 2010). Although their major role lies in regulating apoptosis, a few members of caspases family, like caspases-3, play an important role in cell differentiation. The caspase-3 has no role in cell death. Caspase-2 and -8 play roles in cell proliferation (tumorigenesis also). The differentiation of muscle progenitors into myotubes occurs in the presence of caspase-3 and caspase-9. But it is limited and regulated by Bcl-xL, an apoptosis inhibiting protein. The osteogenic differentiation of MSCs also happens in the presence of caspase-3, absence of which leads to decreased bone density. Caspase-3 also plays a major role in differentiating embryonic and haematopoietic stem cells; macrophage differentiation and absence of the same lead to improper differentiation. Caspase-3 and -7 are involved in liver regeneration and wound healing. They target iPLA2 (phospholipid) activity and cleave it, for the secretion of prostaglandins. These prostaglandins in turn trigger stem cell proliferation, tissue regeneration and repair. They also target the WNT (Wingless-related integration site) pathway and regulate, usually by paracrine functioning. Caspase-8 is also involved in liver regeneration, but it interferes with the NFkB (nuclear factor kappa B) pathway (Shalini et al. 2015).

16.3.5 mTOR

16.3.5.1 mTOR in Cancer

The mammalian target of the rapamycin (mTOR) pathway is composed of two complexes—(1) mammalian target of rapamycin complex 1 (mTORC1), and (2) mammalian target of rapamycin complex 2 (mTORC2). Furthermore, the function of mTORC1 is to promote cell metabolism and growth, while the role of mTORC2 is to control cell division and survival (Zou et al. 2020). The mTOR pathway has been found to have a close link with cancer as it is known to involve in cell proliferation, survival, growth, glucose metabolism, protein synthesis, autophagy and apoptosis (Tian et al. 2019). Moreover, mTOR, in healthy conditions, is a principal controller for the division of cells, whereas under cancer conditions, activated mTOR signals encourage cancer cells to expand, spread and infiltrate healthy tissues (Zou et al. 2020). Also, mTOR is a commonly affected pathway in the case of human cancer and is recognized to have a fundamental part in cancer

development and progression (Tian et al. 2019). The altered level of mTOR is seen to be related to a higher development of tumour and metastasis and generally occurs due to the following mechanism—(1) mTOR gene mutation that leads to a hyperactive mTOR signalling cascade, (2) mTORC1 and mTORC2 mutation leads to dysregulated mTOR signalling and (3) upstream gene mutation can also lead to deregulated mTOR pathway (Tian et al. 2019; Hua et al. 2019). Meanwhile, various mTOR antagonists have been discovered for cancer treatment, and a range of medicines have been demonstrated to have high activity when paired with mTOR antagonists (Zou et al. 2020; Hua et al. 2019). Hence, certain mTOR antagonists have already been certified for the treatment of cancer, whereas others are still being studied in clinical studies (Hua et al. 2019).

16.3.5.2 mTOR in Regeneration

Mammalian target of rapamycin (mTOR) is a member kinase of PI3K-PKC family (phosphoinositide 3-kinase/protein kinase C associated kinase). mTOR has two units—mTORC1 and mTORC2 where the former mTORC1sub-unit is one of the essential components for helping cells to recoup tissue damage by reviving homeostasis. Dysregulations and disruptions in mTOR can cause diseases like cancer, organ dysfunction and degenerative disorders. WNT signalling and TNFα trigger the activation of mTORC1 which then suppresses autophagy and lysosome biogenesis and promotes cell growth, metabolism in regulation with environmental factors and growth factors. mTORC2 regulates cell migration and cytoskeleton remodelling to enhance cell survival and proliferation.

mTOR promotes axial regeneration by inactivation of pTEN and suppressing cytokine signalling 3, which are negative regulators of mTOR. Pathways like JAK-STAT and JNK-MAPK also promote axial regrowth during neuroregeneration. By activating mTOR, astrocytes are inhibited, which in turn leads to promotion of retinal ganglion cells (Wei et al. 2019). Mutations in TSC genes usually cause pathway disruption for mTOR and further lead to cancer. Pathway disruption from RAS-MAPK to inhibit TCS is also shown as an obvious reason for cancer (Guertin and Sabatini 2007).

16.4 Genes Regulating Cell Division

16.4.1 p53

16.4.1.1 p53 in Cancer

Cells respond to various stress signals, such as oncogene activation, DNA damage, hypoxia and reactive oxygen species (ROS), by expressing the tumour suppressor *p53*. *p53* activates a wide range of responses within the cell, from cell cycle arrest, senescence, or ferroptosis to eliminate unrecoverable cells. Therefore, *p53* is supposed to act as the 'Guardian of the genome' by preventing the accumulation of mutations that cause cancer (Lane 1992; Levine 2020). Cancers with *p53* mutations develop in an initial stage or a late stage depending on the type of cancer and strongly

facilitate onset or progression (Levine et al. 2019). As a consequence of *p53* mutations, the remaining wild-type allele of TP53 is usually lost. This results in the loss of wild-type *p53* in late-stage cancers, which further confers a selective advantage to these cancer cells (Song et al. 2007; Amelio and Melino 2020). Many *p53* mutations alter the central DNA-binding domain, and a few points of particular interest have thus far been identified: R175, G245, R248, R249, R273 and R282 (Duffy et al. 2020). Studies have revealed an association between immune checkpoints and *p53*. The program-death receptor PD-1 (PD-1) and programmed death-ligand 1 (PD-L1) are activated by *p53* when cancerous cells and normal T cells are stressed (Thiem et al. 2019). Immune checkpoints such as PD-L1 on tissue and PD-1 on T cells act together to reduce inflammation by reducing the activation signals from T cells after antigen recognition. Utilizing this immune checkpoint mechanism, tumour PD-L1 amplification fosters tumour surveillance and immune tolerance (Alsaab et al. 2017). Thus, mutant *p53* may be useful as a biomarker for the response to immunotherapy in certain contexts and may correlate with better prognosis due to distinct immunogenic signals (Liu et al. 2019). p53-TLR regulatory axis is unique to humans and apes. In consideration of TLR-mediated cancer treatment, this evolutionary gap is significant since mouse models do not mimic the regulatory axis present in humans. Mutations of the *p53* gene not only alter TLR gene expression, but also have other consequences. These alterations modulate TLR3 sensitivity and reactivity to known ligands, resulting in downstream apoptosis (genotoxic stress) (Pradere et al. 2014). P151H and R337H, two transcriptionally active or TLR3-enhancing *p53* mutants that control TLR3 responsiveness, might inhibit TLR3-mediated immune responses, while other *p53* mutations may increase their expression (Taura et al. 2010). Several cancers can be affected by overexpression of microRNA, and another mutant-specific GOF of *p53* may be relevant. Because exosomal-mediated transfer of microRNA is crucial to many kinds of cancer, a second mutant-specific GOF of *p53* can also be important (Mantovani et al. 2019). MicroRNA transfer via exosomes can induce similarly phenotypic changes in macrophages in tumours expressing mutant *p53*. Furthermore, these reprogrammed macrophages increased degradation of the extracellular matrix and were more invasive in comparison to macrophages that were introduced to nonmutated tumour cells (Cooks et al. 2018).

16.4.1.2 p53 in Regeneration

p53 is one of the tumour suppressor genes; dysregulation of which is involved in many cancers (Duronio and Xiong 2013). *p53* acts as a coordinator to promote regeneration and inhibit tumour. It is therefore regarded as the guardian of the genome. During studies, *p53* was observed to contribute majorly in many cellular processes and also it was seen to decide the outcome of cascade of events, pointing towards its role in regeneration. In a normal cell, *p53* and its isoforms trigger regeneration after injury. Here, p53 triggers mitogenic growth factors, promoting cell division. It controls and allows the blastema of differentiated cells for their re-entry into the cell cycle. In progressive stages of wound healing, *p53* was found to suppress growth factors and downregulate cell division. It was concluded through

studies that *p53* regulates the pliability of differentiated cells and their fate during regeneration. *p53* controls cell apoptosis by transactivation of Noxa, an apoptotic protein, and regulates cell cycle arrest by interfering in MAPK and JNK signalling pathways. It can trigger regeneration only under homeostatic conditions especially in the nervous system. Mutations, cellular insults and telomeric shortening for this particular gene alone cause more than 50% of the cancers in humans (Charni et al. 2017). Recent observations have shown that p53 can also act as a cancer inhibitor. When the transcript of *p53* binds at a certain hydrophobic site of MDM2 protein, it results in protein–protein interactions that occur in the domain that inhibits cancer. As one of the strategies to prevent cancer because of *p53*, it can be inactivated by dysregulating the DNA-binding function through point mutations or other strategies. p53-DNA binding domain (DBD) is very flexible which can direct towards partial unfolding of p53 even at slightest changes of normal body temperature range, pointing towards mutation. Ligands which bind to BDB can remodel the protein folding into normalcy and stabilize it (Selivanova 2010).

16.4.2 p21

16.4.2.1 p21 in Cancer

Molecular studies of knockout mice and biochemical and functional analysis of cultured cells have been crucial to understanding the role of p21 in cancer. Initially, *p21* was found to be a potential mediator of p53's tumour suppressor activity, leading to ground-breaking research (El-Deiry et al. 1993). Further work showed that, despite eliminating DNA damage-induced and p53-dependent growth arrest, deletion of Cdkn1a did not affect p53-dependent apoptosis. Because of this, p21 can't explain the entire spectrum of p53's tumour suppressor activity (Efeyan et al. 2007). Despite that, p21 is a major determinant of tumour protection by p53, since cysteine methylation abolished the ability of p53 to induce apoptosis but preserved partial growth arrest in mice expressing a mutant form of *p53* (Trp53R172P+/+) (Barboza et al. 2006). An important insight into the role of p21 in tumour suppression was provided by a study by Shen et al. 154 that showed the existence of a prominent tumour suppressor role for this gene in genomically unstable tumours. Ataxia-telangiectasia mutated was induced by a Cdkn1a deficiency in conjunction with DNA damage checkpoint protein loss, resulting in aneuploidy prior to tumour development (Shen et al. 2005). There is an inverse correlation between *p21* downregulation in colorectal cancer and microsatellite instability regardless of *p53* status. This supports the notion that the loss of genomic stability protection by *p21* contributes to the development of human malignancies (Edmonston et al. 2000; Minucci et al. 2002). Several mouse genetic studies have also revealed that *p21* may act as an oncogene, as shown by a study that demonstrated deletion of Cdkn1a suppressed the development of spontaneous lymphomas in Trp53−/− and Atm−/− mice, as well as radiation-induced lymphomas in nuclear-type 138 and Trp53−/− mice (De la Cueva et al. 2006; Wang et al. 1997). Cdkn1a−/− mice develop lymphomas demonstrating a high level of apoptosis, suggesting that *p21*'s

anti-apoptotic activity promotes tumour growth. Apoptosis can be suppressed by *p21* in lymphocytes, but it is not clear why this activity can only be found in lymphomas. In the absence of *p21*, restricted tumour formation in the absence of cellular differentiation may result from blockage of proliferation at a phase when cells are not capable of proliferating. It has also been shown that *p21* can also promote oncogenesis without inhibiting its anti-apoptotic activity by promoting the assembly of complexes of cyclin D with CDK4 or CDK6, yet independent of its anti-apoptotic activity. p21 can also promote tumour growth independently of its antiapoptotic activity by promoting the assembly of complexes between cyclin D and CDK4 or CDK6 without negatively affecting their kinase activity (LaBaer et al. 1997).

16.4.2.2 p21 in Regeneration

p21 is involved majorly in cell cycle exit during development (Duronio and Xiong 2013). It is also observed to play a role in various cell responses for DNA damage, cytokine activities, cellular insults like oxidative stress. It is to be noted that *p21* is regulated by *p53* for tumour suppression activity and also inhibits progression of cell cycle. Another major role of *p21* is to inhibit the expression of Cdk, because of which, there is a block in the proliferation, by preventing phosphorylation, which in turns leave Rb not phosphorylated (Arthur and Heber-Katz 2011). With the understanding that p53 regulates p21, inhibitory molecules targeting sequence of *p21* were identified. A sequence of *Cdkn1a* gene was bound to p53 and was targeted to regulate 21. Though results proved to be positive, more work has to be done on this for better understanding (El-Deiry 2016).

16.4.3 Yamanaka Factors

The Yamanaka cocktail, Nobel prize–winning concept, was described by Kazutoshi Takahashi and Shinya Yamanaka. It states that adult fibroblast cells can be reprogrammed into embryonic-like states by triggering four factors—Oct-3/4, Sox2, c-Myc and Klf4. They express marker genes of embryonic stem cells as well (Takahashi and Yamanaka 2006).

The first wave is led by c-MYC/Kruppel-like factor, while the second is propelled by OCT4/SOX2/KLF4. OCT3/4, SOX2, KLF4 and c-MYC are the four transcriptional factors that make up OSKM. Overexpression of these four major transcriptional factors induces pluripotent qualities in somatic cells by modulating the signalling network required for pluripotent features in somatic cells (Takahashi and Yamanaka 2006; Huang et al. 2009; Holczbauer et al. 2013; Fiorenza and Rava 2019).

16.4.3.1 Yamanaka Factors in Cancer

Yamanaka factors reprogramme somatic cells into pluripotent cells in two phases. After the first discovery of Yamanaka factor reprogramming in mouse embryonic fibroblasts by retroviral transduction, a number of changes were made, and different

delivery techniques were tried to improve the process' sensitivity. OCT4 is a transcription factor that has been linked to CSC stemness and has been used as a biomarker in breast, colon, oral and lung cancer. Self-renewal potential, chemoresistance and a poor prognosis are also caused by it (Roy et al. 2019). Overexpression of *Sox2* has been linked to squamous cell cancer and has the ability to regulate self-renewal in embryonic stem cells (Islam et al. 2015). KLF4 is a CSC marker that is involved in a variety of tumour-related activities and has a controversial role in cancer aetiology. It is both a tumour suppressor and an oncogene, and it is required for the survival of CSCs (Roy et al. 2019). When a subset of Yamanaka factors, OCT4 and KLF4, as well as NANOG, are overexpressed in a subpopulation of radioactively challenged cancer cells, cancer stem-like cells are generated (Lagadec et al. 2012). KLF4 was discovered to be increased in cancer cells, and knocking it out inhibited tumour growth (Yu et al. 2011). According to Wolfer et al. (2010), c-MYC can aid in the preservation of cancer stemness, allowing cancer cells to survive for longer periods of time. Misregulation of c-MYC can lead to abnormal cell behaviour and accelerate the stemness process in cancer cells. CSCs are regressed, and tumour cells' apoptotic behaviour is increased when *c-Myc* expression is knocked down or silenced. If c-MYC is reactivated, the tumour may relapse. Even in the absence of c-MYC, further mutations can accumulate and aid in the self-renewal capacity of CSCs (Wang et al. 2008). Overexpression of *klf4* in the xenograft model results in higher levels of both KLF4 and EpCAM proteins, according to Firtina Karagonlar et al. (2020). Overexpression of KLF4 in the Huh 7 cell line results in considerable protein binding to the EpCAM promoter, upregulating EpCAM and E-Cadherin expression and increasing hepatic CSCs. Circular RNA was found to be important in the CSC-rich hepatoblastoma microenvironment in another investigation. Circular RNA suppresses miR-7-5p and decreases its sponge activity on KLF4, upregulating KLF4 expression and liver CSCs as a result (Chen et al. 2020).

16.4.3.2 Yamanaka Cocktail in Regeneration

Oct4, Sox2 and *NANOG* are the core components of the pluripotency circuitry. The octamer binding transcription factor 4, *Oct-4*, is one of the important genes that are present in the primordial germline cells. One of the unique properties of *Oct-4* is that it can trigger itself with the help of transcription factor Sox2. This transcription factor, *Oct-4*, has a POU domain which can interact at different sites to determine the pluripotency factor. Reduction on *Oct-4* and *Nanog* expressions leads to reduction in pluripotency and vice versa. The expression of *Nanog* is regulated by *Oct-4*, and the triggering of *Sox2* is sometimes required for *Oct-Sox* enhancers. The *Oct-4* is hence called the master switch for totipotency-pluripotency in the lifespan of humans. It is not only expressed in embryonic conditions but also expressed in adults. It is observed in the kidneys, the pancreas, the endometrium, the thyroid, the brain, the skin, and the peripheral blood. Here, *Oct-4* expression is mandatory for maintaining self-renewal property in somatic cells and maintaining homeostasis in the cells (Wu and Schöler 2014).

Sex determining region Y-box2, Sox2, is a prerequisite for the formation of various tissues and organs during the development of an embryo. Its major function is to maintain pluripotency and self-renewal of embryonic stem cells. It regulates master transcription factors, miRNA, signalling pathways without intricacy and dysregulations lead to cancer (Feng and Wen 2015, p. 2). It means that overexpression can affect cell proliferation, invasion, apoptosis and metastasis. It plays a role in pathways like WNT, PI3K, JAK-STAT3 and is shown to function in the stemness factor of adults as well as embryonic stem cells. This gene also contributes by playing an important role in deciding cell fate of pluripotent stem cells. It plays an important role in the final phase of reprogramming major fate decisions in induced pluripotent stem cells (Kretsovali 2017, p. 2).

c-Myc is a proto-oncogene which monitors and co-ordinates a variety of cellular processes like angiogenesis, apoptosis, cell growth and cell differentiation. *C-Myc*, in association with Max, forms dimers and regulates its target genes. One set of genes is triggered and activated by Myc/Max through promoters. *C-Myc* is very important for cell-lineage committed haematopoietic cell proliferation and can create equilibrium between self-renewal of cells and their differentiation (Wilson et al. 2004). By targeting G-quadruplexes in the promoter, Myc/max dimerization is prevented, leading to control of cell proliferation. This strategy is also being used and being studied further to inhibit cancer (Chen et al. 2014).

Krüppel-like factor 4, Klf4, controls cell proliferation and neural stem cell differentiation. This blocks the STAT3 pathway and contributes to regenerate the injured ganglion cells. It majorly phosphorylates STAT3 by cytokine signalling (Qin et al. 2013). Klf4 can also inhibit the proliferation in the cell cycle by inhibiting cyclins and upregulating p21 expression. This can result in loss of proliferation (Moore et al. 2011).

16.4.4 NANOG

16.4.4.1 NANOG in Cancer

NANOG is a diverse homeobox domain protein that plays a canonical role in transcriptional regulation of pluripotency and self-renewal in embryonic stem cells (ESCs). The expression of NANOG is highest in pluripotent cells, such as embryonic stem cells (ESCs), and embryonic germ cells (EGs) as well as embryonic carcinoma cells (ECs) (Chambers et al. 2003; Mitsui et al. 2003). Immunohistochemistry (IHC) in a variety of primary human tumour samples demonstrates that NANOG protein is expressed in both the nucleus and cytoplasm. There is evidence that NANOG is involved in tumour development or progression when present in neoplastic cells. Several studies suggest that NANOG influences CSC properties by imbuing subsets of cancer cells with self-renewal potential, which ensures the immortality of the entire tumour population. The NANOG mRNA and protein are found in many CSC subpopulations, including CD44+ breast cancer cell and prostate cancer cell, as well as CD133+ prostate, brain cancer and ovarian cancer cell, as well as CD24+ liver cancer cells. It is of clinical significance that higher

NANOG expression has often been linked to worse outcomes in tumours of the epithelium (Bourguignon et al. 2012; Zbinden et al. 2010). The second effect of forced *NANOG* expression is to promote the accumulation of breast cancer cells that are CD133+ and AldeFLUOR+ (Jeter et al. 2011). The RNAi-mediated NANOG knockdown, on the other hand, leads to reduced CSC properties such as sphere formation, which promotes cancer cell proliferation (Jeter et al. 2009). Immunity is another critical hurdle cancer cells must overcome. It is purported that cancer cells expressing *NANOG* can evade the immune system. NANOG generated by hypoxia protects against tumour cell killing by cytotoxic T lymphocytes, possibly by way of signal transducer and activator of transcription 3 (STAT3). A *NANOG* knockdown rendered xenograft tumours susceptible to immune surveillance when exposed to vaccine-induced evolution and immunity evasion of human papillomavirus cervical cancer cells, as the knockdown of *NANOG* reduced the growth of TC-1 cells (Hasmim et al. 2011). Proliferation of cancer cells appears to be positively correlated with NANOG levels. Increased proliferation is a hallmark of neoplastic disease despite not yet knowing whether this phenomenon is linked directly to a cell's fate (as in ESCs). Knockdown of NANOG reduced the proliferation, invasion, migration and apoptosis of human gastric cancer cells in conjunction with cell cycle arrest in the S phase during cell cycle progression (Ji and Jiang 2013).

16.4.4.2 Nanog in Regeneration

Although, in cancer, there are many downstream pathways which *NANOG* regulates, the role of *NANOG* in regeneration is majorly concerned with post-translational modification of proteins and interferes with transcriptional activity. It regulates DNA binding and sub-cellular localization as well as protein stability. *NANOG* re-activates transcription along with Oct4. Although *Oct4* and *Sox2* are stable, *NANOG* fluctuates extensively in stem cells during embryonic conditions. Also, *NANOG* requires OCT4 and SOX2 for its upregulation. *NANOG*, along with its 11 pseudo genes, participates in regulating the modifications of pluripotency and stem-cell renewal, primarily through phosphorylation (Saunders et al. 2013).

16.4.5 Hox Genes

16.4.5.1 HOX Genes in Cancer

Studies of HOX upregulation have been reported in various HOX-related fields, including leukaemia, where haplotype translocations or altered regulation lead to the malignancy known as trithorax homologue myeloid lymphoid leukaemia. A majority of the studies that have looked at invasion and metastasis caused by abnormal *HOX* gene expression have focused on solid tumours and have found that mutations in upstream regulators or loss of function mutations usually result in *HOX* gene deregulation (Del Bene and Wittbrodt 2005). Apoptosis and proliferation of cancer cells are also affected by HOX molecules, which regulate cell cycle-related proteins. Numerous studies indicate that many HOX transcription factors are abnormally expressed in cancer, and that these abnormalities contribute to tumour invasion

and metastasis. Inhibition of ZEB1 by HOXD9 suppresses HCC cell migration, invasion, as well as EMT in part by interacting with the zinc-finger Ebox binding homeobox (ZEB)-1 promoter region (Lv et al. 2015). In epithelial ovarian cancer cells, HOXA9 induces intraperitoneal dissemination by activating transcription of the *cadherin3* gene encoded by P-cadherin within the enhancer region of the gene encoding growth factor b (TGFb)-2 (Ko et al. 2012; Quéré et al. 2011). Studies have suggested HOXA9 deregulation in the temporal and spatial domains is related to primary tumours and specific histological subtypes. HOXA9 has therapeutic potential; however, its effectiveness is restricted by low membrane permeability. HOXC10 upregulates VEGFA transcription by attaching to its promoter via protein arginine methyltransferase 5 and WD repeat domain 5. These mechanisms govern the posttranslational modification of histones to promote angiogenesis (Tan et al. 2018). HOXA5 boosts Akt1 mRNA and protein expression, as well as Akt activity, through coordinating the downregulation of *PTEN*, enhancing the capsular patellar junction's stability (Feng et al. 2017). In malignancies, such as myeloid leukaemia, HCC, breast cancer and lung cancer, HOX proteins are involved in treatment resistance. HOX proteins influence cancer cell treatment resistance by regulating a variety of non-coding RNAs (ncRNAs). HOXB13 is shown to mediate chemotherapy resistance in lung cancer as an example. Through direct binding to promoters of drugs-transfer and drug-resistance-related genes, the cisplatin-HOXB13-ABCG1/EZH2/Slug network, which includes ATP Binding Cassette Subfamily G Member 1, Enhancer of Zeste 2 Polycomb Repressive Complex 2, and Slug, upregulates several genes associated with drug resistance and drug-transfer (Barresi et al. 2016; Zhan et al. 2019). In cancer, posttranslational changes of HOX proteins primarily affect protein stability, DNA binding, transcriptional activator-like effector interaction, transcriptional activation capability and unexplained cellular effects (Yu et al. 2020). The molecule networks IGF1-HOXA13-ACLY/IGF1R and CXCL12-HOXB5-CXCR4/ITGB3, which target blocking the downstream protein with small molecular drugs, are promising anticancer therapies in colorectal cancer (Qiao et al. 2021). HOXA4, HOXA9 and HOXD10 expression alterations promote aberrant colorectal cancer cell proliferation and differentiation, which contributes to tumour growth (Zhou et al. 2018; Mohr et al. 2017; Miller et al. 2018; Cheng et al. 2018). The discovery of chemical compounds that target downstream molecules has increased the possibility to precisely target the *HOX* genes intermediated network and prevent cancer development, thereby improving cancer clinical outcomes.

16.4.5.2 HOX Genes in Regeneration

Homeobox genes or HOX genes are required for embryonic development, regulating cell fate and repair of the adult body. They decide the pattern of each region of the body through Hox code. Hox genes are majorly responsible for embryonic and adult stages of life during stem cell differentiation (Seifert 2015). They can be programmed to make or change any tissue through a proper pattern, but it cannot be erased or change a fixed pattern, thus making it a limiting factor in regeneration. On the other hand, epigenetic mechanisms of transcripts of HOX make it stronger for tissue homeostasis and cell plasticity. The retention of a particular position in adult

cells for regeneration holds good for homeostasis but does not hold good for cell plasticity. This is one of the reasons for the loss of regenerative ability in humans and other mammals. In order to retain plasticity, organisms must be able to undergo changes in its polarity through specific pathways. Modulation of the WNT pathway and silencing of β-catenin results in regeneration, although it could lead to inappropriate polarity. It is essential for the organism to remember the position of the injured tissue, missing structures of the wound and understanding what type of cells, like totipotent/somatic, must be placed in those wounded sites. These functions are done by HOX genes (Wang et al. 2009). HOX genes also play a role in apoptosis, when they are expressed in normal conditions. But during an abnormal condition, when they are overexpressed, they cause abnormal development and malignancies (Shah and Sukumar 2010).

16.5 Pathways Regulating Cell Division

16.5.1 Hippo Pathway

The Hippo pathway is a revolutionary preserved pathway present in higher-level vertebrates that facilitate vital target genes in a variety of biological functions, including differentiation, cellular proliferation, cellular fate determination, survival, organ size and tissue homeostasis (Calses et al. 2019; Harvey et al. 2013). Fundamentally, it consists of MST1/2 (mammalian Ste20-like kinases 1/2), LATS1/2 (large tumour suppressor 1/2), YAP (yes associated protein) and/or its paralog TAZ (transcriptional coactivator with PDZ binding motif, also called as WWTR1 (WW domain-containing transcription regulator)) (Han 2019). The Hippo pathway mechanism involves the initiation of the Hippo kinase cascade via MST1/2 automatic phosphorylation. Furthermore, with the aid of MOB1A/B, NF2 and SAV1, active MST1/2 phosphorylates LATS1/2, which then phosphorylates YAP/TAZ. Finally, it causes the cytoplasmic retention of 14-3-3 and the destruction of YAP/TAZ by SCF. YAP/TAZ is a transcriptional coactivator that interacts with TEAD to control gene transcription (Yang et al. 2021).

16.5.1.1 Hippo Pathway in Cancer

The Hippo pathway modulates a variety of cellular activities important in cancer, such as proliferation and apoptosis (Harvey et al. 2013). The activity of the Hippo pathway has been reported to be regularly dysregulated in a broad range of cancers in humans including NSCLC (non-small-cell lung carcinoma), breast carcinoma, gastric carcinoma, hepatocellular carcinoma, renal cell carcinoma, colorectal carcinoma, osteosarcoma, rhabdomyosarcoma and angiosarcoma (Harvey et al. 2013; Han 2019). Furthermore, in human tumours, the level of components of the Hippo pathway is commonly changed, which is related to worse prognosis and shorter patient survival (Nakatani et al. 2017). According to studies, the dysregulation of YAP/TAZ and TEAD can enhance cancer (Han 2019). But the mutations within the

pathway are relatively rare and present in <10% of cancer cases (Cunningham and Hansen 2022).

In addition to this, Hippo pathway dysregulation in human tumours could be caused by molecular processes such as susceptibility to tumour mechanical characteristics and interaction with other tumour pathways (Harvey et al. 2013). Studies over the last two decades have revealed that the Hippo pathway has both tumour-suppressing and tumour-promoting functions. Studies have shown that YAP/TAZ and LATS have tumour-suppression and tumour-promoting functions, respectively, in several types of cancer, including haematological cancer, colorectal malignancies and ERα positive breast tumours. Moreover, YAP/TAZ activity in the tumour environment, such as immune cells and peritumoral cells, can influence tumour development, and their effect differs from that of YAP/TAZ activity in cancerous cells. As a result, a complex link has formed between the Hippo signalling pathway and cancer growth (Li and Guan 2021).

16.5.1.2 Hippo Pathway in Regeneration

This pathway is essential in development, homeostasis and also in regeneration of most organs of the body. It controls cell function, cell size and cell proliferation, and is also a crucial pathway for the suppression of tumour. G-protein coupled receptors play a vital role in regulation of subcellular localization of YAP and TAZ. Both YAP and TAZ are essential for defining cell structure, shape and polarity. During the embryonic stem cells phase, YAP promotes pluripotency by binding directly to the target. When YAP expression is enhanced in the embryonic stage, cells turn to be pluripotent whereas when YAP is inhibited, cells lose pluripotency. Also, studies show that YAP is increased when cells are being reprogrammed. Both YAP and TAZ are overproduced in most cancers, resistant to apoptosis. With small molecule modulators and inhibition of YAP and TAZ, suppression of cancer can be strategized. Defects in Hippo directly affect YAP and TAZ which also contribute to cancer initiation and prognosis (Johnson and Halder 2014).

16.5.2 JAK-STAT Pathway

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is the highly preserved signalling pathway present in a wide range of species that are involved in an array of cytokines and growth factors. This pathway is important for cell differentiation, proliferation, survival and embryology (Luo and Balko 2019; Bousoik and Montazeri Aliabadi 2018). Furthermore, this route transports extracellular signals to the cell and begins the gene transcription involved in the proliferation and differentiation of the cell by a sequence of phosphorylation cascades. The JAK/STAT pathway is mainly involved in cytokine signalling, which includes erythropoietin, thrombopoietin, interferons, interleukins and granulocyte-colony stimulating factors (Luo and Balko 2019). In addition to this, the family of JAK has four members (JAK1, JAK2, JAK3 and TYK2), each with 7 preserved JAK homology domains (JH1–7); meanwhile, the family of STAT has 7 members

(STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) (Pencik et al. 2016). The OG (oligomerization), CC (coiled-coil), DB (DNA-binding), LK (linker), SH2, phosphotyrosine tail (Y) and transcriptional activation (TA) domain are all found in STAT family members (Lim and Cao 2006). The downregulated controllers of the pathway include (1) tyrosine phosphatases (SHP1 and SHP2), which dephosphorylate JAKs, (2) suppressors of cytokine signalling (SOCS), which compete with STAT binding to the cytokine receptor and (3) the PIAS family, which obstructs with STAT binding to DNA. The downregulated regulators of the pathway suppress the JAK/STAT pathway by turning it off, hence modulating the amplitude and temporal regulation of pathway signalling (Luo and Balko 2019).

Janus kinase (JAKs) are members of non-receptor tyrosine kinases. Downstream to JAKs, signal transducer and activator of transcription (STATs) are present. They are activated together as a receptor when a ligand, such as a cytokine, binds to the site of the reception. When a receptor activates, associated JAKs are phosphorylated for specific residues. Activated JAKs then phosphorylate and select STATs. Activated STATs disintegrate from the receptor and dimerize to move into the nucleus where transcription factors regulate the target genes. This pathway is known to participate in cell proliferation, differentiation and haematopoiesis. This is one of the stable stress-response pathways that promote injury-induced apoptosis and compensatory proliferation. Activation of transcription genes due to tissue damage is associated with compensatory proliferation (La Fortezza et al. 2016).

16.5.2.1 JAK-STAT Pathway in Cancer

JAK/STAT signalling pathway is a critical component of cancer development, either as a tumour-specific driver of cancer growth and metastasis or a mediator of immune surveillance (Brooks and Putoczki 2020). This pathway is part of 12 other cancer pathways (Vogelstein et al. 2013). JAKs usually engage the receptor of tyrosine and then go into a dormant state until the binding of ligand occurs. Because of genetic abnormalities or polymorphisms, improper activation of the JAK/STAT system results in chronic activation of JAKs in the absence of cytokine signalling, which can lead to cancer or carcinogenic activity. Furthermore, while this mechanism is involved in many cellular processes relevant to cancer, each of the JAK/STAT pathway components can be activated in different ways in different forms of cancer, and the molecular and cellular environment can have diverse consequences on cancer characteristics. Moreover, whereas the importance of the JAK-STAT signalling mechanism in haematological malignancies is very well known, there is growing recognition of its role in solid tumour development (Luo and Balko 2019). JAK-STAT dysregulation has been linked to loss- or gain-of-function in certain malignancies, resulting in alterations that can begin and promote carcinogenesis (Villarino et al. 2017). Abnormal JAKs can stay active, resulting in uncontrollable transduction of the JAK/STAT mechanism. Conversely, numerous mutations in *STAT*s, including *Stat1*, *Stat3* and *Stat5*, have been discovered. Diverse haematological malignancies have been linked to somatic mutations in *Stat3*. CD4+ T-LGL oncogenesis has been associated with Stat5b mutations, with abnormal CD4+ T cell proliferation being observed in more than 55% of patients. The

V617F mutation is linked to a threefold greater risk of early death in myeloproliferative cancer patients as compared to those who do not carry the mutation (Nielsen et al. 2011). While JAK-STAT mutations are less common in solid tumours, the JAK2 V617F mutation has been found in non-small cell lung cancer (NSCLC), with GOF mutations connected to PD-L1 changes in tumour cells (Jeong et al. 2008; Li et al. 2017). Furthermore, LOF mutations in JAK1 and JAK2 have been associated with the decrease of PD-L1 expression in the TME as a result of dampened tumour-inherent IFN signalling in melanoma patients, which may lead to bad patient response to checkpoint inhibitors (Shin et al. 2017). STAT3 and STAT5 expression abnormalities in the TME are linked to tumour growth and dissemination. Upregulation of STAT5 has been related with early recurrence and worse prostate tumour patient survival, in contrary to this, a high level of STAT3 is found to be linked with low patient survival and increased recurrence risk in cancers such as renal cell tumour, glioblastoma, cervical tumour, colorectal tumour and melanoma (Owen et al. 2019).

16.5.2.2 JAK-STAT Pathway in Regeneration

JAKs and STATs are activated together as a receptor when a ligand, such as cytokine, binds to the site of reception. When a receptor activates, associated JAKs are phosphorylated for specific residues. Activated JAKs then phosphorylate and select STATs. STATs, when activated, dissociate from its receptor to move into the nucleus in dimerized form, to the target gene sites where transcription factors are regulated. This pathway is essential for the cell division, their differentiation and haematopoiesis (Jang and Baik 2013). This is one of the stable stress-response pathways that promote injury-induced apoptosis and compensatory proliferation. Activation of transcription genes due to tissue damage is associated with compensatory proliferation (La Fortezza et al. 2016).

16.5.3 PI3K Pathway

16.5.3.1 PI3K Pathway in Cancer

Phosphatidyl-inositol-3-kinases (PI3K) are a family of plasma membrane-associated lipid kinases made up of three subunits: p110 catalytic, p85 regulatory and p55 regulatory. PI3K is divided into three categories based on structural differences and specificity of substrate: classes I, II and III (Yang et al. 2019). Class I PI3Ks are the group of heterodimers that is made up of a four CAT (catalytic) and eight regulatory subunits (Porta et al. 2014, p. 27; Jean and Kiger 2014). Class I is further categorized into two isoforms—class IA and class IB PI3Ks. Class IA PI3K is composed of the p110α, p110β and p110δ catalytic subunits that are produced by distinct genes PIK3CA, PIK3CB and PIK3CD, respectively, whereas class IB PI3K consists of only p110γ catalytic subunit which is generated by PIK3CG (Yang et al. 2019). Furthermore, class II PI3K consists of only catalytic subunits—PI3KC2α, PI3KC2β and PI3KC2γ produced by PIK3C2A, PIK3C2B and PIK3C2G, respectively, whereas class III is composed of catalytic and regulatory subunits synthesized by

PIK3C3 and PIK3R4, respectively (Jean and Kiger 2014). Moreover, activation of Akt/PI3K is done by growth factor receptor protein kinases that eventually results in the regulation of various cell activity (Porta et al. 2014).

In recent years, PI3K has been investigated to be dysregulated in a variety of cancers (Yang et al. 2019). PI3K is known to have a fundamental part in human malignancies as shown by the decreased activity of PTEN and/or mutations of the kinases (Hennessy et al. 2005; Dillon et al. 2007). Nevertheless, PI3K dysregulation has been described by a number of mechanisms, including (1) inhibition of the PTEN, a tumour suppressor, (2) PI3K mutation and (3) stimulation of tyrosine kinase growth factor receptors or oncogenes upstream of PI3K (Aziz et al. 2009; Stemke-Hale et al. 2008; Zhou et al. 2000). Moreover, PI3K is considered as a fundamental therapeutic target for cancer treatment (Yang et al. 2019). PI3K antagonists are mainly classified into—(1) dual PI3K/mTOR inhibitors, (2) pan-PI3K inhibitors and (3) isoform-specific inhibitors (Yang et al. 2019). Additionally, other than PI3Ks, a range of therapies have also been discovered to suppress various proteins in the PI3K/AKT/mTOR pathway such as mTORC1, mTORC2 and AKT. Many of these medications have shown considerable therapeutic improvements in ER+ metastatic breast tumours that have progressed on previous lines of endocrine therapy, with better progression-free survival (PFS) (Nunnery and Mayer 2020).

16.5.3.2 PI3K Pathway in Regeneration

Phosphoinositide-3-kinase (PI3K) is one of the members of the lipid kinase family which phosphorylates 3′ hydroxyl groups of phosphoinositide. It is characterized by PIP3, an essential component for cell growth, cell survival, and cell proliferation (Yuan and Cantley 2008). This is a controlled process, and the pathway involves many steps. A completely activated AK strain transforming mediator (Akt) triggers angiogenesis, metabolism, proliferation, transcription, apoptosis (Hemmings and Restuccia 2012). Various oncogenic genes and tumour suppressor proteins are regulated by the AKT pathway. The proteins and transcription factors in this pathway behave oncogenic when they are either upregulated or downregulated. Most cancers formed in this manner are sporadic (Altomare and Testa 2005).

16.5.4 Ras-Raf-ERK Pathway

16.5.4.1 Ras-Raf-ERK Pathway in Cancer

The ERK is a kinase cascade pathway and one of the well-studied pathways in human cancers (McCubrey et al. 2007). The pathway is initiated either by growth factors or mutation of proteins involved in the pathway; the most common proteins are RAF and RAS. Dysregulation of this pathway is common, and it performs a vital function in malignancies namely pancreatic, melanoma, colorectal and lung cancer (Neuzillet et al. 2014). Targeting these kinases has already been found to be fruitful in cancer treatment (Huang et al. 2017).

16.5.4.2 Ras-Raf-ERK Pathway in Regeneration

This pathway is one of the first kinase cascade pathways to be discovered and characterized in pathways. This pathway is a part of various cellular processes which include proliferation, cell cycle inhibition, terminal differentiation of stem cells and apoptosis. They signal from the cell membrane into the nucleus for many physiological processes such as mitogenic signals and differentiation signals. Raf proteins activate MAP kinase kinases (MAPKK) and MAPK or ERK kinases (MEK) pathways which in turn activate MAPKs and ERKs. These ERKs move into the nucleus and phosphorylate transcription factors to control them. The Ras and Raf are also linked with oncogenes (Peyssonnaux and Eychène 2012).

16.5.5 Wnt Pathway

16.5.5.1 Wnt Signalling Pathway in Cancer

WNT is considered as a crucial signalling pathway that is known to regulate tissue morphogenesis during embryogenesis and repair and has also been firmly linked with cancer (Patel et al. 2019; Sharma et al. 2021). This pathway has been predominantly shown in the case of human colorectal cancer, but an altered WNT signalling pathway has already been reported in other kinds of human tumours namely oral, lung, breast, cervical and haematopoietic malignancies (Zhan et al. 2017). The WNT pathway is a vast network of proteins that coordinates the regulation of molecular processes in a systematic manner. Furthermore, any disturbance in the regulation results in the emergence of various diseases and tumorigenesis (Bugter et al. 2021). Also, the important characteristic of the dysregulated WNT pathway is the integral nuclear localization of β-catenin. Several studies have investigated the mutations in β-catenin's serine and threonine residues that prevent phosphorylation by GSK3 and CK1 kinases, resulting in WNT signalling pathway hyperactivation (Sparks et al. 1998). Moreover, high throughput cancer genomics studies have revealed that the WNT pathway also gets activated by gene mutation, receptors, ligands and intracellular components which include APC, CTNNB1 and AXIN1 (Sharma et al. 2021; Patel et al. 2019). Additionally, the essential components of the WNT pathway, namely, (1) receptor–ligand interface of WNT, (2) destruction complex of β-catenin and (3) transcription complex of TCF/-catenin have been found to be a prospective target for cancer treatment and are now being studied in preclinical and clinical trials. Hence, targeting the WNT/β-catenin pathway has been identified as a possible candidate for customized cancer treatment techniques. To confirm the feasibility, effectiveness, patient stratification and drug delivery of new WNT/β-catenin focused therapies in human cancer, more research is needed (Zhang and Wang 2020).

16.5.5.2 Wnt Signalling Pathway in Regeneration

Wnt are a group of secretory factors, which along with frizzled receptors make a pathway in cells which are majorly involved in cell homeostasis and cell development. It regulates many cellular activities in adult tissues like axis patterning, cell division, cell fate decisions, self-renewal of somatic cells (Jansson et al. 2015). It is

essential in tissue regeneration with signals between adjacent cells (Clevers et al. 2014). Studies also show that signals from the WNT signalling pathway induce epidermal and haematopoietic stem cells development and dysregulation might lead to abnormality in self-renewal of progenitors thus directing towards cancer. Inhibiting the Wnt pathway also showed that uncontrolled cell renewal and proliferation can be interrupted (Reya and Clevers 2005).

16.6 Influence of Microenvironment

16.6.1 DNA Methylation and Epigenetic Regulation in Cancer

To sustain normal growth and development, as well as gene expression in various organs, epigenetic mechanisms are essential. Gene expression and function can be altered by abnormal epigenetic regulation, which can lead to illnesses like cancer. Human tumours are essentially a genetic illness, as a huge number of genes are altered or improperly active during cancer growth. Cancer is caused by a combination of accumulative genetic mutations, epigenetic modifications and environmental influences. Much research has focused on describing the genomic landscape of malignancies, ranging from oncogene-driven signalling pathways to the mutation spectrum in various cancer subtypes. Epigenetic effects, unlike genetic mutations, refer to changing gene expression without causing permanent changes in the genomic sequence. They're used more frequently in cancer cells because epigenetic changes are reversible and easier to control than genomic evolution (Easwaran et al. 2014). DNA methylation is the most well-studied epigenetic mechanism, and it primarily occurs in CpG islands (CGIs), which are found in the 5′ promoter region of more than half of all human genes (Lister et al. 2009). It plays a key role in X chromosome inactivation, embryonic development, genomic imprinting, epigenetic reprogramming, cell identity establishment and lineage specification, among other things. It works by covalently attaching methyl groups from *S*-adenosylmethionine (SAM) to the 5 position of the cytosine pyrimidine ring to silence genes. The 5-methylcytosine (m5C) structure can either restrict transcriptional factors (TFs) from accessing DNA-binding sites or recruit methyl-binding domain proteins (MBDs) to remodel chromatin in conjunction with histone changes, resulting in repressive gene expression (Robertson 2005). The catalysis of DNA methylation is regulated by three DNA methyltransferases (DNMTs), notably DNMT1, DNMT3a and DNMT3b. The maintenance DNA methyltransferase, DNMT1, has a greater catalytic activity and is primarily responsible for maintaining the DNA methylation status by preferentially methylating hemimethylated DNA during replication (Goyal et al. 2006). While 'de novo' methyltransferases DNMT3a and DNMT3b generate and support the precise DNA methylation status in the genome, they both prefer to bind to previously unmethylated DNA regardless of replication (Liang et al. 2002). DNA demethylation, on the other hand, is a reversible process that restores silenced genes damaged by DNMTs. It is catalysed by a family of ten-eleven translocation methylcytosine dioxygenases (e.g. TET1, TET2 and TET3), which can convert 5mC

to 5-hydroxymethylcytosine (5-hmC) and then further oxidise 5-hmC to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (Scourzic et al. 2015; Zhao and Chen 2013). In numerous types of cells, homeostasis between demethylation and methylation of the genome occurs as a dynamic mechanism of gene expression.

16.6.2 Role of DNA Methylation and Epigenetics in Regeneration

Gene expression is largely dependent on one or many of these factors—transcription regulators such as lncRNA, miRNA, DNA modifications and epigenetic code. It was understood through experimentation that because of tissue damage or injury, a change in the transcriptome can be observed. This change is due to epigenetic changes in the new, regenerating tissues. It is observed that the polycomb repressor complex, PRC, is playing a major role in regeneration of skin and intestine, regulates the regeneration when the repressive mark H3K27me3 pattern is expressed on the gene (Zhang et al. 2021).

16.6.3 Role of Extracellular Matrix in Cancer

An extracellular matrix (ECM) is an environment that surrounds a tumour and consists of cellular components (fibroblasts, endothelial cells, adipocytes, immune cells etc.) as well as noncellular components. Cancer progresses as carcinoma cells recruit host stromal cells, altering their metabolism and characteristics, and together they form a unique microenvironment within which they collaboratively remodel the surrounding matrix and facilitate tumour invasion (Rowe and Weiss 2009). Proteolytic enzymes (such as matrix metalloproteinases) remodel the ECM, increasing stiffness and changing its composition (Piperigkou et al. 2021) and enzymes that influence the modification and cross-linking of extracellular matrix proteins (such as lysyl oxidases (LOX)) (Ye et al. 2020). These vesicles, which contain nucleic acids, lipids and proteins, play a significant role in tumour progression and behaviour, including reshaping the environment around tumours, stimulating fibroblast activity, stimulating angiogenesis, modulating immunity and forming neoplastic niches (Dong et al. 2021; He et al. 2021). In addition to matrix degradation, the release of matrix-bound growth factors and matrikines regulates signal transduction and tumour growth and migration by interacting with surface receptors (Manou et al. 2019; Niland and Eble 2020).

16.6.4 Role of Extracellular Matrix in Regeneration

Extracellular matrix usually regulates the behaviour of cell proliferation, shape and survival. It remodels the cell as well as degrades the cell. Sometimes, its contributions, when mis-utilized or overpowered, become a reason for diseases

like cancers. The ECM is composed of structural proteins, specialized proteins, matricellular proteins, glycoproteins etc. These help it to build and rebuild cells for repair and regenerate. The ECM is cleaved by proteolytic members such as proteases. They release small signalling molecules and growth factors to degrade the matrix proteins. These proteases must always be regulated and, when not under control, the tissue homeostasis is disturbed. It can be therefore inferred that when there is a mis-regulation of protease activity, it could lead to many diseases such as cancers (Daley et al. 2008).

16.6.5 Inflammation

16.6.5.1 Role of Inflammation in Cancer

In the tumour microenvironment, IL-6 encourages the recruitment of immune cells, which further stimulates the production of proinflammatory cytokines. As a result, IL-6 contributes to the growth of chronic inflammation and tumour. The presence of elevated IL-6 levels is observable in a variety of cancer types, similar to patients with arthritis and those with Castleman disease. Elevated IL-6 levels are observed in a variety of different forms of cancer, just as they are in patients with arthritis, Castleman disease, or following infection (Dethlefsen et al. 2013; Kotowicz et al. 2016; Chung and Chang 2003; Chen et al. 2013; Jinno et al. 2015; Riedel et al. 2005; Macciò and Madeddu 2013; Sanguinete et al. 2017; Miura et al. 2015; Culig and Puhr 2012; Altundag et al. 2005; Chang et al. 2013). The Cancer Genome Atlas (TCGA) (Gao et al. 2013; Cerami et al. 2012) has detected no clinically relevant alterations in IL-6, IL-6R, or gp130 genes in tumour types studied to date. A majority of surgical specimens from patients with inflammatory hepatocellular adenomas were found to contain activating mutations in gp130. An additional polymorphism ($-174G > C$) in the promoter region of the IL6 gene has been shown to result in an increase in its expression. IL-6/IL-6R/JAK/STAT3 pathway activation occurs due in large part to epigenetic alterations, and transcription factor expression and activation might play a major role in elevated levels of IL-6 in cancer (Rebouissou et al. 2009). Epigenetic alterations are believed to play a major role in aberrant activation of the IL-6/IL-6R/JAK/STAT3 pathway in cancer, and transcription factor expression and/or activation may play a major role in abnormal expression of IL-6 in cancer. Over 70% of human cancers exhibit abnormally elevated STAT3 activity (Frank 2007; Roeser et al. 2015). STAT3 hyperactivity has been observed in malignancies including acute myeloid leukaemia (AML), multiple myeloma and solid tumours of the bladder, breast, brain, cervix, colon, oesophagus, head-and-neck, kidney, liver, lung, ovary, pancreas, prostate, stomach and uterus. Many of these cancers have been shown to have high levels of phosphorylated and/or activated STAT3. An exogenous form of STAT3 encoded by a constitutively active form confers growth and tumour-inducing properties on fibroblasts, demonstrating the ontogenetic properties of this protein (Chen et al. 2008; Sonnenblick et al. 2012; Takemoto et al. 2009; Zhang et al. 2016). STAT3 activation and phosphorylation have been associated with poor clinical prognosis in multiple

types of cancer. STAT3 expression leads to anchorage independent growth and tumorigenicity in fibroblasts, thus proving STAT3's oncogenic activity (Bromberg et al. 1999).

16.6.5.2 Role of Inflammation in Regeneration

Inflammation is defined with its five characters like dolor, calor, rubor, tumour, functio laesa (Karin and Clevers 2016). It is a part of one of the initial lines of defence of the body against pathogenic organisms. This mechanism is a response to infection. Inflammation is a kind of hallmark for cancer, but its role in regeneration is not very prevalently known.

Certain signalling pathways are triggered during inflammation that leads to regeneration. Pro-inflammatory cytokines like IL-6 and IL-11 trigger the activation of STAT3 and enhance intestinal epithelial cell proliferation and cell regeneration. IL-6 also triggers YAP and Notch pathways, through STAT3 independent signalling cascade, suppressing cell death and promoting cell proliferation (Pesic and Greten 2016).

Macrophages play a crucial role in regeneration. They activate surrounding cells to trigger cytokines (Crupi et al. 2015). They perform phagocytosis which later results in scar tissue formation. The most accepted hypothesis for macrophages playing a role in healing of wounds and regeneration is the transition of pro-inflammatory phenotype (M1) to a regulatory anti-inflammatory phenotype (M2). This mechanism leads to a complete regeneration unlike the scar formation. After an injury, macrophages differentiate among themselves and turn into M1 phenotype. This is because they get exposed to pro-inflammatory cytokines and necrotic cells. Because of this initial response and shift into M1, dormant satellite cells are triggered for activation and are expanded into the injured site. Once the initial inflammatory response is subsided, they transit into M2 phenotype. This transition could be because of IL-10. By transition to M2 phenotype, it subsequently releases anti-inflammatory cytokines and promotes tissue repair and remodelling (Brown et al. 2014). Any abnormality in M2 would, therefore, promote tumour than M1, in comparison (Mills 2015).

Along with STAT3 and macrophages, NF-κB and AP1 are also important for regulating immune responses. They are also primary inflammatory response regulators that control regeneration and wound healing. TNF inhibits apoptosis by activating NF-κB as NF-κB inhibits necrosis. IL-6 and TNF together stimulate intestine and liver regeneration by targeting epithelial cells. TNF triggers epithelial cells in the notch pathway. IL-6, a cytokine member, stimulates cell proliferation and cell subsistence. IL-22 stimulates proliferation, prolongs the differentiation of cells in final cell division, and constrains cell death. Also, IL-22 directly activates STAT3 and MAPK.

Reactive oxygen species (ROS) that are produced during inflammation trigger the proliferation of intestinal stem cells by activating Jun-N-terminal kinases (JNK) and other transcription factors. The transcription factors are activated by the growth factor EGF family. This pathway also interacts in cell migration during tissue injury for wound closure.

Self-limiting acute inflammation is essential for proper regeneration and restoration for injured tissue since it is the primary stage of a wound to heal and it is followed by tissue repair and tissue remodelling (Karin and Clevers 2016).

16.7 Growth Factors

Growth factors are polypeptide molecules, which are secreted by cells, to aid them in regulating, proliferating, differentiating and migrating for healing. They usually bind to very specific target molecules and regulate the downstream processing of the cell. Although these molecules are receptor specific, their function is cell specific since their action is much localized. Off-target effect or over expression of growth factors is one of the major contributing reasons for cancers (Cheah et al. 2021).

16.7.1 Growth Factor Receptor in Cancer

Growth factors and their receptors (GFR)s, which are located on cell membranes or in the cytoplasm, play important roles in cell growth, survival, angiogenesis and metastasis. GFR amplification results in both innate and acquired resistance to traditional and targeted chemotherapies and drugs. To prevent apoptosis, or programmed cell death, escalating growth impulses interact differently with death signals. Role of different GFRs in cancer has been depicted in Table 16.2.

16.7.2 Growth Factors in Regeneration

Growth factors play a role in regeneration through growth factor gradients causing chemotaxis. This process is observed as an illustration in platelet-derived growth factors where growth factors scatter out from clots of blood platelets and macrophages, and this scatter behaves as primary chemotactic signal for fibroblast cells. Also, the gradient in growth factors is the reason for cell migration (Cheah et al. 2021).

16.7.2.1 Transforming Growth Factor Beta (TGF-β)

The TGFβ is a signal transducer that has a key role in healing of wounds and regeneration specific to tissues. It transcribes three isoforms—TGFβ1, TGFβ2 and TGFβ3—which are structurally similar to cytokines and regulate embryonic development through autocrine and paracrine mode. They are usually inactive in state and are activated in the extracellular matrix. Once it is in active state, TGFβ binds to its target ligand's transmembrane receptor through kinase activity. It plays a pleiotropic role by behaving as a crucial factor in cell proliferation, differentiation, migration, invasion and chemotaxis during inflammation and in endothelial cell proliferation, migration, invasion, cell maturation during angiogenesis. It acts as a very able chemo-attractant and inflammatory mediator for neutrophils, basophils and mast

Table 16.2 Role of different GFRs in cancer

GFR family	Role in cancer	References
EGFR/ ERB/HER	*HER2* gene associated to poor prognosis was observed in diverse cancers; aggressive metastatic breast (15–30%), gastric (10–30%), ovarian (20–30%), endometrial (1–47%), esophageal (0–83%), lung (20%) and invasive urothelial bladder (20%) (0–80%) carcinomas In breast cancer (20–78%), constitutive expression of active truncated *EGFR* vIII lacking the extracellular domain was identified, which was linked to tumour aggressiveness. Patients had mutations in the *HER2* gene in lung cancer, the *Her3* gene (somatic) in breast, colon and gastric cancer and the *Her4* gene in melanoma, colorectal, gastric, lung and breast cancer	Witsch et al. (2010)
TGF-βR	*TGF-RII* gene mutations are found in 58–82% of colon and pancreatic cancers, are absent in 24% of prostate cancers, and are downregulated in breast and lung cancers	Elliott and Blobe (2005)
IGFR	*IGF1R* gene amplification has been found in a small number of breast and melanoma cases Squamous cell carcinomas of the lung were shown to have mutations in the *IGF2R* gene	Witsch et al. (2010)
PDGFR	Amplification of the *IGF1R* gene has been discovered in a small proportion of breast and melanoma cases Mutations in the *IGF2R* gene have been discovered in lung squamous cell carcinomas	Demoulin and Essaghir (2014)
VEGFR	*VEGFR1-3* gene expression has been found to be high in a variety of cancers, including bladder, brain, breast, colon, gastric, lung, ovarian, prostate and head-and-neck carcinomas	Goel and Mercurio (2013)
FGFR	*FGFR1-3* gene amplification has been seen in a variety of cancers. For example, the *FGFR1* gene is found in 20% of lung cancer cases, 10% of breast cancer cases, 5% of ovarian cancer cases and 3% of bladder cancer cases; the *FGFR2* gene is found in 10% of gastric cancer cases and 4% of breast cancer cases in triple negative cases; and the *FGFR3* gene is found in bladder and salivary adenoid cystic cancer cases Mutations in the *FGFR1-4* genes have been discovered. For example, *FGFR1* gene in melanoma (rare), gliblastoma; *FGFR2* gene in endometrial (12%), lung (5%), gastric (rare); *FGFR3* gene in bladder (50–60% nonmuscle invasive, 10–15% muscle invasive), cervical (5%), prostate (3%), colorectal; *FGFR4* gene in rhabdomyosarcoma (7–8%) cancer	Dieci et al. (2013)

cells. It can also act as an antagonizer for other neutrophil chemo-attractants like IL-8 to neutralize immune cells and to migrate to the injured site of the tissue.

During an injury, margins are more activated to get altered phenotypically to alter their cytoskeleton and dissolute cell–cell interactions. Through their autocrine and paracrine signalling, it instructs cells from the margin to migrate and proliferate for

healing. If these TGFβ isoforms and their signals are absent at the injury site, then the wound healing is disturbed and diminished. TGFβ plays a vital role as a growth suppressant also. It is regulated by SMAD3, and decline in SMAD3 also results in loss in growth suppressive effect of TGFβ. Hence, they both are proportional in expression. It also interacts in the Hippo pathway to ensure wound healing properties. Dysregulations in *SMAD3* interactions and Hippo pathway lead to cancer. Another important role of TGFβ is to facilitate and regulate the capillary sprout creation during angiogenesis at the site of injury. It helps in production of haemopoietic effector cells and promotes angiogenesis. Ideally, TGFβ signals margin cells from dermis at site of injury to promote fibroblasts and produce extracellular matrix. By the end of wound healing fibroblasts turn into myofibroblasts and undergo apoptosis leaving a scar. But in mammals, a scar-free cutaneous healing is observed in fetal development due to the established role of TGFβ. The TGFβ1 and TGFβ2 are high, but TGFβ3 is low in adults at the wound site but it is inverse in case of foetus. Also, to prevent fibrosis, various integer-mediated non-proteolytic activation of TGFβ1 is required.

TGFβ also has a crucial role in tissue regeneration. Stage one of tissue regeneration is the formation of blastema. Blastema has a mass of undifferentiated cells, from a heterogeneous pool of lineage restricted progenitor cells. Wound epithelium forms the epidermal layer around the site of injury and allows cells to migrate for healing. As the epithelium begins to cap, by thickening, it becomes stratified in appearance with basal keratinocyte polarity and basal lamina. Studies have shown that blastema induction and proliferation are triggered and controlled by wound epithelium (Gilbert et al. 2016).

16.7.2.2 Fibroblast Growth Factor (FGF)

Fibroblast growth factors (FGFs) act through tyrosine kinases to play a vital role in cell proliferation, survival, metabolism, morphogenesis, cell differentiation, tissue repair and regeneration. Pathways like MAPK and PI3K-AKT are regulated by these growth factors. Basic FBF (bFGF) promotes angiogenesis, an important wound healing character. Also, it catalyses tissue remodelling. Granulocyte macrophage colony stimulating factor (GM-CSF), a bFGF, was studied and was shown to be most prominent in wound healing as it contains higher bFGF, TGF-1 in wound fluids. FGF 15/19, FGF21, FGF23 are hypothesized to promote regeneration. FGF 11 contributes to angiogenesis in hypoxia conditions, while FGF21 is involved in spine repair.

FGFR1 and FGFR2 delay wound healing. Cancer associated FGF23 is a biomarker and is observed to be upregulated in osteoclasts (Farooq et al. 2021).

16.8 Autophagy

16.8.1 Autophagy in Cancer

Autophagy is a cytoplasmic catabolic mechanism of cells that have evolved over time and is driven by ATG (autophagy-related genes). It takes part in the removal of degraded cell organelles, abnormal proteins, pathogens and mass cytoplasm from the cell, while aids in recycling the cell nutrients (Yun and Lee 2018). There are four main phases in the process of autophagy. The first phase, induction, the mTOR pathway is involved in the initiation of this phase along with a few autophagic proteins, namely, ULK1, ATG13, ATG101 and FIP200. The second phase, phagophore, which is generated by BECLIN1-linked PIP3 kinase III. The third phase, autophagosomal membrane, which is guided by 2 ubiquitin-like conjugation systems and requires autophagic components such as ATG3, ATG4, ATG5, ATG7 and ATG12. The cellular machinery is recycled until the maturation stage, with the exception of a piece of LC3II that attaches to the membrane. The fourth phase, maturation, is involved in the merging of the autophagosome and lysosome. ATG6 or BECLIN 1 has been known to govern early autophagosome development (Barnwal et al. 2022). As a result, autophagy is involved in both tumour initiation and control (Yun and Lee 2018).

Furthermore, in the case of healthy cells, autophagy helps in maintaining biological function homeostasis, cell quality control and the clearance of old proteins and degraded organelles (Mizushima 2007; Yu et al. 2018). But in the case of cancerous cells, autophagy has been reported to perform a dual role—(1) under certain conditions, it involves in spreading of cancer by inducing proliferation of cancer cells via catabolic processes during situations like drugs administration and deficiency of nutrients and (2) in another condition, it involves in inhibiting cancer cell progression by inhibiting the survival of cancer cells, which eventually results in apoptosis (Yun and Lee 2018; Barnwal et al. 2022). Moreover, mTOR and AMPK have been found to negatively control tumour suppressor factors, resulting in the activation of autophagy and prevention of cancer development (Comel et al. 2014). On the other hand, cancer genes can be stimulated by mTOR, class I PI3K, and AKT, which in turn suppress autophagy and promote cancer growth (Choi et al. 2013). Nevertheless, it is known that mutations in essential autophagy proteins limit tumour growth. For example, BIF-1 proteins, which are connected to BECN1, have been found to become aberrant or absent in a range of cancer types, including colorectal and gastric cancer. UVRAG proteins are likewise related to *BECN1* and serve as the controller of autophagy. UVRAG mutation is known to impair autophagy, which causes an increase in the proliferation of colorectal cancer cells. On the contrary to this, certain forms of RAS-activated malignancies, such as pancreatic tumours, have a high level of autophagy. Further, high-level autophagy inhibition reduces cell proliferation and facilitates tumour suppression in certain malignancies. In conclusion, autophagy is a promising therapeutic cancer target, and researchers are exploring the utilization of autophagy stimulators as adjuvant therapy (Lim et al. 2021).

16.8.2 Autophagy in Regeneration

Autophagy is the process of degradation of cytoplasmic contents in lysosomes. It is one of the major approaches, by cell, to remove cellular waste. This process prevents cells from becoming toxic or damaged. It is to be noted that apart from playing a crucial role in tissue regeneration and cell reprogramming. It promotes induction of pluripotency by counteracting with cellular senescence and apoptosis. Also, degradation of mitochondria through autophagy is believed to improve the reprogramming of cells and promote tissue regeneration. Since autophagy is also important for protein control, maintenance of tissue homeostasis, it is implied that cellular aging is controlled by autophagy (Pan et al. 2013). The JNK pathway, when dysregulated, increases phosphorylation and further induces autophagic cell death, thus preventing cell from tissue homeostasis and reprogramming. Also, NFκB upregulates certain genes like *Beclin 1*, which stimulates autophagy.

mTOR negatively regulates autophagy. mTORC1 acts downstream of the Ras-PI3K pathway, signalling Ras to suppress autophagy, while mTORC2 also has a negative feedback loop, inhibiting transcriptional activity, in Ras senescence. Irregularity in autophagy contributes to tumour survival (Young et al. 2009) (Table 16.3).

16.9 miRNA

16.9.1 miRNAs in Cancer

miRNAs are small, endogenous, single-stranded and revolutionary conserved ncRNAs (non-coding RNAs) of 19–25 nucleotides in length encoded by introns, exons, intersection between introns and exons, or their genes. They can be either cancerous or tumour-suppressor, and contribute to silencing of RNA and post-transcriptional gene regulation. However, multiple reports have found that numerous miRNAs are inhibited in tumour phenotypes because of defective miRNA synthesis (Paul and Banerjee 2022). Studies have also reported that expression of miRNA is aberrant in human cancer due to a variety of mechanisms, which include (1) miRNA gene amplification or deletion, (2) aberrant miRNA transcriptional control, (3) abnormal epigenetic changes, (4) alteration in the biogenesis of miRNA, (5) genomic abnormality and (6) epigenetic factors (Peng and Croce 2016; Di Leva et al. 2014). Moreover, investigations have shown that miRNAs can be carcinogenic or tumour suppressive under certain circumstances (Peng and Croce 2016). Hence, numerous researches have illustrated that miRNAs have a function in tumour cell drug resistance by either targeting drug-resistance-related genes or affecting genes present in apoptosis, cell division and cell cycle. An individual miRNA can modulate multiple genes and has tissue-specific effects (Si et al. 2019). A list of miRNAs involved in cancer and regeneration has been provided in Table 16.4.

Table 16.3 mRNAs/proteins involved both in cancer and regeneration (identified by KEGG pathway)

mRNA/protein	Target	Role of mRNA/protein
WNT-4, WNT5A, WNT10B, β-CATENIN, LRP5/6, PAX7 GSK3β, DKK-1 SURVININ	WNT signalling	Promotes cell proliferation Inhibits Wnt signalling Helps in evading apoptosis in cancer cells
TGF-β, CCN2, SMAD-4, C-myc, BMP2, ACTIVIN SMAD-7 TGFβR,SMAD-2/3 inhibition	TGF-β signalling	Promoting differentiation, angiogenesis, gonadal growth, embryo differentiation Inhibits TGF signalling Causes insensitivity of anti-growth signals in cancer
SHH, GLI, PTC, HHIP, CCND, BCL2 GLI inhibition	Hedgehog signalling	Activates cell proliferation, tissue patterning, stem cell development and maintenance Promotes cell proliferation in cancer
MAP2K1, CREB, NFKB, RAS, FOS, C-myc, ERK, RAF Suppression of DAPK-mediated ERK inhibition	MAPK signalling	Promotes cell proliferation and anti-apoptosis Causes sustained angiogenesis in cancer
YAP/TAZ, TEAD	Hippo signalling	Helps in controlling cell proliferation, apoptosis and fate
m TOR, RHO, PKC, SGK1, AKT, CASP9, PTEN Suppression of AKT inhibition	mTOR signalling	Promotes cytoskeletal organization and cell survival Helps cancer cells in evading apoptosis
P13K, AKT, MYC, CCND, CDK, FASL, BIM, BCL-2, MCL-1, C-myb, BRCA1 Suppression of PTEN-mediated inhibition	P13K-AKT signalling	Promotes cell survival and cell cycle progression Promotes evading apoptosis, proliferation in cancer
JAK, STAT, SOCS, PIMI, BCL2, MCL 1, BCL-XL, C-myc, cyc-D	JAK-STAT signalling	Promotes cell cycle progression and anti-apoptosis

16.9.2 miRNAs in Regeneration

It was identified through sequencing that embryonic stem cells which have a different expression of miRNAs when compared with epiblast stem cells, like miR-290-295, miR-17-92, miR-302/367 clusters are expressed differentially along with another repetitive cluster on chromosome 2 (Sen and Ghatak 2015).

miRNAs play roles in different types of cells. In stem cells, a few miRNAs, especially from miR-290 family, are expressed for regulation of regeneration. The cell proliferation is observed to be seemingly low in the G1 phase when DICER was inhibited and DROSHA was deleted. miRNA like let-7, when expressed in proliferating and self-renewing cells like embryonic stem cells, its stemness is blocked. *Let-7* is also observed in differentiated cells, but regeneration depends only on the stemness and, therefore, it can be concluded that *let-7* is switched off when stemness is required. miR-1 and miR-133 are expressed in cardiac muscles and

Table 16.4 List of miRNAs involved in cancer and regeneration

miRNA	Target	Role of miRNA	Reference
miR 17-92, miR-21	*pTEN*	Oncogenic role in cancer	Farazi et al. (2011)
Let-7/miR-98, miR-141/200	*Ras*	Tumour suppressor in cancer	Farazi et al. (2011)
miR-34a, miR-48,miR-49	*TP53*	Transcriptional regulation of cancer and regeneration	Farazi et al. (2011)
miR-21,miR-29,miR-50,miR-51	Ras pathway	Tumour suppression in cancer	Farazi et al. (2011)
miR-17-92 cluster	*Myc*	Suppress apoptosis in regeneration (usually)	Farazi et al. (2011)
miR-34	*TP53*	Cell cycle arrest in cancer	Farazi et al. (2011)
miR-290-205, 17–92, 302/367	*Bcl*	Anti-apoptosis in cancer and regeneration	Sen and Ghatak (2015)
miR-290-295	*Myc*	Suppressing autophagy in regeneration	Thatcher and Patton (2010)
Let-7	*Ras*	Loss of self-renewal in cancer	Thatcher and Patton (2010)
miR-1	Cardiac muscle	Cell cycle termination in cancer and regeneration	Thatcher and Patton (2010)
miR-133	Skeletal muscle	Promoting differentiation in regeneration	Thatcher and Patton (2010)
miR-27b	Skeletal muscle	Muscle cell differentiation in regeneration	Thatcher and Patton (2010)
miR-15a, miR-16, miR-195	*Ngn3*	Downregulation of translation in regeneration	Thatcher and Patton (2010)
miR-23	*Hes-1*	Downregulation of Hes-1 in regeneration	Thatcher and Patton (2010)
miR-17, miR-302 families	Yamanaka cocktail	Induction of pluripotency in regeneration	Pedroza-Torres et al. (2019)
miR-290	Yamanaka cocktail	Enhance pluripotency in regeneration	Pedroza-Torres et al. (2019)
miR-93, miR-106b, 302a-d	Ras-induced senescence	Cell programming in regeneration	Pedroza-Torres et al. (2019)
miR-200c, miR-302a-d, miR-369	Somatic cells	Activate fibroblast in regeneration	Pedroza-Torres et al. (2019)
miR-29b	AMPK pathway	Tumour proliferation in cancer	Pedroza-Torres et al. (2019)
miR-125, 33, 190	*HIF-1*	Suppressing tumour in cancer	Pedroza-Torres et al. (2019)
pre-miR-17, miR-34a, miR-96, miR-125b	Pro-apoptosis caspase	Translational reduction in cancer	Reddy (2015)
miR-143/145	*Ras* gene	K-Ras activation in cancer	Reddy (2015)

skeletal muscles. The miR-1 expression terminates cell cycle in mammals, thus promoting differentiation in progenitors. While miR-206 is expressed only in muscle cells, its induction by MyoD and myogenin promotes muscle differentiation. miR-27b is essential for the initiation of muscle differentiation. miR-15a, 16, 195 downregulates the translation of *ngn3*, a gene which transcribes NGN3 (neurogenin 3 protein). This protein is called the master controller of pancreatic development. miR-23 downregulates *hes1*, an enhancer of Notch signalling effector. Improper amount of *hes1* results in improper cell count and premature differentiation. *miR-34* when overexpressed, it induces apoptosis and inhibits cell growth and tumour invasion. When the same miRNA is downregulated, the pancreas escapes the cell cycle and it leads to cancer (Thatcher and Patton 2010). miR-17 and miR-302 families are found to be expressed in Yamanaka genes, and their induction of pluripotency is disrupted when these miRNAs are disrupted. It is hypothesized that miR-290 may increase the efficiency of Yamanaka factors in inducing pluripotency, majorly as a substitute for *c-Myc*. miR 290–295 are predicted to bind to c-Myc. It was also found that miR-93, 106b, 302 also enhance cell reprogramming. It is also observed that the absence of miR-302a-d does not allow epithelial marker expression on Yamanaka factors. A group of miRNAs 200c, 302a-d, 369 activate the human fibroblast and reprogramme it as an induced pluripotent stem cell. While miR-133 controls unnecessary ossification, miR-675, 221 promote collagen-II expression in chondrocytes. When satellite cells of the human genome are induced with miR-1, 206, the cells were seen to improve in their differential potential (Sen and Ghatak 2015).

miRNAs not only regulate genes but also regulate metabolic pathways. miR-320, 123a, 422, 506, 136 regulate the glucose uptake and cancer metabolism, influencing the PI3K pathway. miR-33a/b regulate enzymes of the AMPK pathway, fatty acid metabolism pathway, while miR-29b regulates amino acid metabolism that helps in regulating metabolism of tumour growth and cell proliferation. Also, Hypoxia inducible factor-1 (HIF-1), a pathway that suppresses cancer growth, can be controlled by miR-125-5p, miR-33-5p, miR-190-5p (Pedroza-Torres et al. 2019).

In Fig. 16.2, each node represents one protein and the lines connecting the circles represent the interactions. It is evident that cancer may be caused by dysfunction of at least 200 proteins, whereas regeneration is ensured when at least 60 proteins work properly and interact with each other. The network comparison shows that almost 80% of proteins involved in regeneration are shared in cancer-causing pathways, and cancer is a much more complicated system than the process of regeneration. mRNAs/proteins found to be associated both in cancer and in regeneration (identified by the KEGG pathway) have been provided in Table 16.3 and corresponding references of each molecule and pathways have been provided in the respective sections of the chapter.

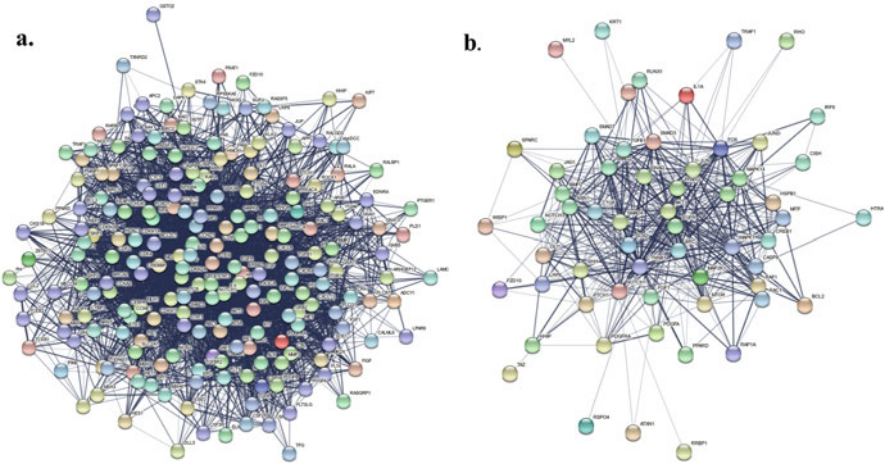


Fig. 16.2 Protein–protein interaction (PPI) network of proteins corresponding genes identified in KEGG pathway database for (**a**) cancer and (**b**) regenerative processes using string database (https://string-db.org/)

16.10 Applied Therapeutics

16.10.1 Regenerative Medicine

The human body has the ability to regenerate complex tissues to a limited extent. For example, the skin and the blood are continuously restored, and the bone, the muscle, the liver and the blood vessels have capacity to self-repair. However, they often fail to regenerate after extensive trauma or injury as they are capable of repairing only unicell lining like epidermis or intestinal mucosa. Regenerative medicine involves comprehending the complex regenerative mechanism in nature to enhance the restoration of tissue damage in the human body (Gurtner et al. 2007; Petit-Zeman 2001). Regenerative medicine is often mediated by the micro-environment that is mostly an extracellular matrix (ECM). ECM plays a significant role in the restoration by providing cell adhesion and transducing signals to dictate their function of cell migration, proliferation and cellular communication by various signalling mechanisms (Walker et al. 2018). For example, integrins, a transmembrane receptors bind to ECM by receptor-ligand binding regulates epidermal stem cell fate by regulating ERK and MAPK signalling and also by controlling orientation of spindle fibres (Watt and Fujiwara 2011). In addition, the sub-types of β integrin contribute to osteoclast migration in bone remodelling. Apart from this, the ECM-Cell adhesion mechanism, variation in signalling pathways, cytokines, growth factors, or other biomolecules lead to developing interesting therapeutic strategies for regenerative medicine which has to be evaluated in detail.

16.10.2 Cancer Therapy

As discussed earlier, mutations or defects of certain transcription factors (TF), signal modulators and other regulatory proteins cause cancer worldwide. Moreover, reports suggest that microenvironment homeostasis has a major role in tumour cell progression. Minimum change in concentration of ECM proteins especially collagen leads to cancer cell proliferation. During initiation of cancer progression, adjacent cancer-associated fibroblast secrete collagen and cancer cells at the site release lysyl oxidase (LOX) which causes cross-linking of collagen at tumour site and eventually increased integrin signalling leads to cancer proliferation over the matrix region (Walker et al. 2018). This makes an effort to use regenerative medicine in treating cancer to restore tissue homeostasis. Ongoing therapies like chemotherapy have been used to treat cancer for decades along with surgery and irradiation to eliminate cancer. These have several limitations in selection of the tumour cell over the normal cells, leading to deficient drug concentrations in tumours, systemic toxicity and development of drug resistance. Lately various strategies and formulations have emerged for cancer therapy per se, liposomal formulation, resistance modulation (e.g. PSC833), antidotes/toxicity modifiers (e.g. ICRF-187), monoclonal antibodies, cell-based therapy and gene therapy. Recent literature on targeted therapy using biomaterial-based delivery systems claimed the improved effectiveness in terms of specificity towards the tumour cells as well as their regulatory factors. These broad strategies in both preclinical and clinical therapeutics are classified into categories.

16.11 Biomaterial-Based Therapies

Synthetically engineered materials which mimic the native cellular environment can be considered as the biomaterials. These materials play an extensive role in providing a matrix environment in the damaged site to enhance cell attachment and proliferation. However, some of the earliest attempts of regenerative medicine included decellularized tissues and organs obtained from allogeneic or xenogeneic sources. Extracellular matrix remains the active component of such practices; of course, it is a biomaterial. Recently, in vitro grown tissue-constructs using organ specific cells grown on decellularized tissues were developed and propounded to be a more promising alternative to decellularized matrix alone in regenerative medicine. These decellularized grafts can further be transplanted into hosts to improve accurate function of the diseased organ. Decellularized materials without recellularization can also be used for the treatment of regeneration as they contain the matrix components like collagen and elastin to facilitate the growth of cells. The studies suggest that fish skin decellularized by hypertonic and hypotonic and triton solutions is similar to human skin properties and involves enhancing epithelial cell adhesion in decellularized material (Kamalvand et al. 2021). Recently, researchers reported perfusion-seeding and culture technique for decellularized liver matrix to attain a vascular matrix which are involved in circulation and facilitate oxygen and nutrient supply after transplantation (Uygun et al. 2010). This proves that decellularized

materials can be a potential strategy for regenerative medicine. However, due to the degradation of ECM proteins during decellularization, a loss in mechanical strength is shown.

Hence, fabrication of scaffold or hydrogel-like biomaterials using naturally derived ECM polymers (collagen, elastin etc.) or synthetic polymers (poly (ethylene glycol), Poly (lactic acid-*co*-glycolic acid) etc., act as ECM analogues and help in attaining the structural and functional properties of tissues. Additionally, these can also be utilized as a cargo medium for the persistent release of drug or growth factors. For example, injectable poly (ethylene glycol) PEG hydrogel was developed to deliver Avastin for ocular treatment (Yu et al. 2014). Polymers are biodegradable, enabling its replacement with the host tissue.

The field of tissue engineering and regenerative medicine is seeing a new age. The field of 3D bioprinting has emerged as a technique with high control over cell placement in the engineered structures (Mao and Mooney 2015). These structures have a definite altitudinal patterning of cells and other biomolecules which is based on computer-aided assembling in layer-by-layer tactic to develop a tissue or organ structure. Although 3D engineered structures attain the cellular assembling and structure of aimed tissue or organ, they fail to mimic the kinetics of usual vascularization and other cellular functions (Ozbolat 2015).

Moreover, biomaterial-based therapies have several limitations such as low engraftment and micro vascularization in engineered structures; lack of cellular signalling and function and limited organ donors are also rendering this therapy unattainable. Hence, incorporation of biomimetics with immune modulators will be a potential strategy to attain regenerative medicine feasibly.

16.12 Newer Therapeutic Aspects

16.12.1 Gene Therapy

The European Medicines Agency (EMA) explains gene therapy-based products or biological medical products as active substances consisting of nucleic acids in order to regulate or repair the genetic sequence and should also possess therapeutic, prophylactic or diagnostic effects on the host (Ma et al. 2020). In practice, this is a complex operation where the transgene should overcome several barriers to express in the target cell-nucleus. The ultimate goal of the regenerative medicine is to attain biocompatibility at the damaged tissue without immune rejection. It clearly emphasizes that modulating the immune system plays a prominent role in regulating regeneration by augmenting the healing process and engraftment (Eming et al. 2007). Processing of cells in regeneration indirectly induces immune response by releasing growth factors and cellular signalling. Cell therapy and gene therapy predominantly fall under the category of altering the immune system. Gene therapy includes techniques for passing the transgene through the plasma membrane and its entry into the cell nucleus without degradation (Luo and Saltzman 2000). Gene therapy is divided under two main subtypes based on the nature of target cells as

germline gene therapy and somatic gene therapy where former includes the transfer of functional gene in reproductive cells and the latter in non-reproductive cells which means modified expression is within the generation and will not pass on to the next generation as in germline gene therapy (Stribley et al. 2002). Nucleic acids involved in gene therapy are majorly plasmid DNA (pDNA) and various types of RNAs. Although pDNA-based studies have shown extensive results for decades, there are difficulties such as nuclear trafficking and low efficiency. Unlike pDNA, RNAs exercise their function only in cytoplasm thereby effectively transfecting cells (Wang et al. 2019b). Therefore, gene therapy using RNA is found to be more effective, and a number of researches are carried out on the same.

16.12.2 RNA-Based Therapy

RNA-based therapies involve both positive and negative regulation of RNA in cancer or regeneration as mentioned. Delivery of messenger RNA (mRNA) encoding regulatory proteins or growth factors of pathway end up in accelerating the pathway or the mechanism, whereas micro-RNA (miRNA) and small interfering RNA (siRNA) follow RNA interference (RNAi) mechanism of gene silencing. RNAi is the phenomenon of sequence-specific degradation of mRNA followed by suppression of associated biological function (Davidson and McCray 2011).

miRNA mediates post-transcriptional gene silencing, but before that it is processed via a complex engagement of endogenously expressed transcripts like drosha and dicer. This processed strand enters an RNA-induced silencing compartment (RISC) guided towards mRNA targets where it binds to the 3′UTR, and the pairing is highly complementary at seed regions of 2–8 base pairs. The miRNA–mRNA complex will be transported to cytoplasmic processing bodies where deadenylation and mRNA degradation takes place. Although miRNAs have an effective role in gene silencing, they have numerous target mRNAs. Hence, it is essential to screen the miRNA and their targets to attain specific function rather than targeting other targets (Elbashir et al. 2001).

The siRNAs are processed from double stranded RNA by dicer. They are 20–24 nucleotides in length. One strand is ‘passenger’ strand, and the other strand is ‘guide’ strand which initiates silencing by binding to mRNA with full complementarity. The cleavage occurs at the 10–12 base pairs from 5′ end of the siRNA binding site. siRNA differs from miRNA by their high complementarity to mRNA that helps in increased specificity towards the target (Zamore et al. 2000).

In most cases, nucleic acids are delivered with cationic or lipid-based complexes to the target site for gene silencing inside the cell. For example, ligand-targeted injection of siRNA with PEGylated poly-plex in tumour bearing mice shows anti-tumour effect with decreased uptake in the lung and the liver (Li and Huang 2006). Similarly, an appropriate delivery system for RNA therapeutics will help in achieving target-specific immune response by either enhancing or supressing the proliferation of cells.

16.12.3 Gene Delivery System

As gene therapy involves the transfer of nucleic acids, it is essential to ensure its reach in the target site without degradation as they are very sensitive to nucleases and their expression after binding to the site. The pertinent delivery system which gives the cellular environment will help in preventing denaturation of nucleic acids. It also satisfies several features.

- It should not involve in immune response and deliver genetic cargo in sustained manner which leads controlled expression.
- It should prevent biodegradation and help in target-specific release.
- It should be inexpensive and commercially available (Ibraheem et al. 2014).

16.12.3.1 Viral Vectors

A virus is a pathogenic microorganism that can survive by releasing their genetic material in the host organism to replicate and transmit to the adjacent cells (Kay et al. 2001). Researchers use their mechanism to carry therapeutic RNA into the nucleus as vectors. These viruses should be modified by genetic engineering to use as a vector. The pathogenicity of the virus is killed and replaced by therapeutic genes (Bouard et al. 2009). However, it retains the ability to infect the cell. These modified viruses are known to be viral vectors which have high transfection efficiency and thus are used till date. But they have certain drawbacks as follows:

1. It causes acute immune responses which can be fatal.
2. The large-scale production of viral vectors is very challenging and highly expensive.
3. The limitations in the size of genes that can be transported by the virus (Nagasaki and Shinkai 2007).

The commonly used viral vectors are retroviruses, adenoviruses, adeno-associated viruses (AAV) and simple herpes virus (Walther and Stein 2000).

16.12.3.2 Non-Viral Vectors

The limitations of viral vectors specifically to severe acute reaction have led to finding safer replacements. Non-viral vectors are safer, cause lower acute response, are inexpensive, and can be prepared in large quantities efficiently. Additionally, they have larger stability and can transfer genes of various sizes (Munier et al. 2005). But because of their low transfection efficiency, they cannot be produced on a large scale. Non-viral vector systems are classed into two groups as physical methods in which nucleic acid (DNA, -miRNA, siRNA) is delivered without any carrier, by preparing competent cell to enhance the permeability of transgene using physical forces (Gao et al. 2007) and chemical methods wherein nucleic acid is transfected into the vector of biological or synthetic origin. The principle of physico-chemical methods of biomolecule delivery which are emphasized in RNA theraupeutics are further elucidated in detail.

Electroporation

This physical technique termed as electro-permeabilization or electric pulse-based gene transfer is a technique in which an electric pulse is used to infuse DNA into viable cells (Golberg 2020). Electroporation is a process of increasing the cell permeability under controlled electric fields to stimulate the uptake of injected DNA into the cell (Niidome and Huang 2002). This effect of electric pulse causes temporary destabilization of the cell membrane, allowing DNA to enter the cell easily. Both physical factors like pulse duration, field intensity and electrode geometry and biological factors such as cell size, shape and density affect the efficacy of gene transfer. Although electroporation in the nucleic acid delivery system has advantages of safety and efficiency, its use in vivo is limited because of difficulties in transferring DNA to large tissue areas, requires surgery to apply electrodes in organs and causes incurable harm in the treated tissues.

Gene Gun

Gene gun technology was first employed in 1987 to present genes into cells (Gehl 2003). It's basically a technique which delivers transgene into a cell or tissue using accelerated particle carriers which are biocompatible heavy metals like gold, tungsten and silver. These carriers should be inactive and smaller in size of about 1–1.5 μm (Mehier-Humbert and Guy 2005). These carriers are treated with plasmid DNA by providing needed acceleration by either vaporizing water under high-voltage electric spark or using helium discharge (Ibraheem et al. 2014). This method can be hired in most fields such as genetic vaccination, immune therapy and suicide gene therapy to treat cancer (Lin et al. 2000). Gene gun transfer method offers many advantages like higher and sustained gene expression (Ajiki et al. 2003) as well as it is achieved without injury to the surrounding cells (Muangmoonchai et al. 2002). Due to its poor competence while transferring the entire gene into tissue by penetration of metal particles, surgery is often needed for deep tissues.

Inorganic Particles

Inorganic particles involve nanomaterials that can be synthesized in varying their physical nature in order to make entrapped molecules survive from degradation by the immune system. Calcium sulphate, silica, gold and magnetic compounds are some of the most commonly studied inorganic particles.

1. Calcium: Initially used delivery systems are calcium phosphate particles as it has a vital role in cellular signalling, it has the benefit of passing through the membrane by receptors, and also has high binding affinity in the cell. They are highly biocompatible and biodegradable. However, calcium phosphate nanocrystals cannot be stored as it grows with time, a drawback which was later overcome by combining other particles.
2. Silica: One of the major components of sand and glass, which are widely used in our daily life. It is used as a gene delivery vehicle because it is relatively easy to functionalize with amino silicanes due to its minimal toxicity. However, the limiting factor is its reduced delivery efficacy in media containing serum as due to non-specific binding.

3. Magnetic nanoparticles, such as (like magnetite), soluble carbon molecules, carbon nanotubes, quantum dots and supramolecular systems, are claiming some promising results in in vitro as well as in animal models. In these nanoparticles, DNA binding is aided by the coating which is applied on the surface. Small particle size helps in passing most of the cell membrane barriers to generate excessive transfection efficiency. However, reviews are required on chronic reactions of safety, plane functionalization effect on physical characterization, its transfection efficiency etc., to improve its clinical application (Al-Dosari and Gao 2009).

Lipid-Based Therapy

Several classes of lipid-based formulations are opted for the delivery of RNA notably liposomes, solid lipid nanoparticles, and nanostructured lipid carriers. Unlike other lipid-based systems, liposomes can be used for loading hydrophilic and ionic molecules. It undergoes similar transportation of fusing into plasma membranes like other biomolecules to reach the target cell then bear endocytosis process to release their drug or nucleic acids in cytoplasm. Cationic liposomes complexed with anionic siRNA forms 'lipoplex' which provides stability of the siRNA. However these cationic liposomes react with negatively charged proteins causes non-specific interactions. Hence, to reduce these complications, PEGylations can be applied on the surfaces that help in enhancing half-life siRNA. Also, pH-sensitive liposomes can be formulated by phosphatidylethanolamine (PE) with pH-sensitive lipids (Fan et al. 2017). This formulation helps in delivering the molecules or drug in low pH microenvironments like tumour sites. RNAi molecules can be incorporated into solid lipid nanoparticles (SLNs) to provide a sustained release to the targeted cell. Nanostructured lipid carriers are modified formulations of SLN where lipid is either solid phase or in liquid phase but in SLNs it is solid core. These can be surrounded by surfactants which give positive surface potential to bind with nucleic acids. Compared to SLNs, nanostructured carriers have high loading space so that combinations of siRNA can also be transported by nanostructured lipid carriers.

Polymer-Based Therapy

Polymers of an amine group when modified to cationic can be used for RNA delivery via electrostatic interaction of RNA and polymers to form a complex. These can be chemically modified to attain biocompatibility. For example, nanoparticles synthesized with PEG-grafted PEI (polyethylenimine) deliver mRNA into lung cells (Ke et al. 2020). Another class of cationic polymers, poly (beta amino esters) PBAEs are prepared by coupling amine monomers to diacrylates to improve biodegradation and cytotoxicity relative to amine group polymers. These PBAEs are used to deliver siRNA to tumour mice models (Kozielski et al. 2019). Research on lipid-PBAE's hybrid polymer synthesis shows improved serum stability and delivery. Dendrimers consisting of branched monomers are also used for RNA delivery when synthesized with cationic groups to form a complex. In addition, its structure has been modified to resist enzyme degradation of nucleic acids. Alkyl

substituted amines with dendrimer-based nanoparticles deliver siRNA to endothelial and hepatocytes in vivo (Khan et al. 2014).

Extracellular vesicles secreted by cells are considered as micro-vesicles (100–1000 nm), exosomes (40–100 nm), or apoptotic bodies (1–4 μm) based on their size range. Exosomes can be isolated from mesenchymal stem cells (MSCs), epithelial cells, mast cells, dendritic cells, even cancer cells and purified by ultrafiltration followed by affinity purification. Exosomes include different transmembrane proteins, heat shock proteins and around 1300 types of RNAs and miRNAs. Most of the functional miRNAs found in exosomes undergo RNAi. Hence, it emerged as a natural carrier for RNAi molecule delivery due to their non-immunogenicity and stability. Native miRNA of the exosomes or miRNA and siRNA can be transfected to exosomes, and they are transported to the target cell by ligand-receptor mechanism via endocytosis (Yu et al. 2016). For example, the upregulation of exosomal miR-146a downregulates Erbb4, Notch1 and Irak1, which leads to a decreased metabolism and improper contractile function in cardiomyocyte; it also inhibits angiogenesis by downregulating TNF receptor-associated factor 6 (TRAF6) expression (Ailawadi et al. 2015). Researchers proved the effective delivery of siRNA by exosomes to silence the functional oncogenes in head and neck cancer. Exosome/transient receptor potential polycystic 2 (TRPP2) siRNA complexes inhibit the expression of TRPP2 in FaDu cells, a cell line of human pharyngeal squamous cell carcinoma (Wang et al. 2019a).

16.13 Cell Therapy

16.13.1 Stem Cell Therapy

Stem cell therapy has proved itself as a non-replaceable and unique candidate for cancer therapeutics because of its regenerative potential and enhanced target on tumours. Although iPSC has evolved as compensation for embryonic stem cell (ESC) application because of ethical barriers, enough studies proved the use of adult stem cells like mesenchymal stem cell (MSC), haematopoietic stem cell (HSC) and neural stem cell (NSC) (Chu et al. 2020). The migration of HSC into their defined niches, called the homing process, leads to an engraftment of marrow tissues. This process occurs prior to differentiation into specialized blood cells. The molecular mechanism of HSC homing process involves an association between stem cell CXCR4 receptor and gradient SDF-1 secreted from the cells lining the bone marrow niches. Similarly, another mechanism is the tumour tropic effect, which involves the secretion of various chemoattractant factors that favour the movement of MSC into the tumour microenvironment. Apart from the above two mechanisms, other mechanisms are paracrine factors secreted from stem cells (Vakhshiteh et al. 2019) and CSC signalling. Researchers are using the above properties of stem cells by different means to use for therapeutic applications. The strategy of using stem cells as therapeutics in HSC transplantation, MSC transplantation after cancer treatment, will show the advantages over side effects, efficiency and degradation

due to the intrinsic tumour-targeting effect of stem cells, genetically modified stem cells, stem cell-derived exosomes, nanoparticles carrying stem cells and stem cell-based anti-cancer vaccine which can be created from oncofetal protein, CSC/ESC, or iPSC-based whole cells.

16.13.2 CAR-T Cells Therapy

Chimeric antigen receptor (CAR) T cell therapy is a class of immunotherapy which employs in the cancer treatment domain. CAR T cell therapy edit the immune response of T cell to produce a receptor which is specific for tumour antigen. In this immunotherapy procedure, autologous T cells are isolated from the sufferer then it is harvested in ex vivo. All the harvested cells undergo viral and non-viral transfection by which T cells are genetically transformed to produce antigen receptors. Once CAR-T cells are able to synthesize the product, these cells are transfused back to the patient body (Gross et al. 1989). CARs possess antigen binding moiety and spacer on their outer cell membrane. Single chain fragment variable (scFv) is a variable monoclonal antibodies which derived from human Abs, humanized Abs, or mouse monoclonal antibody(mAbs) which displayed on the outer membrane and are able to act against tumour-associated antigens (TAAs) (Sadelain et al. 2013). CAR T cell does not follow antigen processing and presentation (MHC class I and class II restriction) directly but CD4+ and CD8+ subset recognize processing antigen from the tumour cell. CD4+ and CD8+ identify the carcinogenic cell and do cytolysis by granzyme and perforin exocytosis (Miliotou and Papadopoulou 2018).

16.13.3 NK Cells-Based Therapy

Natural killer cells are group-I lymphocytes, which control many infections, tumours by preventing their spread and damage through their innate cytotoxicity. They detect various cell-surface receptors and identify healthy cells and self-cells from the other cells. The property of cytotoxicity has been exploited in the past few years to develop many mechanisms. They can be activated by distress by stress-induced ligands like KNG2D, Toll-like receptor ligands. NK cells can show MHC class-I-specific receptors and fail to receive inhibitory signals when they interact with class-I-deficient haematopoietic cells. This means that NK cells can recognize the missing 'self'. The inhibitory receptors have killer cell immunoglobulin-like receptors (KIRs), CD94-NKG2A heterodimers (Vivier et al. 2008).

NK cells act as the first line of defence by playing two major roles—cytotoxic effect and immune regulation. Because of their capability to act in the absence of MHC immunosurveillance, they can function as cancer suppressors. But they can also get suppressed because of surrounding factors in a tumour microenvironment. The suppression itself denotes that NK cells are representing a site of tumour or cancer, thus making them promising strategies for immunotherapy. NK cells are

stimulated killing activating receptors (KARs) which in turn induce ligands-like tumour necrosis factor-alpha (TNFα), Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) and lead to activate apoptotic pathway. They diagnose and cause cell death in unhealthy cells by perforin and granzymes. They have higher cytotoxicity as compared to other cells, which makes them more resistant to cancer cells and have higher immune surveillance capability. The different therapeutic approaches using NK cells are allogeneic cell infusion therapy to trigger alloreactivity of NK cells, enhancing activity through cytokines IL-2 or IL-15 and inhibiting checkpoints of immune response by releasing dominant inhibition, blocking immune tolerance signal, or targeting emerging inhibitory checkpoints to induce the activity of NK cells in vivo (Du et al. 2021).

16.14 Conclusion

It is believed that every minute several cancer cells are born in the human body. Cell division events in regenerative processes occur even more frequently. Almost all the molecular tools used for regenerative processes could be misused for cancer development. Analogously, it can be put as cancer rides on the same horse as regeneration does, however, with diminished systemic controls. Yet, all of the human population may not die of cancer. That indicates homeostatic mechanisms of the human body have the ability to pull back the reign to maintain order and reverse the cancerous condition. This discipline is imposed at many scales viz., molecular scale, molecular-network scale, cellular scale and systemic scale.

The general idea is, genetic mutation followed by malfunctioning molecules results in a tumour cell formation. Proteins like p53, cyclins, Wnt, members of Hippo, Ras-raf and PI3K, pathways often promote regeneration and homeostasis. However, such proteins, upon miscoding or malfunctioning, promote cancerous growth. At a cellular scale and systemic scale, immune cells that are generally trained to eliminate cancerous phenotypes start supporting the growth of cancer tissues, when stimulated with certain cytokines that in turn might be the result of malfunctioning proteins sitting inside a cancer cell. However, certain proteins such as certain caspases, mTORs, p21, certain FGFs and some of the Yamanaka factors seem to have an exclusive role in regeneration. They also have a crucial role in embryonic development as well. Talking about embryology, gene and gene products such as hox and polycomb have exclusive roles in early developmental stages. Effectively modulating these genes for the purpose of regenerative medicine is a herculean challenge in recent times.

At an intra-cellular molecular scale, miRNAs have newfound but crucial regulatory roles. They can modulate the overall molecular interaction network and derive desirable epigenetic changes. These changes are brought however by modulating the translation of many of the aforesaid proteins. Many miRNAs are now being discovered having crucial roles in promoting regeneration and inhibiting cancer progression and vice versa. Cellular scale controls, however, include autophagic cell deaths that are usually meant for maintaining tissue homeostasis. Dysregulation of certain

autophagic mediators can cause chaos in the surrounding tissues. Similarly, the pro-tissue role of ECM can turn wry and pro-cancerous if its composition varies. This can happen due to paracrine actions of surrounding cells. Systemic restrictions on cancer cells and promotion of regeneration are mostly exerted by the nervous and immune systems as identified in lower vertebrates like amphibians. However, tumorigenic events in the said systems bring out the most complex challenges.

In cancer therapy and regenerative medicine, the most promising therapy is cell therapy. As discussed earlier, this included stem cells, gene-corrected cells, gene-overexpressed cells and immune cells, with a mode of delivery being primarily local or systemic injection. Immunomodulation or immune-restoration aiming cell therapies generally designed for cancer treatment nowadays are identified as regenerative medicine because the end result is regeneration or restoration of a normal immune system. This is achieved mostly by cell therapeutic strategies. However, cell therapy has not been able to deliver the desired results.

Molecular therapy in both the cases depended on identification of right molecular targets followed by right molecules and right modalities to deliver it. The rawest form appears in the form of ECM therapy, performed in most regenerative medicine approaches. In addition, protein therapies using a varied formulation are explored as an effective mechanism. Proteins, such as FGFs, BMPs, TGFs and monoclonal antibodies targeted to inhibit specific pathways are mostly in use. Gene- or nucleic acid-based therapy, using a viral vector or non-viral delivery systems, has been recently and most successfully explored in COVID19 vaccination in many countries. The scepticism underlying the safety of nucleic acid medication has majorly been cleared up with the pandemic. This opens an opportunity to explore stringently designed pDNA, mRNA, snRNA and miRNA molecules for cancer therapy and regenerative medicine.

The confusions and dilemmas are mostly cleared up. Cancer and regeneration that originally seemed to be a mesh of convoluted crossroads alone actually have certain segregated flyovers. Careful selection of bypasses and therapeutics most likely will land us in mutually exclusive destinations.

References

Ailawadi S, Wang X, Gu H, Fan G-C (2015) Pathologic function and therapeutic potential of exosomes in cardiovascular disease. Biochim Biophys Acta 1852:1–11. https://doi.org/10.1016/j.bbadis.2014.10.008

Ajiki T, Murakami T, Kobayashi Y, Hakamata Y, Wang J, Inoue S, Ohtsuki M, Nakagawa H, Kariya Y, Hoshino Y, Kobayashi E (2003) Long-lasting gene expression by particle-mediated intramuscular transfection modified with bupivacaine: combinatorial gene therapy with IL-12 and IL-18 cDNA against rat sarcoma at a distant site. Cancer Gene Ther 10:318–329. https://doi.org/10.1038/sj.cgt.7700575

Al-Dosari MS, Gao X (2009) Nonviral gene delivery: principle, limitations, and recent progress. AAPS J 11:671. https://doi.org/10.1208/s12248-009-9143-y

Ali MU, Ur Rahman MS, Jia Z, Jiang C (2017) Eukaryotic translation initiation factors and cancer. Tumor Biol 39(6):1010428317709805

Alsaab HO, Sau S, Alzhrani R, Tatiparti K, Bhise K, Kashaw SK, Iyer AK (2017) PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. Front Pharmacol 8:561

Altomare DA, Testa JR (2005) Perturbations of the AKT signaling pathway in human cancer. Oncogene 24:7455–7464. https://doi.org/10.1038/sj.onc.1209085

Altundag O, Altundag K, Gunduz E (2005) Interleukin-6 and C-reactive protein in metastatic renal cell carcinoma. J Clin Oncol Off J Am Soc Clin Oncol 23(5):1044–1045

Amelio I, Melino G (2020) Context is everything: extrinsic signalling and gain-of-function p53 mutants. Cell Death Discov 6(1):1–7

Arthur LM, Heber-Katz E (2011) The role of p21 in regulating mammalian regeneration. Stem Cell Res Ther 2:30. https://doi.org/10.1186/scrt71

Aziz SA, Davies M, Pick E, Zito C, Jilaveanu L, Camp RL, Rimm DL, Kluger Y, Kluger HM (2009) Phosphatidylinositol-3-kinase as a therapeutic target in melanoma. Clin Cancer Res 15(9):3029–3036

Barboza JA, Liu G, Ju Z, El-Naggar AK, Lozano G (2006) p21 delays tumor onset by preservation of chromosomal stability. Proc Natl Acad Sci 103(52):19842–19847

Barnwal SK, Bendale H, Banerjee S (2022) Non-coding RNAs associated with autophagy and their regulatory role in cancer therapeutics. Mol Biol Rep 49(7):7025–7037. https://doi.org/10.1007/s11033-022-07517-8

Barresi V, Ieni A, Cardia R, Licata L, Vitarelli E, Reggiani Bonetti L, Tuccari G (2016) HOXB 13 as an immunohistochemical marker of prostatic origin in metastatic tumors. APMIS 124(3): 188–193

Bergmann A, Steller H (2010) Apoptosis, stem cells, and tissue regeneration. Sci Signal 3:re8. https://doi.org/10.1126/scisignal.3145re8

Bertero A, Murry CE (2018) Hallmarks of cardiac regeneration. Nat Rev Cardiol 15:579–580. https://doi.org/10.1038/s41569-018-0079-8

Boice A, Bouchier-Hayes L (2020) Targeting apoptotic caspases in cancer. Biochim Biophys Acta (BBA)-Mol Cell Res 1867(6):118688

Boilly B, Faulkner S, Jobling P, Hondermarck H (2017) Nerve dependence: from regeneration to cancer. Cancer Cell 31:342–354. https://doi.org/10.1016/j.ccell.2017.02.005

Bouard D, Alazard-Dany N, Cosset F-L (2009) Viral vectors: from virology to transgene expression. Br J Pharmacol 157:153–165. https://doi.org/10.1038/bjp.2008.349

Bourguignon LY, Earle C, Wong G et al (2012) Stem cell marker (Nanog) and Stat-3 signaling promote MicroRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells. Oncogene 31:149–160

Bousoik E, Montazeri Aliabadi H (2018) "Do we know jack" about JAK? A closer look at JAK/STAT signaling pathway. Front Oncol 8:287

Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr (1999) Stat3 as an oncogene. Cell 98(3):295–303

Brooks A, Putoczki T (2020) JAK-STAT signalling pathway in cancer. Cancers 12(7):1971

Brown BN, Sicari BM, Badylak SF (2014) Rethinking regenerative medicine: a macrophage-centered approach. Front Immunol 5. https://doi.org/10.3389/fimmu.2014.00510

Bugter JM, Fenderico N, Maurice MM (2021) Mutations and mechanisms of WNT pathway tumour suppressors in cancer. Nat Rev Cancer 21(1):5–21

Calses PC, Crawford JJ, Lill JR, Dey A (2019) Hippo pathway in cancer: aberrant regulation and therapeutic opportunities. Trends Cancer 5(5):297–307

Canavese M, Santo L, Raje N (2012) Cyclin dependent kinases in cancer: potential for therapeutic intervention. Cancer Biol Ther 13(7):451–457

Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, Antipin Y (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2(5):401–404

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113(5):643–655

Chambers SM, Shaw CA, Gatza C, Fisk CJ, Donehower LA, Goodell MA (2007) Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. PLoS Biol 5(8):e201

Chang CH, Hsiao CF, Yeh YM, Chang GC, Tsai YH, Chen YM, Huang MS, Chen HL, Li YJ, Yang PC, Chen CJ (2013) Circulating interleukin-6 level is a prognostic marker for survival in advanced nonsmall cell lung cancer patients treated with chemotherapy. Int J Cancer 132(9): 1977–1985

Charni M, Aloni-Grinstein R, Molchadsky A, Rotter V (2017) p53 on the crossroad between regeneration and cancer. Cell Death Differ 24:8–14. https://doi.org/10.1038/cdd.2016.117

Cheah E, Wu Z, Thakur SS, O'Carroll SJ, Svirskis D (2021) Externally triggered release of growth factors—a tissue regeneration approach. J Control Release 332:74–95. https://doi.org/10.1016/j.jconrel.2021.02.015

Chen C-H, Poss KD (2017) Regeneration genetics. Annu Rev Genet 51:63–82. https://doi.org/10.1146/annurev-genet-120116-024554

Chen CL, Cen L, Kohout J, Hutzen B, Chan C, Hsieh FC, Loy A, Huang V, Cheng G, Lin J (2008) Signal transducer and activator of transcription 3 activation is associated with bladder cancer cell growth and survival. Mol Cancer 7(1):1–12

Chen MF, Chen PT, Lu MS, Lin PY, Chen WC, Lee KD (2013) IL-6 expression predicts treatment response and outcome in squamous cell carcinoma of the esophagus. Mol Cancer 12(1):1–12

Chen B-J, Wu Y-L, Tanaka Y, Zhang W (2014) Small molecules targeting c-Myc oncogene: promising anti-cancer therapeutics. Int J Biol Sci 10:1084–1096. https://doi.org/10.7150/ijbs.10190

Chen L, Shi J, Wu Y, Qiu R, Zeng L, Lou L, Su J, Liao M, Deng X (2020) CircRNA CDR1as promotes hepatoblastoma proliferation and stemness by acting as a miR-7-5p sponge to upregulate KLF4 expression. Aging (Albany NY) 12(19):19233

Cheng S, Qian F, Huang Q, Wei L, Fu Y, Du Y (2018) HOXA4, down-regulated in lung cancer, inhibits the growth, motility and invasion of lung cancer cells. Cell Death Dis 9(5):1–13

Choi AM, Ryter SW, Levine B (2013) Autophagy in human health and disease. N Engl J Med 368(7):651–662

Chotiner JY, Wolgemuth DJ, Wang PJ (2019) Functions of cyclins and CDKs in mammalian gametogenesis. Biol Reprod 101(3):591–601

Chu D-T, Nguyen TT, Tien NLB, Tran D-K, Jeong J-H, Anh PG, Thanh VV, Truong DT, Dinh TC (2020) Recent progress of stem cell therapy in cancer treatment: molecular mechanisms and potential applications. Cells 9:563. https://doi.org/10.3390/cells9030563

Chung YC, Chang YF (2003) Serum interleukin-6 levels reflect the disease status of colorectal cancer. J Surg Oncol 83(4):222–226

Clevers H, Loh KM, Nusse R (2014) An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science 346:1248012. https://doi.org/10.1126/science.1248012

Comel A, Sorrentino G, Capaci V, Del Sal G (2014) The cytoplasmic side of p53's oncosuppressive activities. FEBS Lett 588(16):2600–2609

Cooks T, Pateras IS, Jenkins LM, Patel KM, Robles AI, Morris J, Forshew T, Appella E, Gorgoulis VG, Harris CC (2018) Mutant p53 cancers reprogram macrophages to tumor supporting macrophages via exosomal miR-1246. Nat Commun 9(1):1–15

Crupi A, Costa A, Tarnok A, Melzer S, Teodori L (2015) Inflammation in tissue engineering: the Janus between engraftment and rejection. Eur J Immunol 45:3222–3236. https://doi.org/10.1002/eji.201545818

Culig Z, Puhr M (2012) Interleukin-6: a multifunctional targetable cytokine in human prostate cancer. Mol Cell Endocrinol 360(1–2):52–58

Cunningham R, Hansen CG (2022) The Hippo pathway in cancer: YAP/TAZ and TEAD as therapeutic targets in cancer. Clin Sci 136(3):197–222

Daley WP, Peters SB, Larsen M (2008) Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci 121:255–264. https://doi.org/10.1242/jcs.006064

Davidson BL, McCray PB (2011) Current prospects for RNA interference-based therapies. Nat Rev Genet 12:329–340. https://doi.org/10.1038/nrg2968

De Benedetti A, Graff JR (2004) eIF-4E expression and its role in malignancies and metastases. Oncogene 23:3189–3199. https://doi.org/10.1038/sj.onc.1207545

De la Cueva E, Garcia-Cao I, Herranz M, Lopez P, Garcia-Palencia P, Flores JM, Serrano M, Fernandez-Piqueras J, Martin-Caballero J (2006) Tumorigenic activity of p21Waf1/Cip1 in thymic lymphoma. Oncogene 25(29):4128–4132. https://doi.org/10.1038/sj.onc.1209432

Del Bene F, Wittbrodt J (2005) Cell cycle control by homeobox genes in development and disease. In: Seminars in cell & developmental biology, vol 16, no. 3. Academic, p 449–460 https://doi.org/10.1016/j.semcdb.2005.02.001

Demoulin JB, Essaghir A (2014) PDGF receptor signaling networks in normal and cancer cells. Cytokine Growth Factor Rev 25(3):273–283. https://doi.org/10.1016/j.cytogfr.2014.03.003

Deshpande A, Sicinski P, Hinds PW (2005) Cyclins and cdks in development and cancer: a perspective. Oncogene 24(17):2909–2915. https://doi.org/10.1038/sj.onc.1208618

Dethlefsen C, Højfeldt G, Hojman P (2013) The role of intratumoral and systemic IL-6 in breast cancer. Breast Cancer Res Treat 138(3):657–664. https://doi.org/10.1007/s10549-013-2488-z

Di Leva G, Garofalo M, Croce CM (2014) MicroRNAs in cancer. Annu Rev Pathol 9:287–314. https://doi.org/10.1146/annurev-pathol-012513-104715

Dieci MV, Arnedos M, Andre F, Soria JC (2013) Fibroblast growth factor receptor inhibitors as a cancer treatment: from a biologic rationale to medical perspectives. Cancer Discov 3(3): 264–279

Dillon RL, White DE, Muller WJ (2007) The phosphatidylinositol 3-kinase signaling network: implications for human breast cancer. Oncogene 26(9):1338–1345. https://doi.org/10.1158/2159-8290.CD-12-0362

Dong Q, Liu X, Cheng K, Sheng J, Kong J, Liu T (2021) Pre-metastatic niche formation in different organs induced by tumor extracellular vesicles. Front Cell Dev Biol 9:733627. https://doi.org/10.3389/fcell.2021.733627

Du N, Guo F, Wang Y, Cui J (2021) NK cell therapy: a rising star in cancer treatment. Cancers 13: 4129. https://doi.org/10.3390/cancers13164129

Duffy MJ, Synnott NC, O'Grady S, Crown J (2020) Targeting p53 for the treatment of cancer. In: Seminars in cancer biology. Academic. https://doi.org/10.1016/j.semcancer.2020.07.005

Duronio RJ, Xiong Y (2013) Signaling pathways that control cell proliferation. Cold Spring Harb Perspect Biol 5:a008904–a008904. https://doi.org/10.1101/cshperspect.a008904

Easwaran H, Tsai HC, Baylin SB (2014) Cancer epigenetics: tumor heterogeneity, plasticity of stem-like states, and drug resistance. Mol Cell 54(5):716–727

Edmonston TB, Cuesta KH, Burkholder S, Barusevicius A, Rose D, Kovatich AJ, Boman B, Fry R, Fishel R, Palazzo JP (2000) Colorectal carcinomas with high microsatellite instability: defining a distinct immunologic and molecular entity with respect to prognostic markers. Hum Pathol 31(12):1506–1514

Efeyan A, Collado M, Velasco-Miguel S, Serrano M (2007) Genetic dissection of the role of p21Cip1/Waf1 in p53-mediated tumour suppression. Oncogene 26(11):1645–1649

Efiok BJ, Safer B (2000) Transcriptional regulation of E2F-1 and eIF-2 genes by α-Pal: a potential mechanism for coordinated regulation of protein synthesis, growth, and the cell cycle. Biochim Biophys Acta 1495(1):51–68

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494–498. https://doi.org/10.1038/35078107

El-Deiry WS (2016) p21(WAF1) mediates cell-cycle inhibition, relevant to cancer suppression and therapy. Cancer Res 76:5189–5191. https://doi.org/10.1158/0008-5472.CAN-16-2055

El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75(4):817–825

Elliott RL, Blobe GC (2005) Role of transforming growth factor Beta in human cancer. J Clin Oncol 23(9):2078–2093
Eming SA, Krieg T, Davidson JM (2007) Inflammation in wound repair: molecular and cellular mechanisms. J Investig Dermatol 127:514–525. https://doi.org/10.1038/sj.jid.5700701
Fan Y, Chen C, Huang Y, Zhang F, Lin G (2017) Study of the pH-sensitive mechanism of tumor-targeting liposomes. Colloids Surf B: Biointerfaces 151:19–25. https://doi.org/10.1016/j.colsurfb.2016.11.042
Farazi TA, Spitzer JI, Morozov P, Tuschl T (2011) miRNAs in human cancer: miRNAs in human cancer. J Pathol 223:102–115. https://doi.org/10.1002/path.2806
Farghadani R, Naidu R (2021) The role of apoptosis as a double-edge sword in cancer. Regulation and dysfunction of apoptosis
Farooq M, Khan AW, Kim MS, Choi S (2021) The role of fibroblast growth factor (FGF) Signaling in tissue repair and regeneration. Cells 10:3242. https://doi.org/10.3390/cells10113242
Feng R, Wen J (2015) Overview of the roles of Sox2 in stem cell and development. Biol Chem 396: 883–891. https://doi.org/10.1515/hsz-2014-0317
Feng F, Ren Q, Wu S, Saeed M, Sun C (2017) Hoxa5 increases mitochondrial apoptosis by inhibiting Akt/mTORC1/S6K1 pathway in mice white adipocytes. Oncotarget 8(56):95332
Fiorenza MT, Rava A (2019) The TCL1 function revisited focusing on metabolic requirements of stemness. Cell Cycle 18(22):3055–3063
Firtina Karagonlar Z, Akbari S, Karabicici M, Sahin E, Tercan Avci S, Ersoy N, Eren Ates K, Balli T, Karacicek B, Kaplan KN, Celiker C (2020) A novel function for KLF4 in modulating the de-differentiation of EpCAM−/CD133− nonStem cells into EpCAM+/CD133+ liver cancer stem cells in HCC cell line HuH7. Cells 9(5):1198
Frank DA (2007) STAT3 as a central mediator of neoplastic cellular transformation. Cancer Lett 251(2):199–210
Fu M, Wang C, Li Z, Sakamaki T, Pestell RG (2004) Minireview: cyclin D1: normal and abnormal functions. Endocrinology 145(12):5439–5447
Gao X, Kim K-S, Liu D (2007) Nonviral gene delivery: what we know and what is next. AAPS J 9: E92–E104. https://doi.org/10.1208/aapsj0901009
Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E, Cerami E (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 6(269):pl1
Gehl J (2003) Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. Acta Physiol Scand 177:437–447. https://doi.org/10.1046/j.1365-201X.2003.01093.x
Geisen C, Möröy T (2002) The oncogenic activity of cyclin E is not confined to Cdk2 activation alone but relies on several other, distinct functions of the protein. J Biol Chem 277(42): 39909–39918
Gilbert R, Vickaryous M, Viloria-Petit A (2016) Signalling by transforming growth factor beta isoforms in wound healing and tissue regeneration. J Dev Biol 4:21. https://doi.org/10.3390/jdb4020021
Goel HL, Mercurio AM (2013) VEGF targets the tumour cell. Nat Rev Cancer 13(12):871–882
Golberg A (2020) Chapter 9: Emerging electroporation-based technologies for wound care. In: Gefen A (ed) Innovations and emerging technologies in wound care. Academic, pp 155–170. https://doi.org/10.1016/B978-0-12-815028-3.00009-2
Goldman JA, Poss KD (2020) Gene regulatory programmes of tissue regeneration. Nat Rev Genet 21:511–525. https://doi.org/10.1038/s41576-020-0239-7
Goyal R, Reinhardt R, Jeltsch A (2006) Accuracy of DNA methylation pattern preservation by the Dnmt1 methyltransferase. Nucleic Acids Res 34(4):1182–1188
Gross G, Waks T, Eshhar Z (1989) Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proc Natl Acad Sci 86: 10024–10028. https://doi.org/10.1073/pnas.86.24.1002Gross4

Guertin DA, Sabatini DM (2007) Defining the role of mTOR in cancer. Cancer Cell 12:9–22. https://doi.org/10.1016/j.ccr.2007.05.008
Gurtner GC, Callaghan MJ, Longaker MT (2007) Progress and potential for regenerative medicine. Annu Rev Med 58:299–312. https://doi.org/10.1146/annurev.med.58.082405.095329
Han Y (2019) Analysis of the role of the Hippo pathway in cancer. J Transl Med 17(1):1–17. https://doi.org/10.1186/s12967-019-1869-4
Hao P, Yu J, Ward R, Liu Y, Hao Q, An S, Xu T (2020) Eukaryotic translation initiation factors as promising targets in cancer therapy. Cell Commun Signal 18(1):1–20
Harvey KF, Zhang X, Thomas DM (2013) The Hippo pathway and human cancer. Nat Rev Cancer 13(4):246–257
Hasmim M, Noman MZ, Lauriol J et al (2011) Hypoxia-dependent inhibition of tumor cell susceptibility to CTL-mediated lysis involves NANOG induction in target cells. J Immunol 187:4031–4039
He C, Wang L, Li L, Zhu G (2021) Extracellular vesicle-orchestrated crosstalk between cancer-associated fibroblasts and tumors. Transl Oncol 14(12):101231
Hemmings BA, Restuccia DF (2012) PI3K-PKB/Akt pathway. Cold Spring Harb Perspect Biol 4: a011189–a011189. https://doi.org/10.1101/cshperspect.a011189
Hennessy BT, Smith DL, Ram PT, Lu Y, Mills GB (2005) Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 4(12):988–1004
Holczbauer Á, Factor VM, Andersen JB, Marquardt JU, Kleiner DE, Raggi C, Kitade M, Seo D, Akita H, Durkin ME, Thorgeirsson SS (2013) Modeling pathogenesis of primary liver cancer in lineage-specific mouse cell types. Gastroenterology 145(1):221–231
Hollern DP, Honeysett J, Cardiff RD, Andrechek ER (2014) The E2F transcription factors regulate tumor development and metastasis in a mouse model of metastatic breast cancer. Mol Cell Biol 34(17):3229–3243
Hounsell C, Fan Y (2021) The duality of caspases in Cancer, as told through the fly. Int J Mol Sci 22(16):8927
Hsu J, Sage J (2016) Novel functions for the transcription factor E2F4 in development and disease. Cell Cycle 15:3183–3190. https://doi.org/10.1080/15384101.2016.1234551
Hua H, Kong Q, Zhang H, Wang J, Luo T, Jiang Y (2019) Targeting mTOR for cancer therapy. J Hematol Oncol 12(1):1–19
Huang J, Chen T, Liu X, Jiang J, Li J, Li D, Liu XS, Li W, Kang J, Pei G (2009) More synergetic cooperation of Yamanaka factors in induced pluripotent stem cells than in embryonic stem cells. Cell Res 19(10):1127–1138
Huang J, Luo Q, Xiao Y, Li H, Kong L, Ren G (2017) The implication from RAS/RAF/ERK signaling pathway increased activation in epirubicin treated triple negative breast cancer. Oncotarget 8(64):108249
Ibraheem D, Elaissari A, Fessi H (2014) Gene therapy and DNA delivery systems. Int J Pharm 459: 70–83. https://doi.org/10.1016/j.ijpharm.2013.11.041
Islam F, Qiao B, Smith RA, Gopalan V, Lam AKY (2015) Cancer stem cell: fundamental experimental pathological concepts and updates. Exp Mol Pathol 98(2):184–191
Jang Y-N, Baik EJ (2013) JAK-STAT pathway and myogenic differentiation. JAKSTAT 2:e23282. https://doi.org/10.4161/jkst.23282
Jansson L, Kim GS, Cheng AG (2015) Making sense of Wnt signaling—linking hair cell regeneration to development. Front Cell Neurosci 9:66. https://doi.org/10.3389/fncel.2015.00066
Jean S, Kiger AA (2014) Classes of phosphoinositide 3-kinases at a glance. J Cell Sci 127(5): 923–928
Jeong EG, Kim MS, Nam HK, Min CK, Lee S, Chung YJ, Yoo NJ, Lee SH (2008) Somatic mutations of JAK1 and JAK3 in acute leukemias and solid cancers. Clin Cancer Res 14(12): 3716–3721
Jeter CR, Badeaux M, Choy G et al (2009) Functional evidence that the self-renewal gene NANOG regulates human tumor development. Stem Cells 27:993–1005

Jeter CR, Liu B, Liu X et al (2011) NANOG promotes cancer stem cell characteristics and prostate cancer resistance to androgen deprivation. Oncogene 30:3833–3845

Ji W, Jiang Z (2013) Effect of shRNA-mediated inhibition of Nanog gene expression on the behavior of human gastric cancer cells. Oncol Lett 6:367–374

Jinno T, Kawano S, Maruse Y, Matsubara R, Goto Y, Sakamoto T, Hashiguchi Y, Kaneko N, Tanaka H, Kitamura R, Toyoshima T (2015) Increased expression of interleukin-6 predicts poor response to chemoradiotherapy and unfavorable prognosis in oral squamous cell carcinoma. Oncol Rep 33(5):2161–2168

Johnson R, Halder G (2014) The two faces of Hippo: targeting the Hippo pathway for regenerative medicine and cancer treatment. Nat Rev Drug Discov 13(1):63–79

Kamalvand M, Biazar E, Daliri-Joupari M, Montazer F, Rezaei-Tavirani M, Heidari-Keshel S (2021) Design of a decellularized fish skin as a biological scaffold for skin tissue regeneration. Tissue Cell 71:101509. https://doi.org/10.1016/j.tice.2021.101509

Karin M, Clevers H (2016) Reparative inflammation takes charge of tissue regeneration. Nature 529:307–315. https://doi.org/10.1038/nature17039

Kay MA, Glorioso JC, Naldini L (2001) Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med 7:33–40. https://doi.org/10.1038/83324

Ke X, Shelton L, Hu Y, Zhu Y, Chow E, Tang H, Santos JL, Mao H-Q (2020) Surface-functionalized PEGylated nanoparticles deliver messenger RNA to pulmonary immune cells. ACS Appl Mater Interfaces 12:35835–35844. https://doi.org/10.1021/acsami.0c08268

Kent LN, Leone G (2019) The broken cycle: E2F dysfunction in cancer. Nat Rev Cancer 19(6): 326–338

Khan OF, Zaia EW, Yin H, Bogorad RL, Pelet JM, Webber MJ, Zhuang I, Dahlman JE, Langer R, Anderson DG (2014) Ionizable amphiphilic dendrimer-based nanomaterials with alkyl-chain-substituted amines for tunable siRNA delivery to the liver endothelium in vivo. Angew Chem Int Ed 53:14397–14401. https://doi.org/10.1002/anie.201408221

Kirkwood TB, Melov S (2011) On the programmed/non-programmed nature of aging within the life history. Curr Biol 21(18):R701–R707

Ko SY, Barengo N, Ladanyi A, Lee JS, Marini F, Lengyel E, Naora H (2012) HOXA9 promotes ovarian cancer growth by stimulating cancer-associated fibroblasts. J Clin Invest 122(10): 3603–3617

Kotowicz B, Fuksiewicz M, Jonska-Gmyrek J, Bidzinski M, Kowalska M (2016) The assessment of the prognostic value of tumor markers and cytokines as SCCAg, CYFRA 21.1, IL-6, VEGF and sTNF receptors in patients with squamous cell cervical cancer, particularly with early stage of the disease. Tumor Biol 37(1):1271–1278

Kozielski KL, Ruiz-Valls A, Tzeng SY, Guerrero-Cázares H, Rui Y, Li Y, Vaughan HJ, Gionet-Gonzales M, Vantucci C, Kim J, Schiapparelli P, Al-Kharboosh R, Quiñones-Hinojosa A, Green JJ (2019) Cancer-selective nanoparticles for combinatorial siRNA delivery to primary human GBM in vitro and in vivo. Biomaterials 209:79–87. https://doi.org/10.1016/j.biomaterials.2019.04.020

Kretsovali A (2017) Dissecting the role of Sox2 in stemness regulation and regenerative medicine. J Stem Cell Res Transplant 4:1026. https://doi.org/10.26420/jstemcellrestransplant.2017.1026

Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. Nature 443(7110): 453–457

La Fortezza M, Schenk M, Cosolo A, Kolybaba A, Grass I, Classen A-K (2016) JAK/STAT signalling mediates cell survival in response to tissue stress. Development 143(16):2907–2919. https://doi.org/10.1242/dev.132340

LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A, Harlow ED (1997) New functional activities for the p21 family of CDK inhibitors. Genes Dev 11(7): 847–862

Lagadec C, Vlashi E, Della Donna L, Dekmezian C, Pajonk F (2012) Radiation-induced reprogramming of breast cancer cells. Stem Cells 30(5):833–844

Lane DP (1992) p53, guardian of the genome. Nature 358(6381):15–16
Levine AJ (2020) p53: 800 million years of evolution and 40 years of discovery. Nat Rev Cancer 20(8):471–480
Levine AJ, Jenkins NA, Copeland NG (2019) The roles of initiating truncal mutations in human cancers: the order of mutations and tumor cell type matters. Cancer Cell 35(1):10–15
Li FL, Guan KL (2021) The two sides of Hippo pathway in cancer. In: Seminars in cancer biology. Academic
Li S-D, Huang L (2006) Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene Ther 13:1313–1319. https://doi.org/10.1038/sj.gt.3302838
Li SD, Ma M, Li H, Waluszko A, Sidorenko T, Schadt EE, Zhang DY, Chen R, Ye F (2017) Cancer gene profiling in non-small cell lung cancers reveals activating mutations in JAK2 and JAK3 with therapeutic implications. Genome Med 9(1):1–11
Liang G, Chan MF, Tomigahara Y, Tsai YC, Gonzales FA, Li E, Laird PW, Jones PA (2002) Cooperativity between DNA methyltransferases in the maintenance methylation of repetitive elements. Mol Cell Biol 22(2):480–491
Lim CP, Cao X (2006) Structure, function, and regulation of STAT proteins. Mol BioSyst 2(11): 536–550
Lim S, Kaldis P (2013) Cdks, cyclins and CKIs: roles beyond cell cycle regulation. Development 140(15):3079–3093
Lim SM, Mohamad Hanif EA, Chin SF (2021) Is targeting autophagy mechanism in cancer a good approach? The possible double-edge sword effect. Cell Biosci 11(1):1–13
Lin MT, Pulkkinen L, Uitto J, Yoon K (2000) The gene gun: current applications in cutaneous gene therapy. Int J Dermatol 39:161–170. https://doi.org/10.1046/j.1365-4362.2000.00925.x
Lin ZP, Zhu YL, Ratner ES (2018) Targeting cyclin-dependent kinases for treatment of gynecologic cancers. Front Oncol 8:303
Lister R, Pelizzola M, Dowen RH, Hawkins RD, Hon G, Tonti-Filippini J, Nery JR, Lee L, Ye Z, Ngo QM, Edsall L (2009) Human DNA methylomes at base resolution show widespread epigenomic differences. Nature 462(7271):315–322
Liu Z, Jiang Z, Gao Y, Wang L, Chen C, Wang X (2019) TP53 mutations promote immunogenic activity in breast cancer. J Oncol 2019:5952836
Loyer P, Trembley JH (2020) Roles of CDK/Cyclin complexes in transcription and pre-mRNA splicing: cyclins L and CDK11 at the cross-roads of cell cycle and regulation of gene expression. In: Seminars in cell & developmental biology, vol 107. Academic, pp 36–45
Luo N, Balko JM (2019) Role of JAK-STAT pathway in cancer signaling. In: Predictive biomarkers in oncology. p 311–319
Luo D, Saltzman WM (2000) Synthetic DNA delivery systems. Nat Biotechnol 18:33–37. https://doi.org/10.1038/71889
Lv X, Li L, Lv L, Qu X, Jin S, Li K, Deng X, Cheng L, He H, Dong L (2015) HOXD9 promotes epithelial–mesenchymal transition and cancer metastasis by ZEB1 regulation in hepatocellular carcinoma. J Exp Clin Cancer Res 34(1):1–13
Ma C-C, Wang Z-L, Xu T, He Z-Y, Wei Y-Q (2020) The approved gene therapy drugs worldwide: from 1998 to 2019. Biotechnol Adv 40:107502. https://doi.org/10.1016/j.biotechadv.2019.107502
Macciò A, Madeddu C (2013) The role of interleukin-6 in the evolution of ovarian cancer: clinical and prognostic implications—a review. J Mol Med 91(12):1355–1368
Malumbres M (2007) Cyclins and related kinases in cancer cells. J BUON 12:S45–S52
Manou D, Caon I, Bouris P, Triantaphyllidou IE, Giaroni C, Passi A, Karamanos NK, Vigetti D, Theocharis AD (2019) The complex interplay between extracellular matrix and cells in tissues. The extracellular matrix. Methods Mol Biol 1952:1–20
Mantovani F, Collavin L, Del Sal G (2019) Mutant p53 as a guardian of the cancer cell. Cell Death Differ 26(2):199–212
Mao AS, Mooney DJ (2015) Regenerative medicine: current therapies and future directions. Proc Natl Acad Sci 112:14452–14459. https://doi.org/10.1073/pnas.1508520112

Mason C, Dunnill P (2008) A brief definition of regenerative medicine. Regen Med 3:1–5. https://doi.org/10.2217/17460751.3.1.1

McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, Chang F, Lehmann B, Terrian DM, Milella M, Tafuri A, Stivala F (2007) Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta 1773(8):1263–1284

McIlwain DR, Berger T, Mak TW (2013) Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 5(4):a008656

Mehier-Humbert S, Guy RH (2005) Physical methods for gene transfer: improving the kinetics of gene delivery into cells. Adv Drug Deliv Rev 57:733–753. https://doi.org/10.1016/j.addr.2004.12.007

Miliotou AN, Papadopoulou LC (2018) CAR T-cell therapy: a new era in cancer immunotherapy. Curr Pharm Biotechnol 19:5–18. https://doi.org/10.2174/1389201019666180418095526

Miller KR, Patel JN, Zhang Q, Norris EJ, Symanowski J, Michener C, Sehouli J, Braicu I, Destephanis DD, Sutker AP, Jones W (2018) HOXA4/HOXB3 gene expression signature as a biomarker of recurrence in patients with high-grade serous ovarian cancer following primary cytoreductive surgery and first-line adjuvant chemotherapy. Gynecol Oncol 149(1):155–162

Mills CD (2015) Anatomy of a discovery: M1 and M2 macrophages. Front Immunol 6:212. https://doi.org/10.3389/fimmu.2015.00212

Minucci S, Monestiroli S, Giavara S, Ronzoni S, Marchesi F, Insinga A, Diverio D, Gasparini P, Capillo M, Colombo E, Matteucci C (2002) PML-RAR induces promyelocytic leukemias with high efficiency following retroviral gene transfer into purified murine hematopoietic progenitors. Blood 100(8):2989–2995

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S (2003) The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631–642

Miura T, Mitsunaga S, Ikeda M, Shimizu S, Ohno I, Takahashi H, Furuse J, Inagaki M, Higashi S, Kato H, Terao K (2015) Characterization of patients with advanced pancreatic cancer and high serum interleukin-6 levels. Pancreas 44(5):756–763

Mizushima N (2007) Autophagy: process and function. Genes Dev 21(22):2861–2873

Mohr S, Doebele C, Comoglio F, Berg T, Beck J, Bohnenberger H, Alexe G, Corso J, Ströbel P, Wachter A, Beissbarth T (2017) Hoxa9 and Meis1 cooperatively induce addiction to Syk signaling by suppressing miR-146a in acute myeloid leukemia. Cancer Cell 31(4):549–562

Moore DL, Apara A, Goldberg JL (2011) Krüppel-like transcription factors in the nervous system: novel players in neurite outgrowth and axon regeneration. Mol Cell Neurosci 47:233–243. https://doi.org/10.1016/j.mcn.2011.05.005

Muangmoonchai R, Wong SC, Smirlis D, Phillips IR, Shephard EA (2002) Transfection of liver in vivo by biolistic particle delivery. Mol Biotechnol 20:145–151. https://doi.org/10.1385/MB:20:2:145

Munier S, Messai I, Delair T, Verrier B, Ataman-Onal Y (2005) Cationic PLA nanoparticles for DNA delivery: comparison of three surface polycations for DNA binding, protection and transfection properties. Colloids Surf B Biointerfaces 43:163–173. https://doi.org/10.1016/j.colsurfb.2005.05.001

Nagasaki T, Shinkai S (2007) The concept of molecular machinery is useful for design of stimuli-responsive gene delivery systems in the mammalian cell. J Incl Phenom Macrocycl Chem 58:205–219. https://doi.org/10.1007/s10847-007-9303-6

Nakatani K, Maehama T, Nishio M, Goto H, Kato W, Omori H, Miyachi Y, Togashi H, Shimono Y, Suzuki A (2017) Targeting the Hippo signalling pathway for cancer treatment. J Biochem 161(3):237–244

Neuzillet C, Tijeras-Raballand A, de Mestier L, Cros J, Faivre S, Raymond E (2014) MEK in cancer and cancer therapy. Pharmacol Ther 141(2):160–171

Nielsen C, Birgens HS, Nordestgaard BG, Kjær L, Bojesen SE (2011) The JAK2 V617F somatic mutation, mortality and cancer risk in the general population. Haematologica 96(3):450

Niidome T, Huang L (2002) Gene therapy progress and prospects: nonviral vectors. Gene Ther 9: 1647–1652. https://doi.org/10.1038/sj.gt.3301923
Niland S, Eble JA (2020) Hold on or cut? Integrin-and MMP-mediated cell–matrix interactions in the tumor microenvironment. Int J Mol Sci 22(1):238
Nunnery SE, Mayer IA (2020) Targeting the PI3K/AKT/mTOR pathway in hormone-positive breast cancer. Drugs 80(16):1685–1697
Olsson M, Zhivotovsky B (2011) Caspases and cancer. Cell Death Differ 18(9):1441–1449
Oviedo NJ, Beane WS (2009) Regeneration: the origin of cancer or a possible cure? Semin Cell Dev Biol 20:557–564. https://doi.org/10.1016/j.semcdb.2009.04.005
Owen KL, Brockwell NK, Parker BS (2019) JAK-STAT signaling: a double-edged sword of immune regulation and cancer progression. Cancer 11(12):2002
Ozbolat IT (2015) Bioprinting scale-up tissue and organ constructs for transplantation. Trends Biotechnol 33:395–400. https://doi.org/10.1016/j.tibtech.2015.04.005
Pan H, Cai N, Li M, Liu G, Izpisua Belmonte JC (2013) Autophagic control of cell 'stemness'. EMBO Mol Med 5:327–331. https://doi.org/10.1002/emmm.201201999
Patel S, Alam A, Pant R, Chattopadhyay S (2019) Wnt signaling and its significance within the tumor microenvironment: novel therapeutic insights. Front Immunol 10:2872
Paul U, Banerjee S (2022) The functional significance and cross-talk of non-coding RNAs in triple negative and quadruple negative breast cancer. Mol Biol Rep 49(7):6899–6918
Pedroza-Torres A, Romero-Córdoba SL, Justo-Garrido M, Salido-Guadarrama I, Rodríguez-Bautista R, Montaño S, Muñiz-Mendoza R, Arriaga-Canon C, Fragoso-Ontiveros V, Álvarez-Gómez RM, Hernández G, Herrera LA (2019) MicroRNAs in tumor cell metabolism: roles and therapeutic opportunities. Front Oncol 9:1404. https://doi.org/10.3389/fonc.2019.01404
Pencik J, Pham HTT, Schmoellerl J, Javaheri T, Schlederer M, Culig Z, Merkel O, Moriggl R, Grebien F, Kenner L (2016) JAK-STAT signaling in cancer: from cytokines to non-coding genome. Cytokine 87:26–36
Peng Y, Croce CM (2016) The role of MicroRNAs in human cancer. Signal Transduct Target Ther 1:15004. https://doi.org/10.1038/sigtrans.2015.4
Pesic M, Greten FR (2016) Inflammation and cancer: tissue regeneration gone awry. Curr Opin Cell Biol 43:55–61. https://doi.org/10.1016/j.ceb.2016.07.010
Petit-Zeman S (2001) Regenerative medicine. Nat Biotechnol 19:201–206. https://doi.org/10.1038/85619
Peyssonnaux C, Eychène A (2012) The Raf/MEK/ERK pathway: new concepts of activation. Biol Cell 93:53–62. https://doi.org/10.1016/S0248-4900(01)01125-X
Piperigkou Z, Kyriakopoulou K, Koutsakis C, Mastronikolis S, Karamanos NK (2021) Key matrix remodeling enzymes: functions and targeting in cancer. Cancers 13(6):1441
Porta C, Paglino C, Mosca A (2014) Targeting PI3K/Akt/mTOR signaling in cancer. Front Oncol 4: 64
Pradere JP, Dapito DH, Schwabe RF (2014) The Yin and Yang of Toll-like receptors in cancer. Oncogene 33(27):3485–3495
Qiao C, Huang W, Chen J, Feng W, Zhang T, Wang Y, Liu D, Ji X, Xie M, Sun M, Fan D (2021) IGF1-mediated HOXA13 overexpression promotes colorectal cancer metastasis through upregulating ACLY and IGF1R. Cell Death Dis 12(6):1–18
Qin S, Zou Y, Zhang C-L (2013) Cross-talk between KLF4 and STAT3 regulates axon regeneration. Nat Commun 4:2633. https://doi.org/10.1038/ncomms3633
Quéré R, Karlsson G, Hertwig F, Rissler M, Lindqvist B, Fioretos T, Vandenberghe P, Slovak ML, Cammenga J, Karlsson S (2011) Smad4 binds Hoxa9 in the cytoplasm and protects primitive hematopoietic cells against nuclear activation by Hoxa9 and leukemia transformation. Blood 117(22):5918–5930
Rebouissou S, Amessou M, Couchy G, Poussin K, Imbeaud S, Pilati C, Izard T, Balabaud C, Bioulac-Sage P, Zucman-Rossi J (2009) Frequent in-frame somatic deletions activate gp130 in inflammatory hepatocellular tumours. Nature 457(7226):200–204

Reddy KB (2015) MicroRNA (miRNA) in cancer. Cancer Cell Int 15:38. https://doi.org/10.1186/s12935-015-0185-1
Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature 434:843–850. https://doi.org/10.1038/nature03319
Riedel F, Zaiss I, Herzog D, Götte K, Naim R, Hörmann K (2005) Serum levels of interleukin-6 in patients with primary head and neck squamous cell carcinoma. Anticancer Res 25(4): 2761–2765
Robertson KD (2005) DNA methylation and human disease. Nat Rev Genet 6(8):597–610
Roeser JC, Leach SD, McAllister F (2015) Emerging strategies for cancer immunoprevention. Oncogene 34(50):6029–6039
Rowe RG, Weiss SJ (2009) Navigating ECM barriers at the invasive front: the cancer cell–stroma interface. Annu Rev Cell Dev 25:567–595
Roy S, Kar M, Roy S, Padhi S, Saha A, Banerjee B (2019) KLF4 expression in the surgical cut margin is associated with disease relapse of oral squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol 128(2):154–165
Sadelain M, Brentjens R, Rivière I (2013) The basic principles of chimeric antigen receptor design. Cancer Discov 3:388–398. https://doi.org/10.1158/2159-8290.CD-12-0548
Sanguinete MMM, Oliveira PHD, Martins-Filho A, Micheli DC, Tavares-Murta BM, Murta EFC, Nomelini RS (2017) Serum IL-6 and IL-8 correlate with prognostic factors in ovarian cancer. Immunol Investig 46(7):677–688
Saunders A, Faiola F, Wang J (2013) Concise review: pursuing self-renewal and pluripotency with the stem cell factor nanog. Stem Cells 31:1227–1236. https://doi.org/10.1002/stem.1384
Scourzic L, Mouly E, Bernard OA (2015) TET proteins and the control of cytosine demethylation in cancer. Genome Med 7(1):1–16
Seifert A (2015) Role of Hox genes in stem cell differentiation. World J Stem Cells 7:583. https://doi.org/10.4252/wjsc.v7.i3.583
Selivanova G (2010) Therapeutic targeting of p53 by small molecules. Semin Cancer Biol 20:46–56. https://doi.org/10.1016/j.semcancer.2010.02.006
Sen CK, Ghatak S (2015) miRNA control of tissue repair and regeneration. Am J Pathol 185:2629–2640. https://doi.org/10.1016/j.ajpath.2015.04.001
Shah N, Sukumar S (2010) The Hox genes and their roles in oncogenesis. Nat Rev Cancer 10(5): 361–371
Shalini S, Dorstyn L, Dawar S, Kumar S (2015) Old, new and emerging functions of caspases. Cell Death Differ 22:526–539. https://doi.org/10.1038/cdd.2014.216
Sharma A, Mir R, Galande S (2021) Epigenetic regulation of the Wnt/β-catenin signaling pathway in cancer. Front Genet 12:681053
Shen KC, Heng H, Wang Y, Lu S, Liu G, Deng CX, Brooks SC, Wang YA (2005) ATM and p21 cooperate to suppress aneuploidy and subsequent tumor development. Cancer Res 65(19): 8747–8753
Shin DS, Zaretsky JM, Escuin-Ordinas H, Garcia-Diaz A, Hu-Lieskovan S, Kalbasi A, Grasso CS, Hugo W, Sandoval S, Torrejon DY, Palaskas N (2017) Primary resistance to PD-1 blockade mediated by JAK1/2 mutations. Cancer Discov 7(2):188–201
Si W, Shen J, Zheng H, Fan W (2019) The role and mechanisms of action of microRNAs in cancer drug resistance. Clin Epigenetics 11(1):25
Siegl-Cachedenier I, Muñoz P, Flores JM, Klatt P, Blasco MA (2007) Deficient mismatch repair improves organismal fitness and survival of mice with dysfunctional telomeres. Genes Dev 21(17):2234–2247
Smetana K, Dvořánková B, Lacina L (2013) Phylogeny, regeneration, ageing and cancer: role of microenvironment and possibility of its therapeutic manipulation. Folia Biol (Praha) 59:207–216
Song H, Hollstein M, Xu Y (2007) p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. Nat Cell Biol 9(5):573–580

Sonnenblick A, Shriki A, Galun E, Axelrod JH, Daum H, Rottenberg Y, Hamburger T, Mali B, Peretz T (2012) Tissue microarray-based study of patients with lymph node-positive breast cancer shows tyrosine phosphorylation of signal transducer and activator of transcription 3 (tyrosine705-STAT3) is a marker of good prognosis. Clin Transl Oncol 14(3):232–236

Sparks AB, Morin PJ, Vogelstein B, Kinzler KW (1998) Mutational analysis of the APC/β-catenin/Tcf pathway in colorectal cancer. Cancer Res 58(6):1130–1134

Stamatakos M, Palla V, Karaiskos I, Xiromeritis K, Alexiou I, Pateras I, Kontzoglou K (2010) Cell cyclins: triggering elements of cancer or not? World J Surg Oncol 8(1):1–8

Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF (2008) An integrative genomic and proteomic analysis of PIK3CA, PTEN, and AKT mutations in breast cancer. Cancer Res 68(15):6084–6091

Stribley JM, Rehman KS, Niu H, Christman GM (2002) Gene therapy and reproductive medicine. Fertil Steril 77:645–657. https://doi.org/10.1016/S0015-0282(01)03233-2

Tadesse S, Caldon EC, Tilley W, Wang S (2018) Cyclin-dependent kinase 2 inhibitors in cancer therapy: an update. J Med Chem 62(9):4233–4251

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663–676

Takemoto S, Ushijima K, Kawano K, Yamaguchi T, Terada A, Fujiyoshi N, Nishio S, Tsuda N, Ijichi M, Kakuma T, Kage M (2009) Expression of activated signal transducer and activator of transcription-3 predicts poor prognosis in cervical squamous-cell carcinoma. Br J Cancer 101(6):967–972

Tan Z, Chen K, Wu W, Zhou Y, Zhu J, Wu G, Cao L, Zhang X, Guan H, Yang Y, Zhang W (2018) Overexpression of HOXC10 promotes angiogenesis in human glioma via interaction with PRMT5 and upregulation of VEGFA expression. Theranostics 8(18):5143

Taura M, Fukuda R, Suico MA, Eguma A, Koga T, Shuto T, Sato T, Morino-Koga S, Kai H (2010) TLR3 induction by anticancer drugs potentiates poly I: C-induced tumor cell apoptosis. Cancer Sci 101(7):1610–1617

Thatcher EJ, Patton JG (2010) Small RNAs have a big impact on regeneration. RNA Biol 7:333–338. https://doi.org/10.4161/rna.7.3.12085

Thiem A, Hesbacher S, Kneitz H, di Primio T, Heppt MV, Hermanns HM, Goebeler M, Meierjohann S, Houben R, Schrama D (2019) IFN-gamma-induced PD-L1 expression in melanoma depends on p53 expression. J Exp Clin Cancer Res 38(1):1–15

Tian T, Li X, Zhang J (2019) mTOR signaling in cancer and mTOR inhibitors in solid tumor targeting therapy. Int J Mol Sci 20(3):755

Uygun BE, Soto-Gutierrez A, Yagi H, Izamis M-L, Guzzardi MA, Shulman C, Milwid J, Kobayashi N, Tilles A, Berthiaume F, Hertl M, Nahmias Y, Yarmush ML, Uygun K (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16:814–820. https://doi.org/10.1038/nm.2170

Vakhshiteh F, Atyabi F, Ostad SN (2019) Mesenchymal stem cell exosomes: a two-edged sword in cancer therapy. Int J Nanomedicine 14:2847–2859. https://doi.org/10.2147/IJN.S200036

Villarino AV, Kanno Y, O'Shea JJ (2017) Mechanisms and consequences of Jak–STAT signaling in the immune system. Nat Immunol 18(4):374–384

Vivier E, Tomasello E, Baratin M, Walzer T, Ugolini S (2008) Functions of natural killer cells. Nat Immunol 9:503–510. https://doi.org/10.1038/ni1582

Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA Jr, Kinzler KW (2013) Cancer genome landscapes. Science 339(6127):1546–1558

Walker C, Mojares E, Del Río Hernández A (2018) Role of extracellular matrix in development and cancer progression. Int J Mol Sci 19:3028. https://doi.org/10.3390/ijms19103028

Walther W, Stein U (2000) Viral vectors for gene transfer. Drugs 60:249–271. https://doi.org/10.2165/00003495-200060020-00002

Wang YA, Elson A, Leder P (1997) Loss of p21 increases sensitivity to ionizing radiation and delays the onset of lymphoma in atm-deficient mice. Proc Natl Acad Sci 94(26):14590–14595

Wang J, Wang H, Li Z, Wu Q, Lathia JD, McLendon RE, Hjelmeland AB, Rich JN (2008) c-Myc is required for maintenance of glioma cancer stem cells. PLoS One 3(11):e3769
Wang KC, Helms JA, Chang HY (2009) Regeneration, repair and remembering identity: the three Rs of Hox gene expression. Trends Cell Biol 19:268–275. https://doi.org/10.1016/j.tcb.2009.03.007
Wang C, Chen L, Huang Y, Li K, Jinye A, Fan T, Zhao R, Xia X, Shen B, Du J, Liu Y (2019a) Exosome-delivered TRPP2 siRNA inhibits the epithelial-mesenchymal transition of FaDu cells. Oncol Lett 17:1953–1961. https://doi.org/10.3892/ol.2018.9752
Wang P, Perche F, Logeart-Avramoglou D, Pichon C (2019b) RNA-based therapy for osteogenesis. Int J Pharm 569:118594. https://doi.org/10.1016/j.ijpharm.2019.118594
Watanabe R, Murai MJ, Singh CR, Fox S, Ii M, Asano K (2010) The eukaryotic initiation factor (eIF) 4G HEAT domain promotes translation re-initiation in yeast both dependent on and independent of eIF4A mRNA helicase. J Biol Chem 285(29):21922–21933
Watt FM, Fujiwara H (2011) Cell-extracellular matrix interactions in normal and diseased skin. Cold Spring Harb Perspect Biol 3:a005124. https://doi.org/10.1101/cshperspect.a005124
Wei X, Luo L, Chen J (2019) Roles of mTOR signaling in tissue regeneration. Cells 8:1075. https://doi.org/10.3390/cells8091075
Whittaker SR, Mallinger A, Workman P, Clarke PA (2017) Inhibitors of cyclin-dependent kinases as cancer therapeutics. Pharmacol Ther 173:83–105
Wilkinson MG, Millar JBA (2000) Control of the eukaryotic cell cycle by MAP kinase signaling pathways. FASEB J 14:2147–2157. https://doi.org/10.1096/fj.00-0102rev
Wilson A, Murphy MJ, Oskarsson T, Kaloulis K, Bettess MD, Oser GM, Pasche A-C, Knabenhans C, MacDonald HR, Trumpp A (2004) c-Myc controls the balance between hematopoietic stem cell self-renewal and differentiation. Genes Dev 18:2747–2763. https://doi.org/10.1101/gad.313104
Witsch E, Sela M, Yarden Y (2010) Roles for growth factors in cancer progression. Physiology 25(2):85–101
Wolfer A, Wittner BS, Irimia D, Flavin RJ, Lupien M, Gunawardane RN, Meyer CA, Lightcap ES, Tamayo P, Mesirov JP, Liu XS (2010) MYC regulation of a "poor-prognosis" metastatic cancer cell state. Proc Natl Acad Sci 107(8):3698–3703
Wong RS (2011) Apoptosis in cancer: from pathogenesis to treatment. J Exp Clin Cancer Res 30(1):1–14
Wu G, Schöler HR (2014) Role of Oct4 in the early embryo development. Cell Regen 3(3):7. https://doi.org/10.1186/2045-9769-3-7
Xanthoulis A, Tiniakos DG (2013) E2F transcription factors and digestive system malignancies: how much do we know? World J Gastroenterol 19(21):3189
Xie D, Pei Q, Li J, Wan X, Ye T (2021) Emerging role of E2F family in cancer stem cells. Front Oncol 11:723137
Yadav P, Yadav R, Jain S, Vaidya A (2021) Caspase-3: a primary target for natural and synthetic compounds for cancer therapy. Chem Biol Drug Des 98(1):144–165
Yang J, Nie J, Ma X, Wei Y, Peng Y, Wei X (2019) Targeting PI3K in cancer: mechanisms and advances in clinical trials. Mol Cancer 18(1):1–28
Yang D, Zhang N, Li M, Hong T, Meng W, Ouyang T (2021) The Hippo signaling pathway: the trader of tumor microenvironment. Front Oncol:11
Ye M, Song Y, Pan S, Chu M, Wang ZW, Zhu X (2020) Evolving roles of lysyl oxidase family in tumorigenesis and cancer therapy. Pharmacol Ther 215:107633
Young ARJ, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot JFJ, Tavaré S, Arakawa S, Shimizu S, Watt FM, Narita M (2009) Autophagy mediates the mitotic senescence transition. Genes Dev 23:798–803. https://doi.org/10.1101/gad.519709
Yu F, Li J, Chen H, Fu J, Ray S, Huang S, Zheng H, Ai W (2011) Kruppel-like factor 4 (KLF4) is required for maintenance of breast cancer stem cells and for cell migration and invasion. Oncogene 30(18):2161–2172

Yu J, Xu X, Yao F, Luo Z, Jin L, Xie B, Shi S, Ma H, Li X, Chen H (2014) In situ covalently cross-linked PEG hydrogel for ocular drug delivery applications. Int J Pharm 470:151–157. https://doi.org/10.1016/j.ijpharm.2014.04.053

Yu X, Odenthal M, Fries JWU (2016) Exosomes as miRNA carriers: formation–function–future. Int J Mol Sci 17:2028. https://doi.org/10.3390/ijms17122028

Yu L, Chen Y, Tooze SA (2018) Autophagy pathway: cellular and molecular mechanisms. Autophagy 14(2):207–215

Yu M, Zhan J, Zhang H (2020) HOX family transcription factors: related signaling pathways and post-translational modifications in cancer. Cell Signal 66:109469

Yuan TL, Cantley LC (2008) PI3K pathway alterations in cancer: variations on a theme. Oncogene 27:5497–5510. https://doi.org/10.1038/onc.2008.245

Yun CW, Lee SH (2018) The roles of autophagy in cancer. Int J Mol Sci 19(11):3466

Zamore PD, Tuschl T, Sharp PA, Bartel DP (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101:25–33. https://doi.org/10.1016/S0092-8674(00)80620-0

Zbinden M, Duquet A, Lorente-Trigos A et al (2010) NANOG regulates glioma stem cells and is essential in vivo acting in a cross-functional network with GLI1 and p53. EMBO J 29:2659–2674

Zhan T, Rindtorff N, Boutros M (2017) Wnt signaling in cancer. Oncogene 36(11):1461–1473

Zhan J, Wang P, Li S, Song J, He H, Wang Y, Liu Z, Wang F, Bai H, Fang W, Du Q (2019) HOXB13 networking with ABCG1/EZH2/Slug mediates metastasis and confers resistance to cisplatin in lung adenocarcinoma patients. Theranostics 9(7):2084

Zhang Y, Wang X (2020) Targeting the Wnt/β-catenin signaling pathway in cancer. J Hematol Oncol 13(1):1–16

Zhang HF, Chen Y, Wu C, Wu ZY, Tweardy DJ, Alshareef A, Liao LD, Xue YJ, Wu JY, Chen B, Xu XE (2016) The opposing function of STAT3 as an oncoprotein and tumor suppressor is dictated by the expression status of STAT3β in esophageal squamous cell carcinoma. Clin Cancer Res 22(3):691–703

Zhang B, Chen Y, Shi X, Zhou M, Bao L, Hatanpaa KJ, Patel T, DeBerardinis RJ, Wang Y, Luo W (2021) Regulation of branched-chain amino acid metabolism by hypoxia-inducible factor in glioblastoma. Cell Mol Life Sci 78(1):195–206

Zhao H, Chen T (2013) Tet family of 5-methylcytosine dioxygenases in mammalian development. J Hum Genet 58(7):421–427

Zhou BP, Hu MCT, Miller SA, Yu Z, Xia W, Lin SY, Hung MC (2000) HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-κB pathway. J Biol Chem 275(11):8027–8031

Zhou L, Wang Y, Zhou M, Zhang Y, Wang P, Li X et al (2018) HOXA9 inhibits HIF-1a-mediated glycolysis through interacting with CRIP2 to repress cutaneous squamous cell carcinoma development. Nat Commun 9:1480

Zou Z, Tao T, Li H, Zhu X (2020) mTOR signaling pathway and mTOR inhibitors in cancer: progress and challenges. Cell Biosci 10(1):1–11