Khawaja H. Haider *Editor*

Stem Cells

Latest Advances

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Editor
Khawaja H. Haider
Department of Basic Sciences
Sulaiman AlRajhi University
Al Bukayriyah, Saudi Arabia

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Dedicated to my wife DIYA who mothered our two sons:
Mowahid who is the love of my life
and
Anas "the angel of paradise" whose departure from my life is a constant source of inspiration for me to do science.

Preface

Stem cell–based therapy has immensely progressed from its initial phase of uncertainty to the current stage of assurance for routine use in the clinical practice. Conceivably, it is one of the fastest-growing and most sought-after fields of research. ***Stem Cells: Latest Advances*** is a continuation of my previously edited books on the same topic. The book is a compilation of 15 chapter contributions by the top researchers involved in stem cell theranostics. It begins with a chapter from Prof David's group, who share their bench experience with multi-microelectrode array (MEA) technology to study induced pluripotent stem cells (iPSCs)-derived cardiomyocytes (CMs) as an experimental model. They have elegantly summarized using MEA technology to assess drug-induced cardiotoxicity and evaluate cardiac-specific diseases' pathophysiology. Chapter 2 is contributed by Prof Hénon, a top scientist in the field of very small embryonic-like cells (VSELCs). The chapter focuses on the regenerative potential of CD34+ VESLCs and discusses their therapeutic potential. Chapter 3 is an elegant contribution from Prof Zorina, a seasoned researcher who provides a synopsis on the structural and functional dynamics of mesenchymal and hematopoietic stem cell axis in physiologic and pathologic conditions, emphasizing their responses to cytoreductive regimens. Chapter 4 is an exceptional contribution from senior researcher Prof Harrell, emphasizing the molecular and cellular mechanisms underlying the beneficial effects of MSC-sourced secretome to treat autoimmune and inflammatory diseases. In Chap. 5, Prof Misra has comprehensively delineated normal and diabetic hearts regarding their metabolic activity and microenvironment and discusses the challenges of cell-based therapy in diabetic patients with myocardial infarction. Prof Raval in Chap. 6 provides an overview of biomaterials with known macrophage interactions in the heart and their cardiac repair implications. While Chap. 7, contributed by Prof Fakoya, discusses in-depth the evolution of stem cell–based therapy with a special emphasis on cardiovascular regeneration, Chap. 8 stresses combining MSCs with pluripotent stem cells (PSCs) to exploit the best of the two cell types for cardiac repair. Chapters 9 and 10 are contributed by Prof Fawzy who is actively involved in the fast-emerging area of stem cell–based dental research. He reviews the osteogenic, hepatogenic, neurogenic, immunomodulatory, paracrine, and dental tissue regenerative potential of dental MSCs compared to the bone marrow MSCs. Continuing on the same note, Chap. 10 discusses stem cell–based emerging approaches in tissue engineering for enamel, dentin, and pulp regeneration. Chapter 11 critically reviews stem cell development for hepatic therapy. The "proof-of-concept" phase, clinical trial initiation, and the required regulatory framework have been addressed as well besides discussing the prospective strategies for their liver-related applications in regenerative medicine. Chapters 12, 13, 14, and 15 all focus on PSCs in the clinics. Chapter 12 is explicit in discussing the effect of diabetes on the functionality of the intrinsic pool of stem cells. It also explores the possibility of using exogenous stem cells, especially iPSCs, as a continuous source of pancreatic β-cells to normalize insulin production. Chapter 13 provides an insight into the recent advances in reprogramming technologies, use of high throughput assays for safety testing, and establishes efficient protocols for iPSC generation. Furthermore, combining "state-of-the-art" bioengineering techniques and organoid technology with iPSCs has been discussed with pediatric use implications. Chapter 14 reviews the feasibility of PSC-derived-CMs for heart cell therapy and study the candidate cues such as time in culture, and its

environments (*e.g.,* extracellular matrices, postnatal hormones, alterations in metabolic substrates, and substrate stiffness), intercellular communications (*e.g.,* physicochemical cues from neighboring non-CMs), and 3-D culture system. The authors provide future perspective of using mature PSC-CMs in disease modeling, drug discovery, and regenerative medicine. Finally, Chap. 15 discusses iPSCs-derived cancer stem cells (CSCs) with xenograft models complementary to patient-derived (orthotropic) xenografts and organoid models with the aid of 3D culture systems. This holds the potential of diverse applications including anti-cancer drug discovery and a renewable source of diverse cell types, that is, CMs, albeit after comprehensive characterization to achieve optimal treatment outcomes. To sum it all, we use the words of Prof Law, who once wrote for me that "stem cell is a viable technology, a technology shared by all animals in the last 500 million years, only to be re-discovered, isolated, purified and to be re-formulated as Cell Therapy."

Al Bukayriyah, Saudi Arabia Khawaja Husnain Haider

Contents

Microelectrode Arrays: A Valuable Tool to Analyze Stem Cell-Derived Cardiomyocytes

1

Sophie Kussauer, Robert David, and Heiko Lemcke

Abbreviations

AP	Action potential
APD	Action potential duration
Ca2+	Calcium
CIPA	Comprehensive in vitro Proarrhythmia Assay
CM	Cardiomyocytes
CSAHI	Consortium for Safety Assessment using Human iPS cells
CVD	Cardiovascular disease
ESC	Embryonal stem cell
FP	Field potential
FPDc	Field potential duration (corrected)
FRET	Fluorescence resonance energy transfer
hERG	Human Ether-a-go-go Related Gene- voltage sensitive potassium channel
hiPSC	Human-induced pluripotent stem cell
K+	Potassium
LMNA	Lamin A/C
MEA	Multi/Micro- electrode array
MYH7	Myosin heavy chain 7
Na+	Sodium
RNA	Ribonucleic acid
SCN5A	Sodium voltage-gated channel alpha subunit 5
TBX5	T-box transcription factor
TdP	Torsade-de-Pointes-Tachycardia
TnnT	TroponinT
VSO	Voltage-Sensitive Optical System
WT	Wild type

1.1 Introduction

Cardiovascular diseases (CVD), like ischemic heart disease and stroke, are the major cause of health damage in all regions of the world (Roth et al. 2017). Hence, there is an urgent need for strategies that allow effective prevention and treatment of CVDs. In 2006, Yamanaka and colleagues first described the generation of induced pluripotent stem cells (iPSC) that are capable to differentiate into any cell type, when cultivated under specific differentiation conditions. This iPSC technology enables researchers to largely produce cardiac cells in vitro without ethical concerns, providing a valuable source for regenerative therapies, disease modeling, and drug testing applications (Takahashi and Yamanaka 2006; Yoshida and Yamanaka 2017; Zuppinger 2019).

The regenerative potential of iPSC-derived cardiomyocytes (CM) has been studied in multiple preclinical studies using small and large animal models. Following transplantation of iPSC-CM, cells were found to improve cardiac function and reverse cardiac remodeling (Guan et al. 2020; Kashiyama et al. 2019; Rojas et al. 2017; Shiba et al. 2016). At the same time, hiPSC-CMs are successfully applied to evaluate the pro-arrhythmic potential of established and newly developed drugs. Considering that cardiotoxicity is one of the main reasons for compound attrition during drug development or withdrawals after approval, hiPSC-CM are a powerful tool for the establishment of predictive drug screening assays (Gao et al. 2018; Rojas et al. 2017; Shadrin et al. 2017; Zhao et al. 2018a, b). The preclinical use of hiPSC-derived CM for drug development has been recently demonstrated by the Comprehensive in vitro Pro-arrhythmia Assay (CIPA) project that was initiated to determine the cardiotoxic effects of cardiac and noncardiac drugs (Edwards et al. 2018; Huo et al. 2018; Izumi-Nakaseko et al. 2018; Pfeiffer-Kaushik et al. 2019). In this regard, it is mandatory to study cellular electrophysiology to receive a comprehensive overview of the impact of tested drugs on cardiac cell function.

Although the iPSC-technology is available for more than 14 years, researchers failed to generate hiPSC-CM matching identical cellular properties as their native counterparts. This immature phenotype is a major obstacle for their use in cardiovascular research and their clinical appli-

S. Kussauer · R. David (✉) · H. Lemcke
Department Cardiac Surgery, Medical Center, University of Rostock, Rostock, Germany

Department of Life, Light and Matter, University of Rostock, Rostock, Germany
e-mail: robert.david@med.uni-rostock.de

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cation. Beside metabolic, mechanical, and ultrastructural changes, cardiac maturation involves the establishment of proper ion channel composition. Many data have been acquired to describe the electrophysiological properties of hiPSC-CM, showing the presence of multiple ion channels like sodium (INa), potassium (IK1, IKr), L-type and T-type calcium channels (Goversen et al. 2018; Lemoine et al. 2017; Zhao et al. 2018a, b). Besides, hiPSC-CM commonly represent a heterogeneous cell population, each with a specific electrophysiological profile (Karakikes et al. 2015). Thus, it is crucial to precisely characterize the electrophysiological phenotype of hiPSC-CM, in particular when subtype-specific differentiation is desired (Hausburg et al. 2017; Zhang et al. 2019).

In the current chapter, we will present techniques that are used to analyze the electrophysiology of hiPSC-derived CM. In particular, we focus on MEA technology and how this platform is applied in drug discovery and disease modeling, and as a valuable tool for the implementation of personalized medicine strategies.

1.2 Methods for the Electrophysiological Characterization of hiPSC-CMs

To assess the electrophysiological features of stem cell-derived CMs, several techniques are available, including patch-clamp analysis, MEA analysis, and fluorescence dye-based visualization of the membrane potential. Limitations and advantages of these different approaches are discussed in the following paragraphs.

1.2.1 Dye-Based Assessment of the Membrane Potential

Fluorescence-based techniques are widely applied in cardiovascular research to visualize molecular structures or to provide insight into the physiological function, for example, by using ion-sensitive dyes (Hou et al. 2017; Roopa et al. 2019). Likewise, they provide the possibility to indirectly measure the electrical activity of cardiomyocytes by changing their emission spectra upon alteration of the membrane potential. Electrophysiological characterization by fluorescence dyes is operationally simple and does not require specialized equipment or devices which make it suitable for a broad range of users. Additionally, as voltage imaging of cells is less invasive, long-term acquisition of the electrical activity can be performed and cells can be further processed in downstream experiments (Storace et al. 2015). Moreover, high-throughput data acquisition and the possibility to obtain spatial information of the membrane potential are achievable by imaging techniques.

In particular, small molecules have been identified to provide a fast and sensitive measurement of membrane potential dynamics (Miller 2016). Several studies have proven the feasibility of voltage-sensitive dyes for drug screening experiments in stem cell-derived CMs (Bedut et al. 2016; del Álamo et al. 2016; Hortigon-Vinagre et al. 2016). Interestingly, dye-based assessment of the membrane potential provides sufficient accuracy to discriminate specific action potential (AP) patterns of cardiac subtypes within a heterogeneous population of hiPSC-CM. In the same study, the authors were capable to detect differences in the AP pattern between hiPSCs derived from patients suffering from long QT syndrome, indicating high sensitivity of these imaging approaches (Takaki et al. 2019). Besides analyzing cultured CM in vitro, fluorescent probes can be applied to study the electrophysiological properties of whole hearts, which enable researchers to monitor electrical wave propagation, for example, to examine the propagation pattern in a trial and ventricular arrhythmias (Herron et al. 2012).

Genetically encoded voltage indicators are another strategy to measure membrane depolarization via fluorescence microscopy. These probes are designed by coupling a voltage-sensitive domain from a phosphatase to either a fluorescent protein in different configurations, a rhodopsin protein or a fluorescence resonance energy transfer (FRET) pair (Han et al. 2013; St-Pierre et al. 2015). Following the alteration of the membrane potential, the voltage sensor undergoes conformational changes that modulate fluorescence emission of the attached fluorescent protein. In contrast to voltage-sensitive dyes, protein-based voltage indicators possess lower phototoxicity, thus, facilitating long-term measurements. Recently developed voltage sensors like QuasAr1, Archer1 or Arclight, demonstrate large fluorescence response when the cell membrane depolarizes (40–80% per 100 mV voltage change) accompanied by fast on/off kinetics (1–10 ms). Multiple studies with hiPSC-CM have revealed that membrane potential sensors can be applied for optical mapping and evaluation of pharmacological compounds as shown for the ArcLight or VSFP-CR protein (Herron 2016; Leyton-Mange et al. 2014; Shaheen et al. 2018; Shinnawi et al. 2019; Song et al. 2015). Considering that the expression of these probes can be coupled to a CM subtype-specific promotor, voltage indicators are suitable to even identify the AP profile of ventricular-, atrial-, and nodal-like cells, respectively (Chen et al. 2017).

However, akin to the dye-based determination of electrical activity, a protein encoded potential indicator allows only the semiquantitative analysis of voltage change (Herron et al. 2012). Despite recent progress in the development of voltage-sensitive proteins, future research needs to improve their biophysical properties, including fluorescence yield, enlarged dynamic range, and faster detection capacity. The latter requirement seems to be important as lower on/off

kinetics result in losing high-frequency AP elements (Herron et al. 2012; Leyton-Mange et al. 2014). Moreover, biocompatibility needs to be carefully addressed since the introduction of voltage-sensitive proteins could have an impact on the electrophysiological characteristics of CMs or might lead to undesired cross talk with endogenous signaling pathways (Kaestner et al. 2018).

1.2.2 Patch-Clamp Recordings

Compared to all techniques currently available to evaluate the electrical activity of individual cells, patch clamping is the gold standard approach for the acquisition of ion current data and precise determination of AP. To characterize the electrophysiological behavior of individual cells, a small-diameter glass pipette is pressed on the cell surface and suction is applied to obtain a tight so-called gigaseal, leading to an electrically isolated membrane patch (Obergrussberger et al. 2015). Ion currents that flux through the channels within this patch can be recorded by a coupled electrode.

Different patch-clamping configurations exist, depending on the scientific question that needs to be addressed. In the cell-attached mode, the cell membrane patch is left intact and the cytoplasm is not influenced (Zhao et al. 2008). The most commonly used patch-clamp configuration is the whole cell mode, where the membrane patch is disrupted by strong suction forces of the attached pipette. As a result, a physical continuity is established between the interior of the pipette and the intracellular lumen. While using this configuration, the operator can tightly control either current or voltage that is applied during measurement. In hiPSC-CMs, voltage patch clamp has been successfully applied to acquire information about ion channel density, voltage dependency, and activation/deactivation properties (Casini et al. 2017). Besides, it is possible to record ion currents only from a small membrane fragment that is removed from the cell by withdrawing the glass pipette. This excised patch mode increases the possibility to study single ion channels. Variations of this configuration allow investigation of effects induced by intra- or extracellular cues (Zhao et al. 2008).

The manual patch-clamp recording is technically challenging and requires high operator skills, including a strong biophysical background for data analysis and interpretation. As measurements are usually performed on the single-cell level, patch clamping is time-consuming and not suitable for high-throughput data acquisition (~10–20 cells per working day), limiting the ability to use patch clamping as a platform for drug screening. To overcome these limitations, automated patch-clamping systems have more recently been developed, improving the efficiency of electrophysiological studies by analyzing 10–700 cells in parallel (Bell and Dallas 2018; Obergrussberger et al. 2018; Scheel et al. 2014). In contrast to manual patch clamping, these automated systems commonly use single-cell suspension to realize high-throughput measurements. Additional features such as temperature control, internal perfusion systems, and the capability for optical stimulation ensure high data quality and reproducibility (Bell and Dallas 2018; Obergrussberger et al. 2018). However, studies revealed that data accuracy and quality was reduced in automated patch-clamp systems if compared to recordings obtained by manual patch clamp (Franz et al. 2017; Yajuan et al. 2012). An important parameter determining consistency and robustness of patch-clamp data is the density and homogeneity of the cell suspension. A fact that is particularly crucial for the analysis of hiPSC-CM. Since these cells are very sensitive to dissociation, bringing cells into suspension can affect membrane integrity or ion channel expression and, thus, alter their electrophysiological properties (Huang et al. 2010; Rajamohan et al. 2016). Also, automated patch-clamp devices lack the possibility of selective cell capturing and targeting. Considering that CM derived from stem cells represents a heterogeneous cell population, it is difficult to acquire patch-clamp recordings from specific cardiac subtypes (Yajuan et al. 2012).

1.2.3 Whole Tissue Measurement with Extracellular Electrodes

Electrophysiological characterization can also be performed on tissue and organ level. The synchronous electrical activity of thousands of cells generates a current outside the cells that can be detected with extracellular electrodes. This strategy is commonly applied in the medical field to detect the electrical behavior of skeletal muscles (electromyogram), heart (electrocardiogram), or brain (electroencephalogram). However, these recordings do not reflect the electrophysiology of individual cells, rather providing information of all cells contributing to the electrical activity of the respective tissue or organ.

1.3 MEA-Based Characterization of Physiological Parameters

A MEA system utilizes multichannel recordings from several electrodes that are embedded in the culture surface, measuring changes of the extracellular field potential (FP) of the attached cell monolayer. This noninvasive technology has been originally used for the detection of electric signals in neural cells, while it has become more popular in the last years to analyze hiPSC-derived CM, especially for the assessment of drug-induced cardiotoxicity (Asakura et al. 2015; Li et al. 2016; Sala et al. 2017; Yamamoto et al. 2016). Commonly, MEA systems record FP data on the

cell population level. However, custom-made MEA platforms have been designed to determine the electrophysiological features of individual cells, which allow a more accurate representation of the CM cell layer (Ryynänen et al. 2018). Due to the small electrodes, resulting in a low signal to noise ratio, conventional MEA systems are less suitable for single-cell recordings. Thus, Ryynänen et al. have developed electrodes that are larger in size, accompanied by an optimized electrode layout for data acquisition of single CMs (Ryynänen et al. 2018). Similarly, agarose-based micro-chambers printed on MEA chips have been shown to facilitate the acquisition of FPs derived from single hiPSC-CM (Kaneko et al. 2018). In addition to cell monolayers, MEA-based electrophysiological characterization can also be performed with slice cultures, as demonstrated for human and murine heart tissue slices (Chowdhury et al. 2018; Kang et al. 2016; Lane et al. 2017; Trieschmann et al. 2019).

As the reader might appreciate, MEA platforms possess high flexibility in terms of electrode number, size, geometry, and alignment. At the same time, this high flexibility is further defined by the possibility to combine MEA arrays with other detection methods to increase the number of parameters describing cellular physiology. While the FP is the main parameter measured by the electrodes, the novel established MEA platforms allow simultaneous measurement of the impedance, which reflects the mechanical movement of the attached cell layer, thus, providing important information about beating behavior, cell death, viability, and proliferation (Doerr et al. 2015; Qian et al. 2017; Takasuna et al. 2017). Correlative analyses of cellular contraction and electrical activity have also been carried out by a combination of MEA and video-microscopy, offering new insights into the electrical and mechanical relationship of hiPSC-CMs (Hayakawa et al. 2014). Likewise, subcellular information can be compared with FP data by the integration of fluorescence microscopy into the MEA setup (Cools et al. 2018). However, this combination with optical methods requires specific structural features of the MEA system in order to obtain proper visualization of the target cell, for example, transparent electrodes (Nagarah et al. 2015; Ryynänen et al. 2018). Additionally, atomic force microscopy and ion conductance microscopy coupled to a MEA system has been established to investigate CMs in the context of both electrophysiology and mechanics. Simultaneous recording of FP and cell morphology in a time-dependent manner allows the reconstruction of three-dimensional motion of the cell surface during a full contraction-relaxation cycle, including the acquisition of contractile parameters such as maximum displacement, time delay and asymmetry factor (Simeonov and Schäffer 2019).

MEA is a cost-effective methodology for long-term measurements of the electrophysiological characteristics of hiPSC-CM at a high-throughput scale. Yet, the recordings of reliable and reproducible FP data require a confluent monolayer to reduce the variability of acquired FP patterns. As cell-cell contacts are crucial for the electrical remodeling of hiPSC-CM, cell density is an important parameter that needs to be carefully addressed by the user when performing MEA analysis (Uesugi et al. 2014).

1.3.1 The Field Potential Describes Cellular Electrophysiology

The cardiac action potential is generated by time-dependent changes in the membrane potential that occur during the contraction of cardiac cells. This requires a tightly regulated interplay between multiple ion channels, mediating ion flux between the extra-and intracellular space. While the initial, depolarization phase is mainly driven by voltage-dependent Na+ channels, the following plateau and repolarization stages are characterized by increased Ca2+ and K+ channel activity.

Classically, the AP of individual cells is analyzed by patch clamping, which contrasts with MEA measurements that reflect the cardiac FP (Fig. 1.1) (Feher 2017; Tertoolen et al. 2018). Both AP and FP represent parameters that describe the membrane potential of cells with electrical activity. However, the FP includes the spatiotemporal electrical activity of hundreds or thousands of cells, attached to the electrode. Therefore, FPs represents a superposition of all ionic processes measured within the respective cell cluster, ranging from slow current fluctuation to fast APs (Buzsáki et al. 2012; Clements 2016). As the FP emanates from the spreading of the AP throughout the cell layer, it is comparable to the clinical electrocardiogram, which corresponds to the time-dependent voltage change based on the electrical activity of the heart (Yamamoto et al. 2016).

The common FP waveform begins with a strong spike based on the influx of Na+ ions from the extracellular space, leading to depolarization of the cellular membrane. The subsequent rise of the intracellular Ca+ level provokes a gentle incline, while K+ efflux promotes repolarization (Fig. 1.1a). As these biophysical processes of FP generation are well understood, it is possible to reconstruct the respective AP pattern and extract specific parameters, defining the electrophysiological behavior of the cells (Fig. 1.1) (Clements 2016). Figure 1.1 illustrates the respective physiological information that can be concluded from the FP pattern. For example, the FP duration (FPD) corresponds to the QT interval found in AP signals. A comparative study of MEA recordings and patch-clamp data revealed that the detected FP duration correlates well with the length of the QT interval of APs (Halbach et al. 2003).

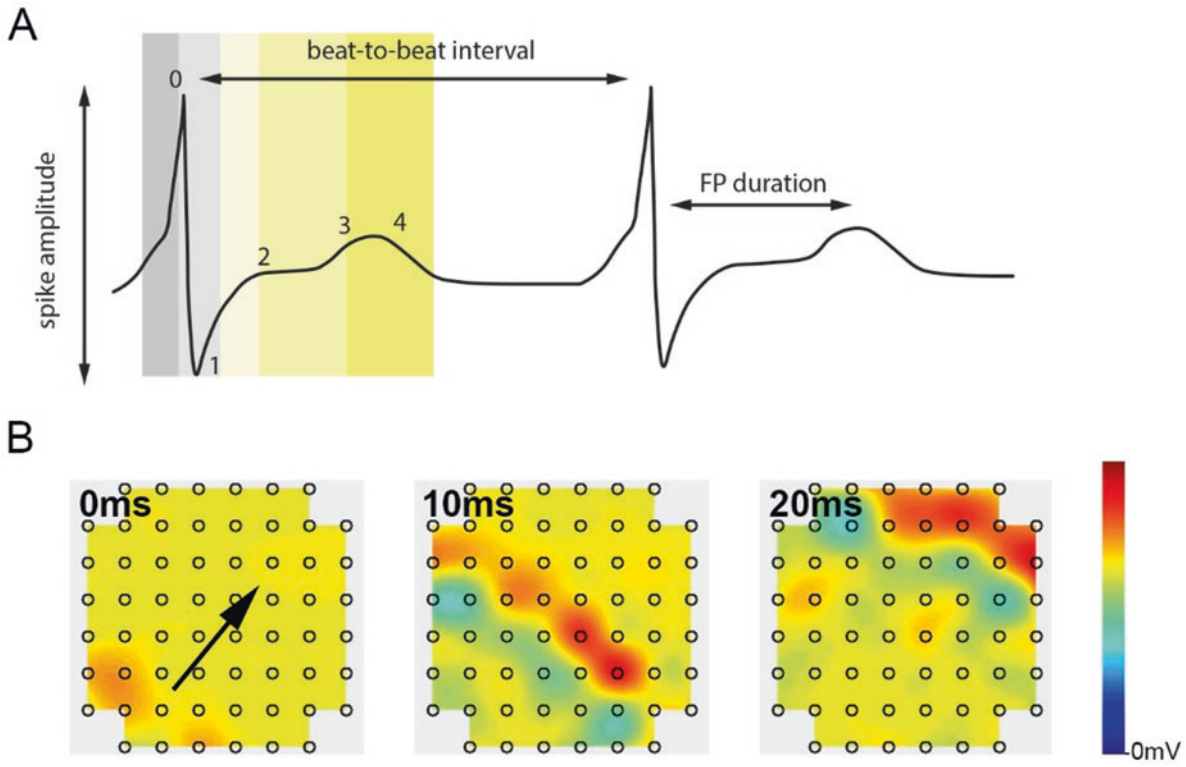

Fig. 1.1 (**a**) Illustration of a common field potential pattern detected by MEA. The different phases of the field potential (0–4) reflect the tightly regulated orchestration of involved ion channels. Following rapid depolarization (0), induced by sodium influx, various ion transporters (Ca2+, Na+, K+) cause a hyperpolarization of the field potential (1). A balanced interplay between calcium influx and potassium efflux results in a plateau phase (2), followed by a repolarization (3), leading to a resting field potential (4). This information allows reconstruction of the corresponding AP signal and extraction of various electrophysiological parameters like beat-to-beat interval, spike amplitude, etc. (**b**) Spatiotemporal data enable the detection of propagation velocity and direction (arrow) of the field potential. Images represent time-lapse analysis of recorded field potential spreading throughout a cell monolayer attached on a MEA chip

Thus, measuring the FP by MEA devices is suitable to determine QT interval prolongation/shortening following drug treatment in hiPSC-CMs (Asakura et al. 2015). In addition, the FP can provide valuable data about AP duration (APD) and the beating frequency of the attached cell layer (Fig. 1.1a). Moreover, when MEA platforms are equipped with multiple electrodes they are capable to collect spatiotemporal information of the FP, including propagation velocity and direction as well as the origin of the AP spread (Logantha et al. 2019) (Fig. 1.1b).

The human heart comprises different cardiac subtypes (atrial, ventricular, nodal), all characterized by specific electrophysiological features due to distinct ion channel composition (Liu et al. 2016). Likewise, hiPSCs-derived CMs represent a mixture of cells that reflect this heterogeneity associated with specific AP and FP patterns (Du et al. 2015; Goversen et al. 2018; Karakikes et al. 2015). Using MEA technology, measured FPs can help to identify the different cardiac subtypes within hiPSC-CM populations. This is particularly important when reprogramming strategies are applied aiming for the differentiation into a certain subtype (Hausburg et al. 2017; Protze et al. 2017).

1.4 MEA-Based Evaluation of Drug-Induced Cardiotoxicity

Cardiotoxicity associated with established, clinically used drugs is a global concern leading to treatment determination and hindering successful drug development. This undesired side effect might induce myocardiopathy, such as arrhythmia, myocardial infarction, and myocardial hypertrophy (Ma et al. 2020). The US department of health and human serviced estimated the number of patients exhibiting adverse drug responses to approximately one million, while drug-induced arrhythmias were found to be the leading side effect (Food and Drug Administration, HHS 2001; Sager et al. 2014).

1.4.1 CIPA—A New Paradigm in Cardiotoxicity Drug Screening

In 2013, the Comprehensive in vitro Proarrhythmia Assay (CIPA) initiative was initiated to evaluate the pro-arrhythmic risk of multiple known drugs and compounds. The new

paradigm, given by the CIPA initiative, involves mechanistically based in vitro assays, associated with in silico simulations of the electrophysiological properties of cardiac cells, followed by cell culture studies. Since its inception, CIPA has led to the evaluation of 28 relevant drugs, for example, Tamoxifen, Vandetanib, Metoprolol, Clarithromycin, and Verapamil, that were classified according to their risk to trigger Torsade-de-Pointes-Tachycardia (TdP), a certain type of abnormal heart rhythm that can lead to sudden cardiac death (Fermini et al. 2016; Millard et al. 2018). More precisely, the CIPA initiative recommends a four-step evaluation for newly developed and established drugs to obtain a more reliable validation of drug-induced cardiotoxicity. (1) Analyzing the impact of respective drugs on specifically defined ion currents. (2) Using computational models, the obtained in vitro data should be reconstructed and recapitulated in silico. (3) In the next step, human stem cell-derived CM represents the platform of choice to verify the data from previous in vitro and in silico assays. (4) Following a positive outcome, the last stage includes a clinical evaluation to investigate the effects on the whole human organism (Fermini et al. 2016).

To assess the cardiotoxicity of listed drugs in compliance to the guidelines recommended by the CIPA initiative, several commercially available stem cell-derived CM (iCell Cardiomyocytes (Fuji), Pluricytes (Pluriomics), Cor4u (Ncardia), Axol Bioscience, i-HCm (Cell applications), ASC Applied Stem Cell, ix Cells Biotechnologies), CDI Cellular Dynamics International, Cellartis (Clontech, Takara), ReproCardio (ReproCELL), ACCEGEN (immortalized from patients or transdifferentiated from hSC) and self-generated cardiac cells have been used for studying cellular electrophysiology. Beside patch clamping and optical-based assessment, MEAs were found to be a valuable platform to analyze electrophysiological changes, provoked by tested compounds. For example, Schocken et al. (2018) have addressed the question whether culture medium conditions can impede proper data acquisition of drug responses in cardiac safety testing, as serum might affect the solubility of the drug, thus, influences free drug concentration. By using a high-throughput platform, 25 compounds have been subjected to MEA-based detection of electrophysiological parameters, showing that the FPD shorting/prolongation can be induced by serum-containing media, depending on the tested drug (Schocken et al. 2018).

1.4.2 hiPSCs—An In Vitro System to Simulate Human Cardiac Physiology

As described above, hiPSCs have become a promising technology for cardiovascular research (Takahashi and Yamanaka 2006). In this regard, the Consortium for Safety Assessment using Human iPS cells (CSAHi) was established by the Japan Pharmaceutical Manufacturers Association in 2013 to propose guidelines for the application of iPSC-derived cardiomyocytes, neurons, and hepatocytes in drug screening assays (Kitaguchi et al. 2017). Based on the CSAHi recommendations, several different parameters need to be determined to predict cardiotoxicity in iPSCs, such as QT prolongation and arrhythmia, while hERG channel activity alone was defined to be insufficient for the reliable prediction of cardiac safety (Nozaki et al. 2017; Sager et al. 2014). In a respective CSAHi study, seven reference drugs were evaluated at 10 testing facilities by using hiPSC cell lines and MEA platform, showing that the detection of extracellular field potentials is suitable to predict QT prolongation and arrhythmogenic liability (Kitaguchi et al. 2016).

One major advantage of hiPSC-derived CM is the possibility to generate them in large quantities, thus, representing a never-fading in vitro source for human cardiac cells (Millard et al. 2018). Unfortunately, they do not possess identical properties as their native counterparts, rather resembling a neonatal or premature state. Compared to adult cardiomyocytes, hiPSC-CM is molecularly and functionally immature, showing a profound difference in gene expression, calcium handling, and ion patterns. Especially the expression pattern of important ion channels, like INa, ICaL, If, Ito, IK1, IKr, and IKs, differs from native cardiomyocytes, isolated from adult tissue (Ma et al. 2011; Paik et al. 2020; van den Heuvel et al. 2014). This different ion channel composition was found to induce different AP properties in atrial and ventricular-like hiPSC-derived CM, while no differences were observed in nodal-like subtypes. To overcome these electrophysiological deficiencies, overexpression of selected ion channels like IK1 and IKs was induced, promoting electronic maturation (Meijer van Putten et al. 2015; Rocchetti et al. 2017; Verkerk et al. 2017). Although a myriad of studies has already been carried out to explore the effect of cardiac and noncardiac drugs on human hiPSC-CM, the aforementioned limitations need to be addressed when transferring data from cell models to human physiology.

To improve data reliability and to better compare the results obtained by MEA and other electrophysiological techniques, Kanda et al. (2016) proposed a standardized protocol for data generation, defining experimental conditions and calibration compounds (Kanda et al. 2016). In this context, CIPA-associated multisite studies have been performed to evaluate the utility of hiPSC-CMs and MEA technology within the CIPA paradigm. The selected hiPSC-derived CM cell lines, test sites, and MEA detection platforms had only minimal impact on drug categorization, while no inter-facility variability was observed (Blinova et al. 2018; Kitaguchi et al. 2016; Millard et al. 2018). A detailed overview about the compounds and their cardiotoxic effects and pro-arrhythmic risk, based on MEA analysis, is provided in Table 1.1, which

summarizes both drugs that are not intended for the treatment of cardiovascular diseases, such as antipsychotic drugs, anti-allergic drugs, or antibiotics, and cardiovascular drugs including cardiac ion channel blockers and anti-arrhythmic/hypertensive compounds. In addition, the substances are sub-categorized concerning their QT-prolonging effects.

To address the clinical transferability of drug testing data, several studies have been performed, comparing

Table 1.1 MEA-based safety testing of drugs using human-induced pluripotent stem cell cardiomyocytes (hiPSC-CM)
Combinations with other techniques such as voltage-sensitive optical system (VSO) are mentioned *CIPA-listed drugs (2016)

SUBSTANCE CLASS	INFLUENCE ON QT INTERVALL	NAME	(SITE OF) ACTION	EFFECT	MIN. EFFECTIVE CONC.	CELL TYPE/ SUBTYPE	METHOD	REFERENCE
NON-CARDIAC MEDICATION	QT-prolonging	Alfuzosin	Drug against prostatic hyperplasia / antihypertonicum	FPDc prolongation/ EAD	30 nM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b; Qu & Vargas, 2015)
		Astemizole*	Antiallergenic / antihistaminergic / multi-channel block	FPDc and repolarization prolongation/ EAD /arrhythmogenic effects	3–10 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Carbachol	Parasympathomimetic / K_{Ach} channel agonist	FPDc prolongation, negative chronotropic effects	10 µM	Double reporter cell line, subtypes: ventricular, atrial, nodal, TBX5 Nkx2.5 (2D protocol) / Commercial hiPSC-CM	Patch clamp, MEA	(Nozaki et al., 2017b; J. Z. Zhang et al., 2019b)
		Chlorpheniramine	Antiallergenic / antihistaminergic	EAD/ FPDc prolongation	270-450 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Chlorpromazine*	Antipsychotic / neuroleptic / multi-channel block	EAD / beating arrest / no effect	0,75 -10 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Nozaki et al., 2017c)
		Chromanol 293B	IKv7.1 (IKs) channel block	FPDc prolongation (control cells)	4 µM	LQTS cells and control (patient- derived cells) n/a (3D protocol) / Commercial hiPSC-CM	MEA	(Ando et al., 2017; Egashira et al., 2012a)
		Cilostazol	Anti- platelet drug / phosphodiesterase inhibitor	FPDc prolongation, beating arrest	14 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Cisapride*	Antimotility agent / multi-channel block	FPDc and repolarization prolongation / arrhythmogenic effects/ EAD Prolongation of QT from patients with long QT syndrome	60-100 nM	Commercial hiPSC-CM / Wt iPSC, and from patient with LQTS (n/a) (3D protocol)	MEA, automated patch clamp	(Ando et al., 2017; Blinova et al., 2018b; Harris et al., 2013; Kitaguchi et al., 2017b; Liang et al., 2013a; Nozaki et al., 2017b; Qu & Vargas, 2015; Scheel et al., 2014b)
		Clarithromycin*	Antibiotic	FPDc and repolarization prolongation / EAD / arrhythmogenic effects	28 µM	Commercial hiPSC-CM	MEA (+VSO)	(Ando et al., 2017; Blinova et al., 2018b)
		Diphenhydramin	Antiallergenic / antihistaminergic	FPDc prolongation / EAD	100 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Dolasetron	Antiemetic	FPDc prolongation / EAD	8-27 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Domperidone*	Antiemetic / dopamine-receptor antagonist / hERG- channel block	FPDc and repolarization prolongation / EAD / arrhythmogenic effects	10-60 nM	Commercial hiPSC-CM	MEA (+VSO)	(Ando et al., 2017; Blinova et al., 2018b; Nozaki et al., 2017b)
		Droperidol*	Antipsychotic / neuroleptic	FPDc and repolarization prolongation / EAD / arrhythmogenic effects	180 nM	Commercial hiPSC-CM	MEA (+VSO)	(Ando et al., 2017; Blinova et al., 2018b)
		Erythromycin	Antibiotic	FPDc prolongation / EAD	2.1 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Fluoxetine	Antidepressant	Clinical QT prolongation	-	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b)
		Gatifloxacin	Antibiotic	FPDc prolongation / EAD	23 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Haloperidol	Antipsychotic	FPDc prolongation / EAD	1-7 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		JNJ303	IKv7.1 (IKs) channel block	FPD prolongation	95 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Moxifloxacin	Antibiotic / multi-channel block	FPDc and repolarization prolongation	10 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Millard et al., 2018b; Qu & Vargas, 2015)
		Nilotinib	Anti-cancer drug (leukemia) / tyrosine kinase inhibitor	FPDc prolongation / EAD	40 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Ondansetron*	Antiemetic / serotonin-receptor block	FPDc and repolarization prolongation / EAD	30 – 950 nM	Commercial hiPSC-CM	MEA (+VSO)	(Ando et al., 2017; Blinova et al., 2018b)
		Paliperidone	Antipsychotic	FPDc prolongation / EAD	950 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)

(continued)

Table 1.1 (continued)

		Pimozide*	Antipsychotic / multi-channel block	FPDc and repolarization prolongation / EAD / arrhythmogenic effects	3–10 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Kitaguchi et al., 2017b)
		Quinine sulfate	Antimalarial	FPDc prolongation / EAD	1.3 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Risperidon*	Anti-psychotic / serotonin-receptor block	FPDc and repolarization prolongation	3–30 nM	Commercial hiPSC-CM	MEA (+VSO)	(Ando et al., 2017; Blinova et al., 2018b)
		Sertindole	Antipsychotic	FPDc prolongation / EAD	23 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Qu & Vargas, 2015)
		Sparfloxacin	Antibiotic	FPDc prolongation / EAD	3 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Sunitinib	Anti-cancer drug (gastro intestinal tumor) / tyrosine kinase inhibitor	FPDc prolongation / EAD	0.3–10 µM	Commercial hiPSC-CM	MEA	(Nozaki et al., 2017b)
		Terfenadine*	Antihistaminergic / H1 receptor block	FPDc and repolarization prolongation / decrease in spike amplitude	100- 1000 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Harris et al., 2013; Qu & Vargas, 2015)
		Terodiline	Anticholinergic / antispasmodic (urology)	FPDc prolongation / beating arrest	5 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Qu & Vargas, 2015)
		Thioridazine	Sedative / antipsychotic / multi-channel block	FPDc and repolarization prolongation EAD / arrhythmogenic effects	100 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Tolterodine	Anticholinergic / antispasmodic (urology)	clinical QT and FPDc prolongation / EAD	20–300 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Kitaguchi et al., 2017b; Qu & Vargas, 2015)
		Vandetanib*	Anti-cancer drug (thyroid tumor) / tyrosin kinase inhibitor	FPDc prolongation / EAD	57 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Vanoxerine	Serotonin-dopamine reuptake inhibitor	Clinical QT prolongation, multiple ion-channel effects, EAD	100 nM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b)
		Ziprasidone	Antipsychotic	FPDc prolongation EAD	230 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
	Non-QT prolonging	Amitriptyline	Antidepressant	no effect on FPDc or EAD / beating arrest at high concentration	570 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Aspirin	Anticoagulant / anti-inflammatoric/ analgetic	no effect on FPDc or EAD or beating arrest	-	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		$BaCl_2$	Digitalis like activity, stimulation of tonic contraction in muscle (contrast agent)	Positive chronotropic effect / K^+ and Ca^{2+} modulation / FPD shortening	-	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b)
		Blebbistatin	Myosin II ATPase inhibitor	Positive chronotropic effects / beating arrest at high concentrations	1–30 µM	Commercial hiPSC-CM	MEA	(Nozaki et al., 2017b)
		Clozapine*	Antipsychotic / multi-channel block	FPDc shortening, positive chronotropic effects	0.3–2.5 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Nozaki et al., 2017b)
		Doxorubicin	Anthracycline / chemotherapeutic	FPDc shortening / positive chronotropic effects, increase in spike amplitude	1 µM	Commercial hiPSC-CM / 50% ventricular, 10% atrial cells patient derived cells (n/a) (2D protocol)	MEA	(Burridge et al., 2016b; Maillet et al., 2016)
		Famotidine	Antihistaminergic / Anti-ulceration drug	No effects on FPDc or EAD or beating arrest	-	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Isoproterenol	Bronchodilator	Positive chronotropic effects / K^+ and Ca^{2+} modulation / FPDc shortening	3–100 nM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Levocetrizine	Antiallergenic / antihistaminergic	No effects on FPDc or EAD or beating arrest	-	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Loratadine*	Antihistaminergic / H1 receptor block / multi-channel block	Positive chronotropic effects / no effect on FPDc or EAD	0.1–3 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Nozaki et al., 2017b)
		Tamoxifen*	Anti-cancer drug / estrogen-receptor modulating	No effect on FPDc or EAD / beating arrest at high concentrations	89 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Tetrodotoxin (TTX)	Neurotoxic drug / Na_v (1.1, 1.7, 1.5)- channel block	Decrease in slope / APD shortening	10 µM	Commercial hiPSC-CM	Automated patch clamp	(Scheel et al., 2014b)
CARDIOVASCULAR	**QT-prolonging**	Ajmaline	Antiarrhytmic class Ia / Na- channel block	FPDc prolongation / EAD	500 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)

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Table 1.1 (continued)

MEDICATION							
	Amiodarone	Antiarrhythmic class III / multi-channel block	Clinical QT / FPDc prolongation / beating arrest	0.4 nM –1 μM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Kitaguchi et al., 2017b; Nozaki et al., 2017b)
	Azimilide*	Antiarrhythmic class III	FPDc prolongation / EAD / negative chronotropic effects	0.3–1 μM	Commercial hiPSC-CM	MEA	(Nozaki et al., 2017b)
	Bay K 8644	Dihydropyridine (DHP; L-Typ) Calcium channel-agonist	FPDc prolongation / negative chronotropic and positive inotropic effects	0.3–3 nM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b)
	Bepridil*	Antiarrhythmic class IV / multi-channel block	FPDc and repolarization prolongation / arrhythmogenic effects / beating arrest	0.1–1 μM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Kitaguchi et al., 2017b; Nozaki et al., 2017b)
	Disopyramide*	Antiarrhythmic class Ia / Na- channel block	FPDc prolongation /EAD	740 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
	Dofetilide*	Antiarrhythmic class III / multi-channel block	FPDc prolongation / arrhythmogenic effects / EAD	0.3–100 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Mulder et al., 2018; Nozaki et al., 2017b; Qu & Vargas, 2015)
	E-4031	Antiarrhythmic class III / hERG- channel block	FPDc prolongation / EAD / severe arrhythmias in LQTS iPSC-CM	2–100 nM	Commercial hiPSC-CM / LQTS cells and control (3D protocol)	MEA, (+ optogenetics)	(Ando et al., 2017; Harris et al., 2013; Lapp et al., 2017; Millard et al., 2018b) (Egashira et al., 2012b)
	Flecainide	Antiarrhythmic class Ic / multi-channel block	FPDc prolongation / EAD / decrease in spike amplitude	100 nM	Commercial hiPSC-CM / CPVT cells and control (2D and 3D protocol)	MEA / Patch clamp	(Ando et al., 2017; Harris et al., 2013; Millard et al., 2018b; Qu & Vargas, 2015) (Acimovic et al., 2018)
	Ibutilide*	Antiarrhythmic Class III / multi-channel block	FPDc prolongation / EAD / arrhythmia like events	0.2–100 nM	Commercial hiPSC-CM	MEA/VSO	(Blinova et al., 2018b; Nozaki et al., 2017b)
	Ivabradin	Antianginal / If- channel block / heart rate reducing	APD prolongation, negative chronotropic effects	1 μM	Double reporter cell line, subtypes: ventricular, atrial, nodal, TBX5 Nkx2.5	Patch clamp	(J. Z. Zhang et al., 2019b)
	JNJ303	IKv7.1 (IKs)- channel inhibitor	FPDc prolongation	300 nM	Commercial hiPSC-CM	MEA (+optogenetics)	(Lapp et al., 2017; Millard et al., 2018b)
	Prenylamine	Antihypertensive, antianginal, L-type Ca- channel block	FPD prolongation / beating arrest	3-15 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
	Procainamide	Antiarrhythmic class Ia / Na- channel block	FPD prolongation/ EAD	12 μM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
	Quinidine*	Antiarrhythmic class Ia / multi-channel block	FPDc and repolarization prolongation / EAD / arrhythmogenic effects / decrease in spike amplitude / reduction in AP upstroke velocity	0.05–10 μM	Commercial hiPSC-CM / Fibroblast-derived iPSC-CM (67% ventricular, 5% nodal, 28% atrial) (3D protocol)	MEA (+optigeneitcs) / Low impedance MEA	(Ando et al., 2017; Blinova et al., 2018b; Harris et al., 2013; Kitaguchi et al., 2017b; Lapp et al., 2017; Millard et al., 2018b; Nozaki et al., 2017b) (Navarrete et al., 2013)
	Ranolazine*	Antianginal / multi-channel block	FPDc and repolarization prolongation / clinical QT prolongation	0.3 μM, clinical conc. <100 μM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Kitaguchi et al., 2017b; Millard et al., 2018b; Nozaki et al., 2017b; Qu & Vargas, 2015)

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Table 1.1 (continued)

		Sematilide	Antiarrhythmic class III	FPDc prologation / EAD	0.1-0.6 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Sotalol*	Antiarrhythmic / beta- adreno-receptor block	Repolarization prolongation / EAD / arrhythmogenic effects / hERG- channel block	1.6- 15 µM; 100 µM	Commercial hiPSC-CM / Fibroblast-derived iPSC-CM (67% ventricular,5% nodal, 28% atrial) (3D protocol)	Low impedance MEA / MEA (+optogenetics)	(Ando et al., 2017; Blinova et al., 2018b; Lapp et al., 2017; Qu & Vargas, 2015) (Navarrete et al., 2013)
		Vernakalant	Antiarrhythmic class III / cardioversion of atrial fibrillation / atrial potassium-channel block	APD prolongation / partly arrhythmogenic effects	-	Double reporter cell line, different subtypes (ventricular phenotype)(2D protocol)	Patch clamp	(J. Z. Zhang et al., 2019b)
		ZD 7288	If- channel block	FPDc prolongation / negative chronotropic effects	3–30 nM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b)
	QT-prolonging	Diltiazem*	Antiarrhythmic / antihypertensive / antianginal / Cav1.2 inhibitor	FPDc shortening	90 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Dronedarone	Antiarrhymic	FPD shortening / beating arrest	100 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		Levocromakalim	Vasodilatant / K_{ATP} opener	FPDc shortening / negative chronotropic effects / membrane hyperpolarization	1–3 µM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Lidocain	Local anaesthetic / antiarrhythmic class Ib / selectiv Na-blocker	Reduction of AP upstroke velocity / FDP not affected	100 µM	Commercial hiPSC-CM	MEA +optogenetics	(Lapp et al., 2017)
		Metoprolol*	Antiarrhythmic / antihypertensive / ß1-adreno receptor block	no effects on FPD or EAD / arrhythmias / hERG block at higher concentrations/	100 µM	Commercial hiPSC-CM / CPVT cells and control (2D and 3D protocol)	MEA (+VSO) / patch clamp	(Ando et al., 2017; Blinova et al., 2018b) (Acimovic et al., 2018)
		Mexiletine*	Antiarrhythmic class Ib / hERG-channel block	Reduction in spike amplitude, Beating arrest and FPD prolongation at high concentrations	1–10 µM,	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Blinova et al., 2018b; Harris et al., 2013; Millard et al., 2018b; Nozaki et al., 2017b; Qu & Vargas, 2015)
		Mibefradil	Antianginal / Antihypertensive / multi-channel block	FPDc prolongation / positive chronotropic effects	0.12–1 µM	Commercial hiPSC-CM	MEA	(Ando et al., 2017; Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Nifedipin*	Vasodilatant / L-type Ca-channel block	FPDc shortening / positive chronotropic effects	0.02–1 µM	Commercial hiPSC-CM	MEA / automated patch clamp	(Ando et al., 2017; Harris et al., 2013; Millard et al., 2018b; Scheel et al., 2014b)
		Nitrendipine*	Antihypertensive / L- type Ca channel blocker	FPDc shortening	5 nM	Commercial hiPSC-CM	MEA	(Ando et al., 2017)
		NS- 1643	hERG-channel activator	FPD shortening / positive chronotropic effects	3 µM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Ouabain	Cardiac glycoside / Na^+-K^+-ATPase inhibitor	FPD(c) shortening / beating arrest	10–100 nM	Commercial hiPSC-CM	MEA	(Kitaguchi et al., 2017b; Nozaki et al., 2017b)
		Propranolol	Antiarrhythmic class II / beta-receptor block	EAD / negative chronotropic effects	10 µM	Commercial hiPSC-CM	MEA	(Nozaki et al., 2017b)
		Verapamil*	Antirrhythmic class VI / multi-channel block	FPDc shortening / Positive chronotropic effects / APD_{20} and APD_{90} shortening	0.02–0.3 µM; 1 µM	Commercial hiPSC-CM	MEA (+optogenetics) / patch clamp	(Ando et al., 2017; Harris et al., 2013; Lapp et al., 2017; Mulder et al., 2018)

results acquired from hiPSC-CM and clinical observations. With increasing interest, the question arose to what extent commercially available hiPS cell lines, generated from a few healthy donors, can represent a whole population with multiple genetic and epigenetic backgrounds. A direct comparison between patient and patient-derived hiPSC-CM regarding their response to cardiogenic drugs was published by Stillitano et al. who explored drug-induced QT prolongation by Sotalol in healthy individuals with CM generated from individual derived-hiPSC. Indeed, hiPSC-CMs recapitulated the phenotype in vitro, specific to the sensitivity (high and low to Sotalol) of each individual (Stillitano et al. 2018). Likewise, a proof of concept and a positive correlation between subject and subject derived hiPSC-CM could be shown for Moxifloxacin-induced cardiac effects (Shinozawa et al. 2017) and also for doxorubicin-induced effects on whole-cell level (Burridge et al. 2016). Despite the fact that hiPSC-CM have been shown to

reflect phenotype and pathology according to their donor specifications, it should be mentioned that cells from healthy donors can only partly predict those drug responses relevant for diseased patients. Blinova et al. have compared the differences of drug-induced QT-prolongation between cells generated.

From diseased (LQT1) and healthy individuals, the latter one not inevitably showing arrhythmic events. Clinical pharmacological and electrophysiological analyses of drug-mediated reactions (dofetilide and moxifloxacin) were compared to in vitro data from individual derived hiPSC-CM, not showing significant correlations for slopes and baseline parameter of the APD. This may be caused by the immaturity and congenital variability of the generated hiPSC-CMs (Blinova et al. 2019).

While developing a new classification to categorize the torsadogenic risk, based on drug testing of 60 substances using hiPSC-CM and MEA platform, Ando et al. focused on the free therapeutic drug concentration. The study considered differences between the free drug concentration in the cell culture medium as an in vitro parameter and the free effective therapeutic plasma concentration as an in vivo parameter. This free drug concentration is assumed to vary based on different protein binding fractions of the tested drugs that in turn relies on divergences in plasma concentrations between human blood and culture medium (Ando et al. 2017).

The influence of cell model variability was further investigated by Zeng and colleagues, who used hiPSC-derived CM of different gender and ethical origin and detected intersex differences in drug responses (Zeng et al. 2019). Similarly, donor-to-donor variability was demonstrated to affect the outcome of cardiotoxicity evaluation. In this study, hiPSC-CM from 43 individuals has been applied to examine the effect of multiple drugs and compounds (Burnett et al. 2019). The authors conclude that this population-based approach should be preferred to circumvent the drawback of donor-to-donor variability and to better reflect results received from clinical trials. Moreover, these data suggest that not all hiPSC-CM models might be suitable for pharmacological analyses and the establishment of preset acceptance criteria is highly recommended before human iPSCs are used for CIPA studies.

1.5 Personalized Medicine

Multiple studies have shown the advantages of using hiPSC-CM and MEA technology, suggesting this approach as a technique to develop personalized medicine as precise therapeutic interference. In the concept of personalized medicine, patient-derived hiPSCs are obtained by reprogramming of somatic cell sources such as fibroblasts, blood cells, or keratinocytes and induced to differentiate into cardiomyocytes, as shown in Fig. 1.2. After Yamanaka et al. have developed the first pluripotent stem cells from fibroblasts, the protocol for the generation of hiPSC remained roughly the same. Delivering the so-called Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc) that are related to ESC development leads to the initiation of pluripotency by decreasing expression of somatic genes followed by metabolic and morphological changes (Takahashi and Yamanaka 2006). During hiPSC maturation, the expression of endogenous pluripotency genes rises, which, if successfully integrated, results in stabilized hiPSC-colonies that represent an optimal source for the generation of cells of all three germ layers (Lodrini et al. 2020). Even though they bear the risk of tumor formation and uncontrolled differentiation or incomplete reprogramming, hiPSCs are a promising candidate for cardiac tissue engineering. Due to their easily accessible cell sources (non-invasive skin biopsy), unlimited proliferation capacity, comparable pluripotency to ESC, while overcoming ethical concerns they are a suitable alternative to adult primary CMs. The latter one was found, to dedifferentiate when cultured in vitro, for example, leading to a decline of sarcomeric structures (Cerbai et al. 2001; Hoppe 1998). Further, the access to human heart tissue is limited and the isolation of a sufficient number of cardiomyocytes for performing in vitro assays is challenging.

Following successful reprogramming and cardiac differentiation of patient-derived hiPSCs, cells are characterized by MEA analyses and effective drug concentrations are evaluated (Fig. 1.2). In parallel, this platform allows cardiac risk assessment during the development of new drugs that in turn can provide important information to establish personalized drug treatments. Using this strategy, not only the most promising pharmaceutical treatments are identified but also adverse drug effects can be detected and prevented. In this approach, hiPSC-derived cardiac cells are aimed to mimic their endogenous counterparts. The triggering of drug-induced life-threatening arrhythmias combined with data generated from disease modeling using patient-derived hiPSC-CM can complete existing clinical diagnostic and therapeutic tools.

Furthermore, those characterized patient-derived cells can be applied for individual cell therapies and transplantation when used as an autologous cell source, overcoming immunological limitations. Since the cardiac muscular cells in humans and other mammals show a very limited capacity of self-renewal in response to injury compared to the high regenerative capacity in lower vertebrates (Choi and Poss 2012), cell therapy is a fundamental and promising therapeutic application. The therapeutic outcome of cell therapies can be enhanced by the addition of methods such as genome editing for the repair of patient-derived cells (van den Brink

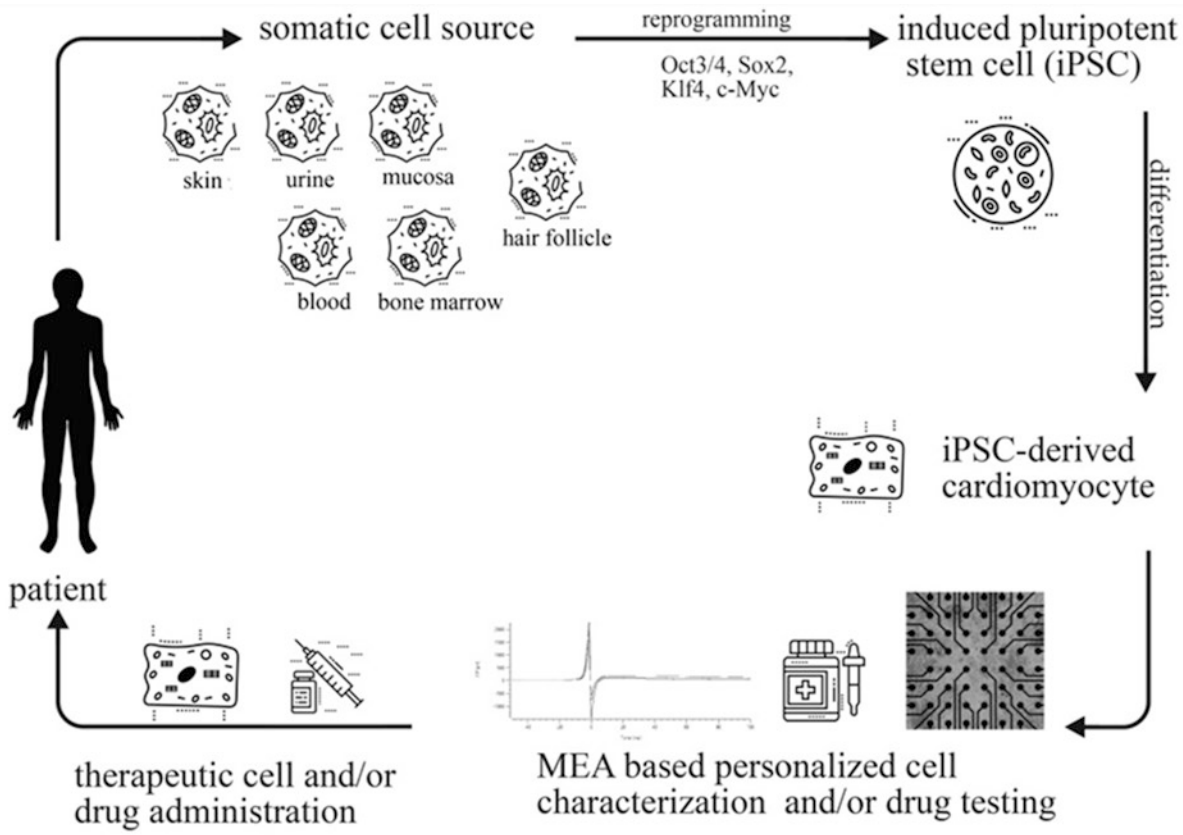

Fig. 1.2 MEA technology in personalized medicine. Schematic use of patient-derived cells for the generation of pluripotent stem cell-derived cardiomyocytes combined with MEA technology. Following iPSC production, MEA systems are applied to characterize hiPSC-CMs and/or to evaluate optimal pharmacological intervention, including the prediction of possible severe side effects

et al. 2019). However, one of the major issues for successful cell therapy is the used reprogramming protocol that is based on integrated gene delivery techniques such as retrovirus or lentiviruses, bearing a higher risk of insertional mutations and uncontrolled side effects compared to non-integrating techniques (Lodrini et al. 2020; Nakao et al. 2020).

All of us, previously known as the Precision Medicine Initiative, was launched in 2015 as a merge of the US National Institutes of Health (NIH) and several research centers aiming to connect/correlate clinical disease phenotypes with genetic data and the influence of different living conditions (lifestyle and environment) to classify subpopulations with comparable incidences, progression and chances of recovery to improve a more appropriated therapy (Chen et al. 2016; Collins and Varmus 2015; Jaffe 2015). Next to genome sequencing, the technological combination of patient-derived hiPSC-CM with the MEA platform is an auspicious tool to achieve those aims. The inclusion of patients even with extreme phenotypic manifestations reflects the variability of disease severity and enhances the understanding of correlations between donors and generated hiPSC-CM (Blinova et al. 2019).

Moreover, individual cardiotoxicity screening as part of the personalized medicine concept is crucial, since healthy individuals do not demonstrate the same drug response as well as probability and dimension of side effects, like OT prolongation (Blinova et al. 2019). Additionally, the genetic and nongenetic variability needs to be considered as it has been shown that not only genes (as whole genome sequencing) but also epigenetic modulations caused by surrounding environmental factors contribute to disease development, risk, and progression, and therefore effect respective therapies (Smith and White 2014).

Finally, personalized medicine will help to understand why patients with certain mutations or a single nucleotide polymorphism have a higher risk of complication or why patients with the same genetic alteration respond differently to the same clinical treatment (Chen et al. 2016).

1.6 hiPSCs as In Vitro Cell Models for CVDs

Since CVDs are the major cause of death worldwide, it is of uttermost importance to understand the underlying mechanism to improve established therapies and to develop novel treatment options. Human iPSC-derived CM, generated from

diseased patients or healthy donors, represent a valuable in vitro model to investigate CVD pathology (Ebert and Svendsen 2010). These hiPSC-cell lines, originated from dermal fibroblasts or blood cells, demonstrate disease-specific pheno- and/or genotype and hence reflect the physiological properties attributed to the respective disease (Liang et al. 2013). This supports our understanding of the connection between the genotype and the cellular phenotype in diseased patients.

The combination of hiPSC-cell models with novel genetic approaches provides further insights into the pathogenic mechanisms. For example, CRISPR/Cas9 genome editing was applied to elucidate the effect of certain mutations associated with long-QT syndrome (Bellin et al. 2013). Reversely, these disease-specific point mutations can be induced and replicated in stem cell-derived CM to study their impact on cellular physiology. While the insertion of the genetic defect caused abnormal AP patterns, corrections of the mutation results in normalization of the electrophysiological parameters (Bellin et al. 2013). Similarly, gene editing approaches facilitate the generation of proper controls that derive from the same hiPSC, just differing in the mutation of interest (presumably causing the disease). Thereby, variations of the genetic and epigenetic background when comparing hiPSC from healthy donors and diseased patients can be avoided (van den Brink et al. 2019). Van den Brink et al. is analytically reviewing different methods of genetic engineering in hiPSC-based disease modeling. In addition, transgenic cell models contribute to revealing the disease-related impact of cardiogenic drugs, showing a positive therapeutic outcome as well as detrimental side effects. The use of patient-specific in vitro cell models also allows the detection of possible differences in drug responses and helps to clarify the clinical vulnerability of high risks groups (Brandão et al. 2017; Liang et al. 2013).

1.6.1 Selective Overview of Current iPSC-Based Cell Models for CVDs

In 2013, Liang and colleagues created a library of hiPSC-derived CM, generated from various patients suffering hereditary CVDs, including familial dilated cardiomyopathy, hereditary long-QT syndrome, and familial hypertrophic cardiomyopathy. Following the evaluation of cardiac drug toxicity, they found that these patients are more susceptible to cardiotropic drugs and have a higher incidence to develop life-threatening arrhythmia if compared to healthy individuals (Liang et al. 2013). Likewise, the Jervell and Lange-Nielsen syndrome is one of the most severe disorders of heart rhythm, associated with delayed repolarization and ventricular tachycardia. To gain new insights into this inherited CVD, a hiPSC in vitro model was established and analyzed by patch clamp and MEA technology, demonstrating enhanced sensitivity to pro-arrhythmic drugs (Zhang et al. 2014).

During the last years, cardiac ion channelopathies have come to the fore in cardiovascular research and several hiPSC-based disease models have been generated for mechanistic in vitro studies. In this regard, the long QT syndrome is the most common one and respective hiPSC cell models were firstly established in 2010, showing significantly increased APDs in ventricular and atrial cells (Moretti et al. 2010). In another study, long QT syndrome hiPSC-derived CM were treated with small molecules, followed by FPDs recordings using an MEA system. The observed changes of FPD upon compound treatment enabled the detailed characterization of the role of specific ion channels, involved in the patho-mechanism (Egashira et al. 2012). Another channelopathy that is simulated in vitro using patient-derived hiPSCs is the catecholaminergic polymorphic ventricular tachycardia that is characterized by abnormal Ca2+ signaling (Liu et al. 2008).

Besides channelopathy, researchers have developed disease models for structural myopathies such as familial dilated cardiomyopathy and hypertrophic cardiomyopathy. The latter one is associated with ventricular fibrillation triggered by ventricular arrhythmias that can result in sudden cardiac death. Although the detailed pathophysiological mechanisms remain elusive, certain mutations were found to contribute to the elaboration of hypertrophic cardiomyopathy. hiPSC-derived CMs, exhibiting this genetic disposition were found to possess altered sarcomere arrangement and impaired electromechanical properties, for example, delayed depolarization and Ca2+ signaling (Carvajal-Vergara et al. 2010; Lan et al. 2013). For dilated cardiomyopathy, several mutations have been detected to be responsible for disease development and, hence, have been used for the generation of respective hiPSC cell models. This includes MYH7, TnnT, LMNA (laminin A/c), Desmin, Titin, and RBM20 RNA-binding motif protein 20 (Hinson et al. 2015; Siu et al. 2012; Streckfuss-Bömeke et al. 2017; Sun et al. 2012; Wyles et al. 2016; Yang et al. 2018). Structural analysis of these mutation-induced cell models showed increased cell size, defective calcium handling, and altered sarcomere organization (Giacomelli et al. 2017; Streckfuss-Bömeke et al. 2017).

Moreover, hiPSC-CMs were generated from patients suffering from Duchenne muscular dystrophy to study the molecular mechanisms underlying the dystrophy. Cardiomyocytes derived from patient hiPSCs showed a phenotypical deficiency of dystrophin, elevated Ca2+ levels, and increased apoptosis (Lin et al. 2015).

In contrast to the generation of hiPSC models from patient-derived cells, novel gene-editing approaches allow the manipulation of cells to simulate the geno- and phenotype of mutations-related CVDs, independent on the avail-

ability of patient tissue material. For example, using CRISPR/Cas9, de la Roche et al. introduced the A735V mutation into the SCN5A gene to create an in vitro model for the Brugada syndrome. Differentiated CMs exhibited irregular electrophysiology, including a decreased upstroke velocity and sodium current density (de la Roche et al. 2019). Similarly, gene editing was successfully applied in patient-derived hiPSC-CMs, suffer from Fabry disease, to analyze the functional consequences of the underlying genetic defects (Birket et al. 2019).

Disease modeling not only helps to reveal the molecular patho-mechanisms but can also elucidate effects on cell morphology and functionality. CMs derived from hiPSCs of hypertrophic cardiomyopathy patients were cultured as microtissue and subjected to contraction force analysis. The induction of mechanical overload revealed that hypertrophic cardiomyopathy reinforces dysfunction of cell contractility (Ma et al. 2018). Similarly, Caluori et al. established a correlative system containing a MEA platform and an atomic force microscope to examine the topography, beating force, and electric events in hiPSC-CM from dilated cardiomyopathy patients (Caluori et al. 2019).

As the CIPA initiative aims to combine in vitro and in silico simulations, computational-based disease modeling is another approach to promote the reliability of the results/predictions obtained in wet lab experiments. For the LQT syndrome, an in silico analysis was carried out to replicate the behavior found in diseased hiPSC-CMs. The acquired in silico data of cellular electrophysiology were comparable to experimental results, highlighting the importance of the synergy of cell culture studies and in silico simulations (Paci et al. 2018).

1.7 Conclusion and Future Perspective

In recent years, human iPSCs have taken on an increasingly important role in studying heart disease and function, heart development, and in the discovery of new pharmacological compounds (Casini et al. 2017; Edwards et al. 2018; Satsuka and Kanda 2019; Zhang et al. 2019). This includes the detailed characterization of the electrophysiological properties of CM derived from these hiPSCs. The MEA technology allows a noninvasive and label-free analysis of electrically active cells and, therefore, has become a commonly applied platform for the assessment of electrical parameters in CMs in vitro. Moreover, MEA-based detection is not only limited to cellular monolayers but can also be used to measure tissue slices from heart and brain or organoids to better reflect in vivo conditions. As such, MEA systems provide the possibility for organ-on-a-chip engineering, facilitating the clinical translation of the obtained in vitro data (Zhang et al. 2018).

MEA technology enables high-throughput measurements, which is helpful for the development and identification of new therapeutic drugs in cardiovascular research. MEA systems and hiPSC-CMs have become a dream team that has already been successfully used for disease modeling and pharmacological studies. In this regard, hiPSC-based MEA platforms offer a promising approach for the establishment of personalized drug administration which provides the possibility of therapeutic intervention, specific to the need of the patient (Chen et al. 2016; Sanzo et al. 2017; Hamazaki et al. 2017).

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CD34+ Stem Cells and Regenerative Medicine

2

Philippe Hénon and Rachid Lahlil

Abbreviations

ABMT	Autologous bone marrow transplant
ALD	Alcoholic liver disease
BMT	Bone marrow transplant
BM-MNCs	Bone marrow mononuclear cells
BMP-4	Bone morphogenetic protein-4
CoREST	Chromatin remodeling complex
CLI	Critical limb ischemia
CRL3	CULLIN3-E3 ubiquitin ligase
ESCs	Embryonic stem cells
EPCs	Endothelial progenitor cells
EPCR	Endothelial protein C receptor
FSH	Follicle stimulating hormone
GVHD	Graft versus host disease
GMP	Good manufacturing process
HGF	Hepatocyte growth factor
HLA	Human leucocyte antigens
iPSCs	Induced pluripotent stem cells
KL	Kit ligand
LH	Leutinizing hormone
LSD-1A	Lysine-specific histone demethylase 1A
MSCs	Mesenchymal stem cells
MI	Myocardial Infarction
MNCs	Mononuclear cells
NO	No-option
PB-MNCs	Peripheral blood MNCs
PBSCs	Peripheral blood stem cells
SDF	Stromal cell derived factor
VSELs	Very small embryonic-like stem cells

P. Hénon (✉)
CellProthera SAS, Mulhouse, France
e-mail: phenon@cellprothera.com

R. Lahlil
Institut de Recherche en Hématologie et Transplantation, Hôpital du Hasenrain, Mulhouse, France

2.1 Introduction

2.1.1 History

The progression from the first attempts of bone marrow (BM) transplantation to the new concept of regenerative medicine, using more or less successfully various types of cells most often originating from the BM—for different therapeutic indications was long and complicated. This progression continues today.

In 1957, American physician E. Donnall (Don) Thomas reported, for the first time, a radical new approach in cancer treatment using radiation and chemotherapy followed by the intravenous infusion of BM (Thomas et al. 1957). One year earlier, at the Mary Imogene Basset Hospital in Cooperstone, New York, USA (affiliated to the Columbia University), he had effectively performed the first bone marrow transplantation (BMT) in humans in a leukemia patient receiving healthy BM from an identical twin. Although he considered this first successful attempt as occasional because of the syngeneic origin of the BM infused, this publication represented the beginning of a series of laboratory and clinical investigations for more than one decade, including those performed by his own team (Thomas 1962).

At the end of 1958, Georges Mathé, a French hematologist and immunologist, performed a human BMT using non-related BM on six Yugoslav engineers who were accidentally irradiated at different degrees after a nuclear reactor incident (Mathé et al. 1959). The two more severely irradiated died without BM engraftment; the four others survived after a long period of aplasia. However, either Mathé was very lucky to find directly compatible donors when the phenomenon of histocompatibility was still unknown, or more probably the BM of these patients less severely irradiated was not permanently destroyed but would get progressively self-renewed over time (Mathé et al. 1958). However, Mathé, for the first time, observed the graft-versus-host-disease (GVHD) in those patients and was the first to study and report the debilitating and wasting conditions that follow BMT in the recipients. He deducted that GVHD must be due

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to an immune reaction of the cells in the donor marrow against the cells in the human patient.

Following his successful syngeneic BMT, Thomas (who had moved to Seattle) attempted to define methods to match the tissues of donor and recipient to minimize the latter's rejection of the donor BM. He also developed drugs to suppress the immune system, thus further reducing the probability of graft rejection by the recipient (Storb et al. 1976).

In 1958, Jean Dausset, a French immunologist identified the first human leukocyte antigens (HLA), which plays a crucial role for the immune system to recognize self from nonself in the human body (Dausset 1999). However, it took a further 10 years to recognize the role of all leukocyte antigens of the HLA system in determining the compatibility between a donor and recipient (Dausset and Rapaport 1966; Dausset 1999). These findings actually acted as a trigger to perform unrelated BMT in the patients. Jean Dausset was Nobel Laureate in 1980 for this discovery of the HLA system and its role in immune reactions.

In 1969, based on this knowledge of human histocompatibility, Don Thomas' group performed the first BMT in a leukemia patient from a relative who was not an identical twin (Buckner et al. 1970). His group further continued to optimize the allogeneic BMT methodology and has performed thousands of procedures over the years, while training physicians from many countries to make BMT routinely carried worldwide and treat people with life-threatening blood disorders. Thus, BMT is a prime example of a successful stem cell therapy. Don Thomas was considered the father of BMT, and in recognition to his contributions in the field, he was awarded the Nobel Prize in 1990.

However, allogeneic BMT is hampered by many disadvantages. First, finding a histocompatible donor is difficult, even if the creation of donor registries in various countries has now consistently improved the chance of finding a suitable donor. Allogeneic BMT is often followed by debilitating and wasting conditions, as first described by Mathé. Although always possible, graft rejection is mostly avoided by posttransplant administration of immunosuppressive drugs. A mild to severe acute GVHD is frequently observed, and even though its antileukemic effect plays an essential role in curing the disease, it can also severely affect different organs (lung, liver, gut, skin, etc.), leading to long-term debilitating morbidities and a patient death rate of up to 40%, depending on the disease. Because BM engraftment takes 3–4 weeks, severe aplasia-related bleedings and infectious diseases may also occur. Thus, for a long time, performing an allogeneic BMT was reserved for patients under 50 years of age, with no comorbidity. In total, no more than 10% of patients were in a position to undergo BMT. Total body irradiation systematically performed at the historical period, also induced secondary effects, for example, bilateral cataracts occurring several years after transplantation, even in children, and sterility.

Thus, intending to avoid such posttransplant severe morbidity/mortality, Norbert-Claude Gorin, a French hematologist having first developed a technology allowing long-term cell cryopreservation (Gorin and Duhamel 1978), performed in 1976 the first autologous BMT (ABMT) from a previously cryopreserved patient's own BM (Gorin et al. 1977). Considering the numerous advantages of the autologous setting (no lack of donor who is the patient him/herself, no graft rejection nor GVHD, more rapid engraftment), this approach progressively supplanted BMT in number, at least for certain indications such as lymphomas or multiple myelomas. In leukemia, however, the absence of GVHD-related antileukemic effect and the risk of persisting contamination of the graft product by residual leukemic cells consistently increased the risk of relapse of the disease.

What is impressive is that, throughout this historical period, clinical investigators were not concerned about the characterization of the hematopoietic stem cells (HSCs). Although the murine HSC had been identified and characterized in the 1960s, the human HSC was not actually investigated. BMT investigators only looked at a maximal number of total nucleated cells in the BM harvest, and because this simplified approach was successful, they did not further pursue knowledge regarding the putative HSCs.

However, with the conjunction of two discoveries in the mid-1980s, this knowledge took a leap forward: these discoveries were the identification of the $CD34^+$ cell and the detection of hematopoietic progenitors circulating into the blood, leading to the first attempts of peripheral blood stem cells (PBSC) transplantation.

2.2 $CD34^+$ Stem Cells and Peripheral Blood Stem Cell Transplantations

$CD34^+$ cells were identified for the first time by Civin in 1984 (Civin et al. 1984). CD34 is a membrane glycophosphoprotein that was discovered due to a strategy to develop antibodies that specifically recognize small subsets of human BM cells, but not mature blood or lymphoid cells (Krause et al. 1996). CD34 antibodies specifically detect approximately 1% of low-density mononuclear cells from BM aspirates of normal donors when there is less than 0.1% CD34 labeling of PB cells (Civin et al. 1996). However, the intensity of CD34 expression on the cell's membrane is heterogeneous and correlates with the stage of cell immaturity/maturity, subdividing cells into $CD34^{bright}$ or $CD34^{dim}$ subgroups. The $CD34^{bright}$ are smaller and less dense than the $CD34^{dim}$ and correspond to the earliest progenitors, that is, stem cells, representing at best one-fifth of the total $CD34^+$ cells. The $CD34^{dim}$ are larger and denser and correspond to

more committed progenitor cells, having lost their clonal growth properties (Herbein et al. 1994).

At the same time as CD34+ cell-type was identified, six groups worldwide, including ours, decided to apply in the humans the results of experimental studies performed and achieved in dogs by Fliedner and others (Fliedner et al. 1979), which showed the appearance of a transient peak of the number of granulocytic progenitors (CFU-GM) circulating in the peripheral blood at the end of post-chemotherapy aplasia. Collecting blood cells by leukapheresis at this time would allow constituting and cryopreserving a cell graft supposed to be capable of reconstituting the BM upon reinjection. Spectacular results were achieved from the very first blood cell transplantations performed almost at the same time by the six independent research groups (one each in Australia, Germany, the United States, and Great Britain, and two in France—Bordeaux and Mulhouse), showing more particularly an unexpected rapid cell engraftment (10–12 days instead of 3–4 weeks with BM) (Juttner et al. 1985; Körbling et al. 1986; Kessinger et al. 1986; Bell et al. 1986; Reiffers et al. 1986; Debecker et al. 1986). Despite these preliminary breakthrough data, the scientific community expressed significant reservations about the future of the technology. Looking at a poster reporting the results of our first three PBSC transplantations displayed by our group in 1987 at a US BMT meeting, Don Thomas said: "absolutely not interesting!". However, when meeting him again 10 years later, after he had received the Nobel Prize, reminding him this sentence, he answered: "even a Nobel Laureate can be wrong!"

So, we had to convincingly prove that the blood not only contains the advanced granulocytic progenitors, capable of solely achieving transient engraftment, but also true stem cells responsible for long-term and sustained BM regeneration. This was made possible once the CD34 antibody was commercially available.

For a long time, CD34+ cells were considered as being solely HSCs giving rise to all hematopoietic lineages (Krause et al. 1996). Because they can be mobilized in large number from the BM into the PB using hematopoietic growth factors, achieving approximately a two-logs higher cell enrichment, such cell enrichment favored the practice of PBSCs transplantation which increased exponentially from the first attempts in the mid-1980s to the point of totally replacing BMT in hematological cancers (Hénon et al. 1991). Determination of the graft content in CD34+ cells came to be routinely performed using an identification/numeration flow cytometry (FCM) methodology that we developed in collaboration with the Gianni's group in Milano (the Mulhouse/Milano method) (Sovalat and Serke 1993; Siena et al. 1993). This protocol was adopted by all groups worldwide for more than 10 years. Looking retrospectively at the data of the first thousand PBSC transplantations that were performed, an optimal amount of CD34+ cells (2×10^6/kg body weight) was further recommended.

Attempts to purify and enrich CD34+ cells at a clinical scale started from the mid-1990s. Various protocols and systems of purification/enrichment using immune-selection methodologies were developed, mainly by Baxter (Isolex 300 i) and by Miltenyi (Clinimax). The goal was to deplete the autologous graft from malignant cells. However, it rapidly became evident that in granulocytic leukemias, most of the leukemic cells were CD34+, thus rendering the CD34+ enrichment not only meaningless but also clinically unsafe for use in the patients. CD34+ cell grafts were also tested in various clinical trials in multiple myeloma without any effect on relapses, which mainly occurred from residual malignant cell clones resisting high-dose chemotherapy and persisting in the patient's body (de la Rubia et al. 1994).

2.2.1 Evolution in CD34+ Stem Cell Knowledge

From the mid-1990s, it was demonstrated that the "true" HSCs population, inducing and sustaining post-aplasia hematopoietic recovery in the long-term, and characterized by a lack of the CD38 differentiation marker (Civin et al. 1996; Hénon et al. 1998), only represents a small portion (≈1%) of the total CD34+ cells thus raising the question of the identity of remaining $CD34^{bright}$ cells. In 1997, Asahara et al. isolated a subpopulation of mice BM-derived endothelial progenitor cells (EPCs) bearing the CD34 antigen capable of inducing neo-angiogenesis, thus demonstrating for the first time the existence of non-hematopoietic CD34+ cells (Asahara et al. 1997). In the following years, researchers progressively established that the CD34 antigen was also a marker of, liver, osteoblastic, cardiac and cartilage progenitor cells (Gordon et al. 2006; Matsumoto et al. 2008; Pasquet et al. 2009); each of these CD34+ subpopulations representing approximately 1% of the total CD34+ cells. In an attempt to explain this diversity in the CD34-expressing subpopulations, several investigators proposed the hypothesis that HSCs could transdifferentiate into various other lineages of progenitor cells, even those normally issued from another germ layer, opening the path for the transgressive concept of "cell plasticity" that persisted a number of years.

2.2.2 CD34+ Stem Cells and Ischemic Diseases

2.2.2.1 Heart Diseases

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide. From the beginning of the 2000s, cell therapy has emerged as a potential treatment to restore heart function after ischemic heart failure. Indeed, in addition

to hematopoietic transplantation, CD34+ cells have been mainly employed to achieve myocardial repair after myocardial infarction (MI) episode. Besides CD34+ cells, other cell types have been tested in more than 80 studies, mostly randomized controlled trials, enrolling patients with ischemic heart disease including MI and heart failure. However, most of these studies have failed to achieve significantly results in terms of cardiac function improvement and attenuation of myocardial remodeling. The so-called BM mononuclear cells (BM-MNCs) are not stem cells but a composite tissue that contains a mix of hematopoietic cells at various stages of maturation, besides stromal cells. Only a very few cells have stem-cell like characteristics, that is, capable of long-term undifferentiated self-renewal or mutilineage differentiation. Mesenchymal stem cells (MSCs) are mainly stromal progenitor cells that do not possess the characteristics required for clonal growth. The existence of cardiac stem cells is highly controversial, excepted in newborns. Supported by experimental and physiological data, CD34+ stem cells have thus emerged as the only "true" stem cells in the BM or in the PB that are capable of positive interaction with an injured heart. For example, CD34+ cells isolated from human PB after HGF-induced mobilization stained positively for c-Troponin-T when transplanted directly into the scar of athymic rats with experimental AMI. These data indicated that CD34+ can also differentiate into cardiomyocytes (Iwasaki et al. 2006). In humans, endogenous CD34+ cells are released into the blood within the first hours following AMI and continues for approximately 1 week, which seems to be a physiological response to limit ischemic scar formation (Theiss et al. 2007).

We were the first to perform direct intracardiac delivery of large amounts of peripheral blood-derived CD34+ cells, previously purified by in vitro immuneselection, in patients suffering from bad prognosis after MI (Hénon et al. 2003). Furthermore, we demonstrated the long-term benefit on regional heart structure and function of this breakthrough approach (Pasquet et al. 2009). We also provided evidence that CD34bright cells could differentiate both endothelial and cardiac progenitor cells after in vitro culture on an appropriate and proprietary medium we developed (Pasquet et al. 2009). Other investigators followed the same direction, using CD34+ cells purified and enriched by immunoselection either from PB after mobilization (Losordo et al. 2011; Vrtovec et al. 2013) or BM aspirates (Quyyumi et al. 2017), but with less significant results due to variations in the dose of cells delivered, route of injection, time of injection after the disease onset, and the stage of the ischemic disease. All these are critical factors to ensure successful therapy and optimal prognosis (Hénon 2020). We have now developed a proprietary automated device (StemXpand®) and disposable kits (StemPack®) allowing a ~20 fold-expansion of PB-CD34+ cells for GMP production on a clinical scale of CD34+ cell grafts (ProtheraCytes®) (Saucourt et al. 2019). We have launched an international multicentered, randomized phase II study (EXCELLENT, EuDract N°20014-001476-63), which is still ongoing, enrolling 44 patients (33 MI patients receiving intramyocardial injection of autologous ProtheraCytes® versus 11 controls [Standard of Care]). The preliminary results of this study are progressing in the same direction as those of our pilot study.

The underlying cardiac repair mechanisms of CD34+ cells are likely multifaceted. Once activated by a complex blend of cardioactive chemokines secreted by the inflammatory scar (Ebelt et al. 2007; Cho et al. 2007), injected CD34+ cells may release soluble paracrine factors and exosomes that can enhance resident cardiomyocytes proliferation (Ratajczak et al. 2019) or support neo-angiogenesis (Sahoo et al. 2011), respectively, thus reducing fibrosis and attenuating remodeling effects. The scar chemokines also chemoattract CD34+ stem cells to home-in to the ischemic zone and induce their commitment along the endothelial and cardiac pathways (Ebelt et al. 2007). These cellular and molecular events are strongly dependent on changes that occur in myocardial stiffness after AMI (Cho et al. 2007). Such commitment cannot occur under steady-state conditions but is crucial for the induction of cardiac tissue repair after ischemic heart disease.

2.2.2.2 Stroke

Stroke is the second most common single cause of death worldwide and is the commonest cause of adult disability. Current treatment options offer only modest benefits. Achieving guidance of stem cells toward regenerating neurons and damaged tissue is a new and innovative clinical research area being currently investigated. Various stem cells have been tested in this context, mainly derived from the BM, primarily MSCs and MNCs. When transplantation of these cells is deemed safe for stroke, their efficacy remains elusive, as they exerted or not a functional recovery, depending on the clinical trials reviewed in (Banerjee et al. 2011) and (Reis et al. 2017). Although purified CD34+ stem cells are not being currently preferred for this therapeutic indication, several lines of evidence would favor their use in the future. First, BM-derived stem cells in preclinical experimental stroke models revealed the cells' ability to display multipotent cell properties (Kocsis and Honmou 2012). Second, as observed after AMI, stroke is followed by a large and bursting spontaneous mobilization of PB-CD34+ cells within at least 1 week duration (Dunac et al. 2007; Gójska-Grymajło et al. 2012, 2018; Borlongan 2019). The extent of this cell mobilization appears to correlate both with acute stroke patients' neurologic and functional status at the onset of the disease and with the degree of neurological and functional recovery (Dunac et al. 2007; Gójska-Grymajło et al. 2012). A smaller increase in CD34/CXCR4 positive cells

correlates well with initially worse neurological status, whereas large increase correlates with better functional neurological status after the 6-month follow-up. Third, CD34⁺ cells have been demonstrated to improve functional recovery and attenuated infarct size in rodent model of stroke (Taguchi et al. 2004; Shyu et al. 2006). Finally, a clinical trial of intra-arterial BM-MNC treatment of acute stroke showed a trend toward a positive correlation between the number of CD34⁺ cells injected and functional outcome at 1 month (Moniche et al. 2012). Despite such favorable outcomes, few clinical trials using purified CD34⁺ cells have been reported. A first-in-man study using CD34⁺ cells, delivered intra-arterially in five patients with acute ischemic stroke, showed improvement in clinical, functional scores and reduction in lesion volume in all patients during 6-month follow-up (Banerjee et al. 2014). A randomized, single-blind, and controlled Phase II study was conducted in 30 patients with mid-cerebral artery infarction, divided in two equal groups: 15 patients underwent stereotaxic injection of 3–8 × 10^6 G-CSF-mobilized and immune-sorted PB-CD34⁺ cells compared with 15 control patients (Chen et al. 2014). Improvements in stroke scales and functional outcome from baseline to the end of the 12-month follow-up period were significantly greater in the CD34 group than the control group. New and larger clinical trials in future (several are currently underway) are required to demonstrate the true potential of CD34⁺ cells in stroke patients.

2.2.2.3 Critical Limb Ischemia

Critical limb ischemia (CLI) is the end stage of lower limb ischemia due to atherosclerotic peripheral disease or vasculitis, including Buerger's disease, with a prevalence of 500–1000 patients/million every year. The first objective in treating CLI is to increase perfusion of the affected limb. However, in 25–40% of the cases, surgical revascularization is not feasible due to lack of autologous vein graft, extensive lesions in the tibial and peroneal arteries, or medical comorbidities. Six months mortality and significant amputation rates are as high as 40 and 20%, respectively, in these "no-option" (NO) patients. In recent years, therapeutic angiogenesis using stem cell therapy has provided a new alternative for these patients. Initially, most clinical studies in this regard, which used BM-MNCs (Matoba et al. 2008; Pignon et al. 2017) or PB-MNCs (Horie et al. 2010), have produced promising limb salvage results. However, as the predominant functional cells for vasculogenesis are EPCs which are CD34-positive (Asahara et al. 2011), the present trend is to prefer using fresh CD34⁺ cells, immuno-magnetically isolated from PB-MNCs. Several clinical studies have tested safety, feasibility, and efficiency of CD34⁺ cell therapy. In a phase II clinical trial of CD34⁺ cell therapy in NO-CLI patients, intra-muscular (IM) transplantation of G-CSF-mobilized CD34⁺ cells, ischemic rest pain scales and physiological parameters improved relatively early after cell therapy, then plateaued later accompanied by recovery of the CLI state (Fujita et al. 2014).

A meta-analysis pooling 10 randomized studies including 479 CLI patients with 234 patients undergoing CD34⁺ cell injections (numbers of CD34⁺ cells injected ranging from 2.93 × 10^6 to 13.61 × 10^7) and 240 controls, seemed to imply a potential therapeutic benefit of CD34⁺ cell therapy for NO-CLI in terms of limb salvage and ulcer healing (Pan et al. 2018). This benefit was more significant in patients having received high doses than low doses of CD34⁺ cells. However, the heterogeneity and the relatively small sample size of most studies, associated with a potential placebo effect of CD34⁺ cell injection, rendered the results of this meta-analysis underpowered to draw a definite therapeutic conclusion. In a more recent prospective randomized single-blinded non-inferiority clinical trial, NO-CLI patients were 1:1 randomized to receive either PB-MNCs or purified CD34⁺ cells (Dong et al. 2018). Cells were implanted under general anesthesia into the calves/arms and feet/hands of the ischemic limb via equidistant IM injections. Although CD34⁺ cells and PB-MNCs globally showed similar efficacies in terms of limb salvage, a significantly higher percentage of the patients had achieved rest pain relief at 2 weeks in the CD34⁺ group than the PB-MNCs group. This suggests that CD34⁺ cells improved perfusion earlier than the PB-MNCs and that the treatment with CD34⁺ cells could be preferred for patients with more critical and progressive ischemia. Again, however, the quantity of CD34⁺ cells delivered is a discriminant factor. Repeated application of stem cells might be performed successfully in the case of CLI relapse (Boda et al. 2011).

2.2.3 CD34⁺ Cells and Non-Ischemic Diseases

2.2.3.1 Liver Insufficiency

Millions of patients worldwide suffer from end-stage liver diseases, whose only curative treatment is liver transplantation. However, the lack of available livers results in the death of most patients while awaiting transplantation. Recent advances in regenerative medicine have brought hope for these patients.

In 2000, Lagasse et al. first reported that purified "HSCs", following intrahepatic transplantation, could give rise to hepatocyte-like cells in vivo and completely repopulate the liver of FAH mice, thus correcting their disease phenotype (Lagasse et al. 2000). Jang et al. further demonstrated in mice that cells that they continue to refer to as HSCs can convert in vitro into functional liver cells within days, and that liver injury-related microenvironmental cues are responsible for the conversion (Jang et al. 2004). In 2006, Gordon et al. reported a phase I study, in which between 1 × 10^6 and

2×10^8 autologous CD34⁺ cells, collected into the blood after G-CSF mobilization, were injected as a single bolus into five patients with cirrhosis-related chronic liver insufficiency either into the portal vein (three patients) or hepatic artery (two patients) (Gordon et al. 2006). No complications or specific side effects related to the procedure were observed. Three patients showed improvement in serum bilirubin and four of five in serum albumin. The disappearance of ascites was observed in one patient. These improvements were sustained for at least 1 year. The same group further performed a small nonrandomized study administering autologous expanded mobilized adult progenitor CD34⁺ cells into the hepatic artery of nine patients with alcoholic liver cirrhosis: they achieved similar significant biological and clinical improvements (Pai et al. 2008).

A retrospective nonrandomized study enrolled 48 patients with end-stage chronic liver diseases (36 post-hepatitis C, 12 post-auto-immune disease) (Salama et al. 2010). All patients were treated with G-CSF-mobilized and immune-selected PB-CD34⁺ cells, further amplified in a hepatocyte differentiation medium, either via their hepatic artery or portal vein. Only 20.8% of these very end-stage liver disease patients—not significantly the sickest when cell therapy was initiated—died during 12 months of follow-up. All the other patients showed marked changes in albumin, bilirubin, INR, and transaminase level and a statistically significant decrease in ascites.

Two more randomized studies were published, but unfortunately, they did not use purified CD34⁺ cells, but BM-MNCs. One enrolled 58 patients with a decompensated alcoholic liver disease (ALD) (28 cell recipients, 30 controls), but did not result in an expanded hepatic progenitor cells compartment or improved liver function, probably because of the meager number of stem cells contained in the BM-MNC product ($0.24 \pm 0.11 \times 10^6$ CD34⁺ cells/kg) (Spahr et al. 2013). On the contrary, the second study, enrolling 47 patients with decompensated liver cirrhosis (32 cell-treated, 15 control), showed a biological efficacy and less severe complications in the transplant group than controls (Bai et al. 2014). However, patients in this study received a three-log higher quantity of BM-MNCs than in the first study (10^{10} versus 10^7, respectively), and even if the number of CD34⁺ cells contained into the cell graft product was not calculated, it is logical to think that it increased in the same proportions, thus explaining the positive results.

It also seems that repeated CD34⁺/133⁺ cell infusions would give more sustained clinical efficacy and improvement in liver functions and quality of life during 22-months follow-up compared with single CD34⁺/133⁺ cell infusion (Zekri et al. 2015). Nonetheless, pushing cell selection to only keep and inject CD133⁺ cells has shown no efficacy in liver function improvement (Newsome et al. 2018).

Liver repair mechanisms using CD34⁺ cells are probably similar to those involved in cardiac repair, that is, cell differentiation and paracrine effects. PB-CD34⁺ cells, bearing the SDF-1 receptor (CXCR4), may differentiate in liver-committed cells and colonize the damaged liver by following an SDF-1 gradient (Hatch et al. 2002; Ratajczak et al. 2004). Hepatocyte growth factor (HGF), matrix metalloproteinase-9 (MMP9)—a fibrosis mediator—and G-CSF can also contribute to CD34⁺ cell recruitment and homing into the liver (Kollet et al. 2003; Higashiyama et al. 2007; Piscaglia et al. 2007).

2.2.3.2 Knee Arthrosis

Knee joint cartilage often suffers defects that cause pain, swelling, functional disturbances, and disability, constituting a serious public health problem. Articular cartilage has a minimal regenerative and self-healing potential due to its avascular, aneural, and alymphatic characteristics and a low number of progenitor cells (Redondo et al. 2018). Stem cell therapy is emerging as an alternative to current treatments, which are more or less effective in lessening symptoms but do not avoid tissue damage. MSCs were first considered a potential candidate for cartilage repair, but despite many experimental studies in small and big animal models, only a few clinical trials have been conducted (Orozco et al. 2013; de Windt et al. 2017). All the clinical outcomes indicated the safety of such an approach and its ability to relieve some symptoms, although their ability to repair the effect of cartilage damage was not always apparent.

A new trend currently favors PBSCs, particularly PB-CD34⁺ cells, because they are easy to obtain and increasing evidence that they are a potential source of chondrogenic progenitor cells (Fu et al. 2014; Wang et al. 2016). Using an experimental rat model, Supartono et al. reported hyaline cartilage regeneration in osteochondral defect after intra-articular injection of human PB-CD34⁺ cells (Supartono 2018). In humans, the use of PB-CD34⁺ stem cells combined with a treatment and rehabilitation algorithm developed for maximum effectiveness of the procedure primarily helps relieve pain, regenerate articular cartilage, and improve joint function (Kubsik-Gidlewska et al. 2018). Chen et al. deducted from a review reporting seven clinical trials using unselected or CD34-selected PBSCs that this approach was safe and seemed to enhance cartilage repair and regeneration by intra-articular injection (Chen et al. 2020). However, considering the deficiency of studies with a high level of evidence in humans, it is still necessary to maintain a conservative attitude toward the positive effect of PBSCs/CD34⁺ cells on cartilage repair, which have to be confirmed by more extensive and larger randomized clinical investigations.

Are CD34⁺ stem cells pluripotent stem cells? Considering their large capacity to differentiate in various cell tissues, it

now seems that some, if not all, CD34⁺ cells might be pluripotent rather than multipotent stem cells, capable of giving rise to such a large panel of organic tissues under specific stimulations.

2.3 Very Small Embryonic-Like Stem Cells

Outside CD34⁺ and their potential of repairing different organs, regenerative medicine remains in search of other stem cells, which would ideally be pluripotent, differentiating into cells belonging to the three germ layers (meso-, ecto-, and endoderm). Therefore, different types of stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have been proposed for therapeutic applications in the past years and represented a great hope during many years of development of effective regenerative medicine. Unfortunately, several drawbacks have hampered their clinical use as pluripotent stem cells. ESCs and iPSCs have exhibited several problems, such as genomic instability, a possibility of teratoma formation, and the risk of immuno-rejection. Currently, only the HSCs show a straightforward, efficient, and safe use in the routine treatment of many hematopoietic diseases and give rise to real therapeutic benefit after transplantation. However, in 2006, pluripotent cells referred to as very small embryonic-like stem cells (VSELs), capable of differentiating and regenerating various organic tissues, were identified and isolated in adult tissues among CD34⁺ stem cells. VSELs have become the most promising avenue to improve the treatment by regenerative medicine of many diseases that have no conventional treatments (Kucia et al. 2006).

The VSELs were first identified in the BM and PB but can also be found in umbilical cord blood and other organs such as the gonads (Kucia et al. 2007), thus diversifying their collection and sampling source. They can support vessel formation in vivo (Guerin et al. 2017b) and be specified to cardiomyocytes (Dawn et al. 2008; Zuba-Surma et al. 2011, p. 201), neurons (Lee et al. 2014), and hematopoietic stem cells (Ratajczak et al. 2011), and differentiate into gametes (Fig. 2.1).

VSELs have similar characteristics to ESCs and are believed to originate from primordial germ cells. They express several markers characteristic of these migrating cells, such as Stella and Fragilis and sex hormone receptors. They escape specification as tissue-committed stem cells, retain their pluripotency by expressing pluripotent transcription factors, that is, Oct-4, Nanog, and Sox-2 at the protein level, and persist throughout life in different organs (Ratajczak 2017). Overall, VSELs are isolated based on their phenotypic features and their small size (5–6 μm) as being CD34⁺, CD133⁺, and/or CXCR4⁺, but lineages (Lin) and hematopoietic markers (CD45) negative (Kucia et al. 2006; Ratajczak et al. 2012). On their surface, they express additional embryonic pluripotent stem cell-specific markers, such as SSEA-4 and TRA-1-81, which could serve as supplementary criteria for sorting.

Their identification and characterization provoked reservations at the time of their discovery but subsequently, several laboratories have then been able to confirm their presence in both animals and humans (Kassmer and Krause 2013; Guerin et al. 2015; Shaikh et al. 2015; Nakatsuka et al. 2016).

Besides, populations similar to or resembling VSELs most likely representing overlapping stem cells have been defined depending on the experimental strategies and diverse markers used for their isolation and their ex vivo expansion conditions. Therefore, different pluripotent and multipotent cells have been reported during the recent decades that include multipotent adult stem cells (MASCs) (Beltrami et al. 2007), multilineage differentiating stress-enduring (Muse) cells (Kuroda et al. 2013), unrestricted somatic stem cells (USSCs) (Kögler et al. 2004), marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al. 2004), multipotent progenitor cells (MPCs) (Cesselli et al. 2009), multipotent adult progenitor cells (MAPCs) (Jiang et al. 2002), omnicytes (Gordon 2008), spore-like stem cells (Vacanti et al. 2001), and the subpopulations of pluripotent HSCs enriched by elutriation and flow cytometry (Orlic et al. 1994).

Outside the hematopoietic system, VSELs are found and isolated from the gonads; and their differentiation into sperm and oocytes in vitro can be achieved in the absence of growth factors/cytokines (Bhartiya et al. 2017). These data were confirmed by excluding possible contamination by reproductive cells during their isolation from gonads, as BM-VSELs cultured on Sertoli cells as feeders can differentiate into gametes (Shaikh et al. 2017). Interestingly, because they are quiescent, VSELs appear to be resistant to oncotherapy and could have potential in the resumption of fertility in patients cured after receiving chemotherapy for cancer (Bhartiya 2016). VSELs were also isolated from other solid organs, such as adult hearts, decreasing frequency during aging (El-Helw et al. 2020), or the pancreas (Zuba-Surma et al. 2008).

In contrast to other pluripotent stem cells, VSELs constitute a rare and quiescent pluripotent fraction of CD34⁺ stem cells that prevents them from over-proliferation and potential risk of teratoma formation. Their quiescence under the steady state could be related in part to their protection from insulin/insulin-like growth factor stimulation due to the erasure of regulatory sequences of paternally imprinted genes such as the Igf2–H19 locus (Ratajczak et al. 2019) and to the low level of expression of genes involved in proliferation and cell signaling, which become upregulated during cell activation. Once activated, VSELs undergo asymmetric division for their self-renewal and give rise to slightly bigger-sized

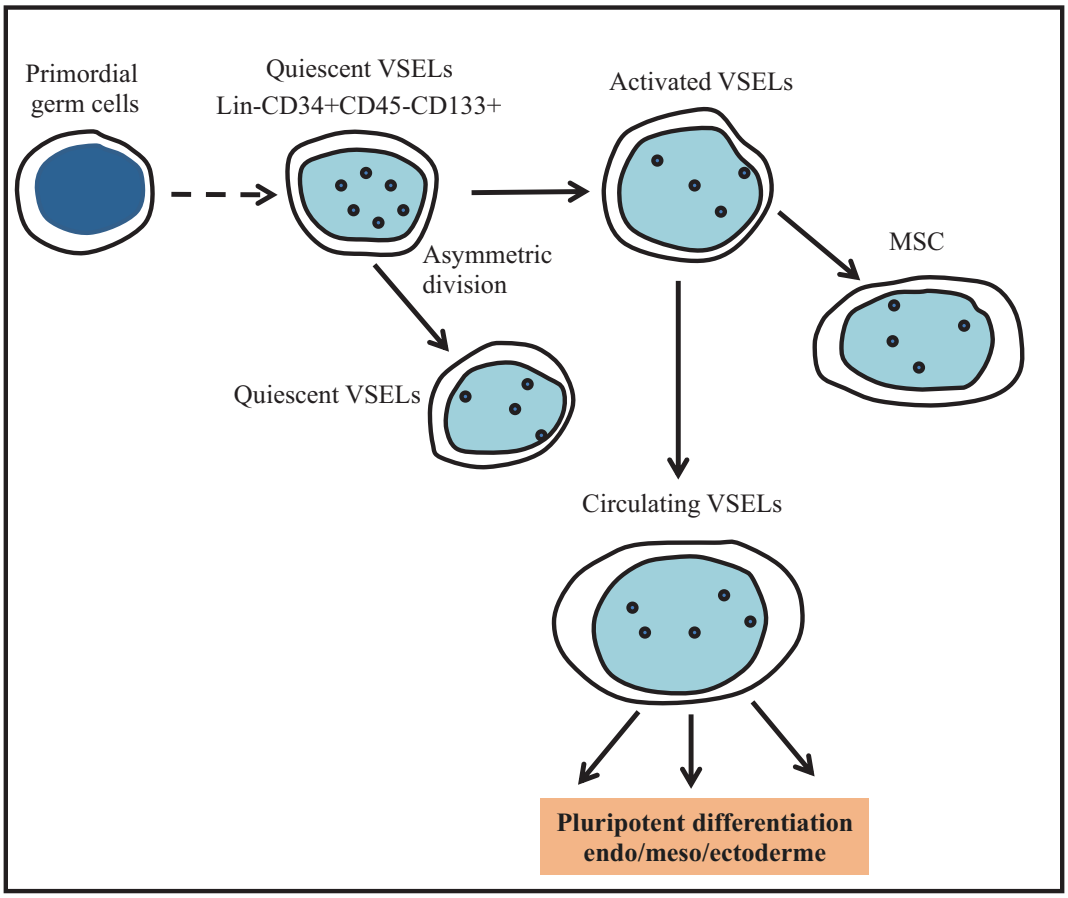

Fig. 2.1 Developmental interrelationship between CD34⁺ very small embryonic-like stem cells (VSELs) and tissue-committed cells. When activated, quiescent VSELs become a source of mesenchymal stem cells (MSCs) and give rise not only to HSCs and EPCs but also to other tissue-committed cells, because they possess an ability to differentiate to the cardiac, neuronal cells, or form gametes which favor their clinical use in many diseases

cells, which undergo symmetric and clonal expansion (Bhartiya et al. 2018).

However, due to their fewness and quiescence (Alvarez-Gonzalez et al. 2013), methods to expand them have been developed by some groups, prompting VSELs to proliferate in the presence of appropriate support without feeders or transduction by DNA or RNA. Therefore, VSELs can be expanded ex vivo by treating the cells with inhibitors of the histone deacetylase sirtuin (Sirt-1), such as nicotinamide and valproic acid. These effects can be enhanced by the addition of pituitary gonadotropins and gonadal sex hormones, such as a cocktail of follicle-stimulating hormone (FSH), luteinizing hormone (LH), bone morphogenetic protein (BMP-4), and kit ligand (KL) (Ratajczak et al. 2017a, 2017b, 2019). On the other hand, alternatively our group determined that the UM171, a pyrimidoindole derivative known to be able to induce the self-renewal of hematopoietic stem cells (Fares et al. 2014) had a positive effect on the expansion and proliferation of different populations of VSELs (Fig. 2.2), thus significantly increasing their number without affecting their ability to differentiate into specific organ cells (Lahlil et al. 2018).

The mechanism by which UM171 induces the expansion of these stem cells remains less well-defined; however, transcriptome analysis of CD34⁺-enriched human CB populations exposed to UM171 treatment revealed induction of endothelial protein C receptor (EPCR) gene expression on a small subset of the cells (Fares et al. 2017). In addition, the epigenetic regulator lysine-specific histone demethylase 1A (LSD1A), in addition to other members of the LSD1-containing chromatin remodeling complex (CoREST), seem to be poly-ubiquitinated and degraded upon UM171 treatment promoting stem cells expansion (Subramaniam et al. 2020). A recent study linked the LSD1 degradation to the UM171 treatment effect on CULLIN3-E3 ubiquitin ligase (CRL3) activity (Chagraoui et al. 2021).

Due to VSELs' properties, and because they are thought to be a reserve source of pluripotent CD34⁺ stem cells, their development could be used to ensure a safe, successful, and efficient regenerative therapy for various non-curable dis-

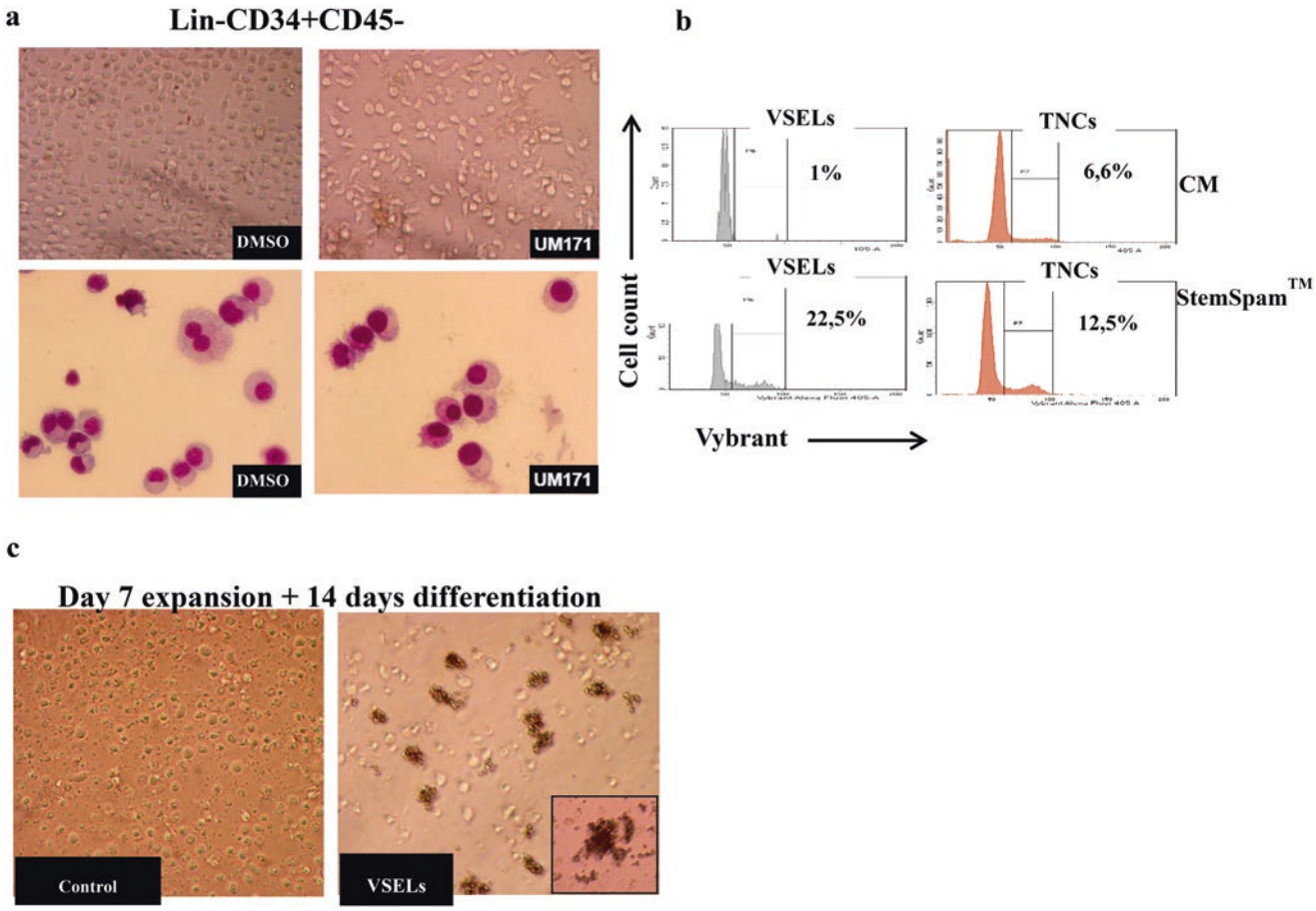

Fig. 2.2 Expansion of VSELs and their differentiation in vitro is now possible. (**a**) The EasySep™ purified $CD34^+$ Lin^- $CD45^-$ cord blood cells morphology as observed by optical microscopy (upper panels) or their May-Grünwald Giemsa cytospin preparations (lower panels) in day 9 cultures in media supplemented with DMSO as control or UM171 (**b**) The $7AAD^+Lin^-CD34^+CD45^-CD133^+$ VSELs cell cycle determination by vybrant labeling after 24h expansion in StemSpan™ containing UM171, in comparison to VSELs cultured in minimal media (conditioned media CM). In the right panels are represented the cell cycles determined from the total nucleated cells (TNCs) in the same conditions of culture as those of VSELs (representative experiment). (**c**) The VSELs and control cells morphologies after 10 days of expansion and 14 days of mesoderm differentiation. (Adapted from Stem Cell Reviews and Reports. 2018;14:510–24)

eases. In vitro and in vivo studies have demonstrated through animal models that they are capable of regenerating several cell types, representing a serious alternative to other stem cell sources in future regenerative trials. However, similar to the other stem cells, the observed improvement inducing a functional repair of damaged tissues or organs in animal models would be mainly due to the direct participation of stem cells in the repair process and their paracrine effects, as VSELs are also a source of growth factors, cytokines, soluble trophic factors, and extracellular matrix. At present, the different infused or locally injected cells have not shown concrete evidence for their significant direct contribution to the repair of injured organs, necessitating for further more careful and in-depth studies.

We previously demonstrated that VSELs circulate in minimal number in peripheral blood under a steady-state throughout life (Sovalat et al. 2016). Still, they can be mobilized in larger numbers by G-CSF from the BM into the PB (Sovalat et al. 2011). By studying VSELs positive for a pluripotent transcription factor, Nanog, isolated from umbilical cord blood, we have found that they contain heterogeneous cells expressing either CXCR4 or CD133 receptors, singly or both of them. Although the differentiation capacities of these subpopulations appear to remain intact, further characterization of their biology in vivo remains necessary (Lahlil et al. 2018).

Over the past several years, different reports indicated that in addition to HSCs, endothelial progenitor, and mesenchymal stromal cells, VSELs are recruited to peripheral blood during MI, likely contributing to the repair of infarcted myocardium (Wojakowski et al. 2004; Kucia et al. 2004; Kränkel et al. 2008; Abdel-Latif et al. 2010). Some clinical studies supported these findings, which demonstrated their therapeutic potential in patients with chronic heart failure (Tendera et al. 2009; Wojakowski et al. 2011).

Moreover, VSELs are actively mobilized from the bone marrow to the peripheral blood in patients with leukemia (Eljaszewicz et al. 2018) or stroke (Borlongan et al. 2011; Grymula et al. 2014). They contribute to bone healing by promoting osteoclastogenesis and bone formation (Leppik et al. 2019). They are also implicated in pancreas regeneration, aging, and carcinogenesis (Bhartiya and Patel 2015). Other studies revealed that in an experimental model of critical limb ischemia, VSELs gave rise to endothelial cells promoting the revascularization (Guerin et al. 2015). Their mobilization also increases in patients with hypoxic chronic obstructive pulmonary disease, possibly promoting lung repair (Guerin et al. 2017a). In patients with Crohn's disease, various populations of stem cells, including MSCs, EPCs, and pluripotent VSELs were mobilized and could be detected in PB during this digestive disease (Marlicz et al. 2012).

Finally, VSELs and EPCs were mobilized to the peripheral blood on the first day of septic shock, concomitant to the concentration of the mediators of chemotaxis CXCL12 and S1P in the blood, whereas the HSCs showed delayed mobilization (Skirecki et al. 2019).

Interestingly, some recent data from the Ratajczak group point to the involvement of the complement cascade in the egress of the majority of stem cells, including VSELs, from the BM to the PB, thus elucidating the mechanism by which stem cells are mobilized from the BM. Indeed, the activation of Nlrp3 inflammasome in an ATP-dependent manner appears to be the most important and primary mechanism in this process of complement activation (Lenkiewicz et al. 2019).

2.4 Conclusion

The affiliation between VSELs and the cells known as CD34⁺ stem cells now appears clearer, and is a more realistic explanation of their role in the repair of various organs than the unlikely concept of "cell plasticity", which should be disregarded. Given their phenotype and unique characteristics, purified CD34⁺ cells are presumed to contain small numbers of primitive CD45 negative cells, with only a tiny fraction containing VSELs, which explains the need for a large number of cells to have a significant effect on organs repair. VSELs represent a minor pluripotent portion of the total CD34 population. Still, their activation by physiologic conditions or by chemokines or soluble factors released by a damaged tissue generates more mature cell stages, which are multipotent and can differentiate along various tissue pathways, paving the way for their use in regenerative medicine. A number of the first therapeutic indications that have already been -or are being—developed (hematological diseases, cardiovascular diseases, liver insufficiency, cartilage defects) have, for certain of them, to be confirmed at a large scale by further studies. However, it is likely that additional therapeutic indications will be determined in the future once the knowledge of the VSELs characteristics and properties has progressed. For example, the use of CD34⁺ cells for post-fractural bone regeneration and healing is already underway. Nevertheless, regardless of the therapeutic indication, the success of CD34⁺ cell therapy depends on the number of cells injected, the chemoattraction of the cells into the damaged organ via the release of specific chemokines, and the route of cell injection. Therefore, the conditions for each therapeutic indication have to be determined and carefully adjusted.

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3 Mesenchymal–Hematopoietic Stem Cell Axis: Applications for Induction of Hematopoietic Chimerism and Therapies for Malignancies

Tatiana Zorina and Labe Black

Abbreviations

5FU	5 fluorouracil
BM	Bone marrow
BMT	Bone marrow transplantation
CAR Cells	CXCL12-abundant reticular cells
CFU-F	Fibroblast colony-forming unit
EC	Endothelial cell
FACS	Fluorescence-activated cell sorting
FC	Facilitating cell
G-CSF	Granulocyte colony stimulating factor
GFP	Green fluorescent protein
GVHD	Graft versus host disease
Gy	Gray (SI unite of radiation)
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
LT-HSC	Long-term repopulating subset of HSCs
mAb	Monoclonal antibody
MAC	Myeloablation conditioning
MAT	Monoclonal antibody therapy
MSCs	Mesenchymal stem cells
MSPC	Mesenchymal stem and progenitor cells
Nesperi	Nestin$^+$ periarteriolar cells
Nesretic	Nestin$^+$ reticular cells
NG2	Neural glial antigen
NMC	Non-myeloablative conditioning
NOD	Non-obese diabetic
OPN	Osteopontin
RIC	Reduced intensity conditioning
SDF1	Stromal cell-derived factor-1
ST-HSC	Short-term repopulating subset of HSCs
T1D	Type 1 diabetes
TBI	Total body irradiation

T. Zorina (✉) · L. Black
Department of Medical Laboratory Sciences & Biotechnology, Jefferson (Philadelphia University + Thomas Jefferson University), Philadelphia, PA, USA
e-mail: tatiana.zorina@jefferson.edu

3.1 Introduction

Cytoreductive regimens are broadly used in different branches of medicine despite significant drawback in patients' well-being caused by associated multiorgan comorbidity. In particular, cytoreduction is still the key component of anti-cancer therapies, as it is the basic means for controlling the growth of highly proliferative malignant tissues. Cytoreductive protocols are also applied as part of the conditioning regimens that are currently a standard component of bone marrow transplantation (BMT) protocols.

BMT has vast therapeutic potential for numerous malignant (Galaverna et al. 2018; Kean et al. 2002) and non-malignant (Wen et al. 2011; Ikehara 2008) diseases. When used for treating malignancies, however, the myeloablative conditioning regimens utilized to secure the donor-derived hematopoietic stem cells (HSCs) engraftment produce the same complications as the direct anti-cancer cytoreductive regimens (e.g., chemotherapy, radiation therapy). In both cases, the cytoreductive therapies are associated with adverse effects on the gastrointestinal tract, reproductive organs, pulmonary, and urothelial and cardiovascular systems and lead to BM aplasia and resulting pancytopenia (Schulenburg et al. 2004; Mohty et al. 2015a, b; Blaise et al. 2015). In addition to its use for the treatment of malignancies, BMT is increasingly being adapted for use as a therapeutic modality for autoimmune, degenerative, and other non-malignant disorders. The complications associated with currently required conditioning regimens preclude its broad clinical adaptation for a large cohort of debilitating disorders that would otherwise benefit from BMT.

A search to ameliorate the side effects associated with cytoreductive therapies for cancers and to develop a BMT protocol without myeloablative conditioning is in progress. In this review, we cover newly emerging insights on BM dynamic structure and the role of the hematopoiesis-supportive function of the mesenchymal compartment within the HSC niches. Potential directions for the exploration of adaptation of the donor-derived mesenchymal stem and

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progenitor cells (MSPCs) into anti-cancer therapies and for the development of the conditioning-free BMT protocols are discussed.

3.2 Hematopoietic Stem Cell and Bone Marrow Transplantation

3.2.1 Bone Marrow Transplantation

The induction of allogeneic hematopoietic chimerism through BMT from a healthy donor has shown promise in the treatment of both malignant (Galaverna et al. 2018; Kean et al. 2002) and non-malignant disorders, including aplastic anemia and numerous autoimmune disorders (Wen et al. 2011; Ikehara 2008). BMT is also therapeutic for a large cohort of blood disorders and pancytopenia of either idiopathic or iatrogenic origins, such as chemotherapeutic or other cytoreductive regimens (Wu et al. 2017; Locatelli and Pagliara 2012; Taniguchi et al. 2011; Ikehara 2008; Wen et al. 2011). BMT can also be used to induce hematopoietic chimerism as a means to secure graft acceptance in transplantation medicine (Yolcu et al. 2017; Sachs 2018; Sayegh et al. 1991; Ildstad et al. 2015; Jacobsen et al. 1994). One of the key features of allogeneic HSCs is their ability to induce tolerance when administered before organ transplantation, provided the BM and the organ graft are from the same donor (Sayegh et al. 1991). Clinical use of tolerance induction via allogeneic chimerism continues to be the focus of medical research and provides viable alternative treatment methods when donor–recipient histocompatibility matching can be achieved. The clinical outcomes and benefits of BMT can be summarized as follows:

- Replacement of malignant clones in the case of hematopoietic malignancies.
- Proliferation and differentiation of the healthy stem and progenitor cells to compensate defective hematopoiesis in aplastic conditions, such as thrombocytopenia, aplastic anemia, and agranulocytosis.
- Induction of tolerance for organ transplantation.
- Suppression of autoimmunity and sustaining unhindered regeneration of the affected tissues.

3.2.2 Bone Marrow Transplantation Challenges

There are two major challenges involved in BMT. The first is graft rejection unlike the cornea of the eye for which transplantation was successfully performed in 1905 without human leukocyte antigen (HLA) matching or immunosuppressive therapy (Fanta 1986). BM is the most sensitive tissue for HLA disparity. In addition to major histocompatibility antigens, numerous minor histocompatibility antigens also play a role in BM graft acceptance (Cattina et al. 2017). The second challenge that limits broad clinical adaptation of BMT is graft versus host disease (GVHD), which occurs when donor-derived effector cells attack and destroy host tissue (Ghimire et al. 2017).

An additional obstacle is due to specific requirements for allogeneic BM engraftment. In solid organ transplantation, successful engraftment means that the donor-derived organ is not rejected and remains vital and functional. In the case of a BMT, engraftment is defined by a chain of events, which include the ability of donor HSCs to "settle" in special microenvironment areas, termed HSC niches, and to display two distinct functions: (i) self-renewal and (ii) differentiation into mature blood cells (Spangrude et al. 1988; Chen et al. 2016; Morrison and Weissman 1994).

HSC niches refer to particular spaces occupied by the host's hemopoietic stem and precursor cells, which must be ablated to create an area where the donor-derived BM can engraft. However, it has been extensively demonstrated in a conventional BMT that the integrity of the HSC niches is affected by myeloablative conditioning, which is administered with the intent to provide a space for the donor stem cells to engraft. A significant drawback of such conditioning is the development of pancytopenia and its associated detrimental complications (Wen et al. 2011; Yolcu et al. 2017). Reducing the adverse effects associated with BMT conditioning regimens continues to be the focus of a large number of research groups. Section 3.3 provides an overview of conditioning regimen types and highlights their limitations.

3.2.3 Hematopoietic Stem Cell: History and Subtypes

Over 100 years have passed since Alexander Maximow postulated the unitary theory of hematopoiesis on which the modern concept of blood cell origins and differentiation is based (Konstantinov 2000). About 50 years later, Till and McCulloch experimentally confirmed Maximow's criteria for defining hemopoietic stem cells (Becker et al. 1963; Till and McCulloch 1961; Siminovitch et al. 1963). In their monumental study, mice were fully reconstituted after a lethal dose of radiation and subsequent intravenous administration of BM. Following this discovery, research on HSC populations has brought to light a breadth of knowledge defining their characteristics.

HSCs represent a small (~0.05%) subpopulation of cells within BM (Bradfute et al. 2005). HSCs were identified over 30 years ago by Berenson and colleagues as $CD34^+$ cells, capable of hematopoietic reconstitution in irradiated primates (Berenson et al. 1988). This finding was further confirmed in other animal models and humans (Bensinger et al. 1966; Link et al. 1996; Kawano et al. 1998; Krause et al. 1994), and the $CD34^+/Lin^-$ phenotype was long used to iden-

tify these cells (Boitano et al. 2010; Baum et al. 1992). Later it was demonstrated that the CD34 marker is predominantly associated with a non-quiescent, short-term repopulating subset of HSCs (ST-HSC) (Ogawa et al. 2001; Yang et al. 2005). Currently, new genetic markers have been defined in the mouse model to represent and purify HSC populations, including Ctnnal^{-}/GFP^{+}/KIT^{+} (Acar et al. 2015) and Hoxb5 (Chen et al. 2016), which are predominantly expressed in long-term HSCs (LT-HSC). To identify and isolate HSCs from mouse BM by flow cytometry and live-cell sorting, the CD150^{+}/CD48^{+}/Sca 1^{+}/c-kit^{+}/Lin^{-} phenotype is commonly used (Acar et al. 2015; Bradfute et al. 2005).

Two basic features of HSCs are their ability to: (i) self-renew (mediated by long-term repopulating, predominantly quiescent cell populations) and (ii) differentiate into mature cells of the peripheral blood (mediated by short-term repopulating, both dormant and actively proliferating cells) (Vaidya and Kale 2015; Sun et al. 2014; Busch et al. 2015; Ji et al. 2010; Seita and Weissman 2010; Orkin and Zon 2008; Li and Clevers 2010). HSC division that generates two differentiated progeny cells eliminates HSC potential (Clevers 2005). A question was addressed as to whether the quiescence (also termed dormant or G0 phase of the cell cycle) is a permanent or reversible state of HSCs. Flow cytometry and label-retaining assays (BrdU and histone H2B-GFP) were used to identify and characterize a population of dormant mouse HSCs (d-HSCs) within the Lin^{-}/Sca1^{+}/c-Kit^{+}/CD150^{+}/CD48^{-}/CD34^{-} cell population. d-HSCs harbor a stock population of multipotent HSCs during homeostasis that can be activated to proliferate and self-renew in response to BM injury or stimulation by growth factors; once homeostasis is achieved, the activated HSCs return to dormancy. Studies suggest that HSCs do not enter the cell cycle stochastically but can reversibly switch from dormancy to a proliferative status in response to physiologic or pathologic stimuli (Wilson et al. 2008; Bhattacharya et al. 2009). Through cellular and molecular methodologies, HSC phenotypes and genotypes have been identified and isolated, allowing the exploration of their unlimited adaptive and clinical potential. However, the process of defining a comprehensive phenotypic profile of human HSC subsets is still in progress.

3.3 Advancement of Conditioning Regimens for Bone Marrow Transplantation

3.3.1 Myeloablative Regimens for Malignant Cell Destruction and Allogeneic HSC Engraftment

Traditional conditioning regimens for BMT aim to create a space referred to as a "niche" for allogeneic HSC engraftment and avoid the onset of GVHD as a possible complication (Choe et al. 2017; Sakaguchi et al. 2019; Chhabra et al. 2018; Shenoy et al. 2016). These conditioning regimens consist of administrating alkylating agents and/or high doses of radiation, which eliminate precursor and undifferentiated HSCs and MSCs. In the case of hematopoietic malignancies, these regimens also beneficially reduce the pool of the malignant cells in BM. However, the morbidity caused by such regimens often outweighs the therapeutic benefits. In some cases, the use of total myeloablation conditioning (MAC) is not necessary. For instance, identical twins have matching HLA genotypes, and so if they are used as a donor–recipient pair, the removal of effector lymphocytes is often not needed. In these specific cases, often only a low dose of chemotherapy and/or radiation is used. Low-dose regimens are referred to as non-myeloablative conditioning (NMC) (Ho et al. 2006). Researchers are also exploring alternative, reduced intensity conditioning (RIC) regimens for BMT to minimize the side effects currently associated with conditioning (Choe et al. 2017; Shenoy et al. 2016; Madden et al. 2016). RIC incorporates chemotherapeutic doses that are intermediary to MAC and NMC. However, even with the use of reduced intensity conditioning regimens, the therapeutic outcomes of BMT continue to be limited by the failure of HSC engraftment and by the potential onset of GVHD.

3.3.2 Myeloablative vs Non-myeloablative vs Reduced Intensity Conditioning

Conditioning for BMT can be grouped based on drug cut-off levels. Dose levels are specific for therapeutic regimens concerning to the type of conditioning (MAC, NMC, or RIC). MAC consists of total body irradiation (TBI) ≥5 Gy with a single reagent (e.g., busulfan ≥8 mg/kg, cyclophosphamide ≥120 mg/kg, fludarabine ≥120 mg/m^2, melphalan ≥140 mg/m^2) (Sakaguchi et al. 2019; Waldhuter et al. 2019). The goal of MAC is complete pancytopenia before BMT. NMC is used to induce lymphopenia with little or no general cytopenia. It usually consists of low doses of fludarabine (90 mg/m^2) or melphalan (70–140 mg/m^2) with TBI ≤ 2Gy (Ho et al. 2006). RIC uses intermediary dosing levels, which are thought to be better tolerated by patients who are more susceptible to comorbidity (Choe et al. 2017; Shenoy et al. 2016; Madden et al. 2016). Some examples of dosage cut-off values include busulfan ≤8 mg/kg and melphalan ≤140 mg/m^2.

Currently, BMT is most commonly applied clinically for the treatment of hemopoietic malignancies. In these cases, myeloablative regimens not only create the sought at space for allogeneic HSC engraftment, but they also contribute to reducing the pool of malignant clones within the host's hematopoietic tissues. Although myeloablative conditioning regimens are standard in clinical practice, an optimal treatment regimen remains to be defined. Studies exploring con-

ditioning regimen modifications are in progress; these are aimed at tailoring treatment to the individual patients' condition.

3.3.3 Incorporating Monoclonal Antibody Therapy into RIC

To facilitate BMT engraftment, immunosuppressive monoclonal antibody therapy (MAT) has recently been incorporated into RIC treatment plans. The most commonly studied is rituximab, a human–mouse chimeric IgG that targets CD20 on B cells. Incorporation of rituximab into modified RIC treatments has led to a decrease in fatal complications. However, the development of acute or chronic GVHD is still similar to that in patients treated with traditional conditioning protocols. Other mAb's that are being utilized in RIC treatment plans include anti-thymocyte globulin, alemtuzumab, ibritumomab, and tiuxetan, although the clinical response to treatment with these antibodies is still part of ongoing studies (Cabrero et al. 2017; Epperla et al. 2017; Auger-Quittet et al. 2014; Briones et al. 2014; Jo et al. 2012).

MAT has been demonstrated to be a powerful tool for treating a variety of conditions. With sufficient progress and development, it is considered to be a viable addition to RIC regimens and to aid in BM graft acceptance. However, MAT incorporation into RIC does not alleviate the severe adverse side effects associated with conditioning (Cabrero et al. 2017; Epperla et al. 2017). Also, the antibodies can cause a diverse range of serious-to-fatal adverse effects because they are not cell type-specific and their target molecules are usually ubiquitously expressed in a variety of cells.

3.4 Bone Marrow Dynamic Structure: HSC and MSCs Niches, Cells, Mechanisms, and Cross-Talk

3.4.1 Hematopoietic Stem Cell Niches

In an attempt to make BMT a side-effect-free therapeutic regimen, researchers have sought to create suitable microenvironments or niches for sustaining donor-derived HSC engraftment. To re-examine the aptness of this notion in the light of the newly accumulated knowledge about HSC niches, we offer a brief synopsis on the topic evolved during the last 40 years. Currently, a new direction is emerging related to the role of the stromal components of HSC niches in physiologic and pathologic conditions. As such, targeting integrity of BM stromal cell populations as an immunomodulatory approach in sustaining hemopoiesis has two potential therapeutic outcomes: (i) sustaining the autologous hemopoietic reconstitution under cytoreductive regimens and (ii) supporting the engraftment of allogeneic HSCs.

Raymond Schofield coined the term "niche" in 1978 to describe the HSC microenvironment required for the stem cell physiological function (Schofield 1978). HSC niches are complex, functionally dynamic structures defined by the cellular, extracellular, and humoral components (Schofield 1978). HSC niches have two major functions: (i) sustaining HSC self-renewal and (ii) supporting their differentiation into specific cell lineage subsets. These functionally different niche types have distinct spatial-structural locations within the BM (Klamer et al. 2018; Jacobsen et al. 1994; Jenq and van den Brink 2010). Although the mechanism by which MSC–HSC axis in these niches supports hemopoietic homeostasis has not yet been fully elucidated, it is clear that it has a critical role in both autologous and allogeneic hematopoiesis. Information on the current understanding of specific HSC niche types, their components, and the functional differences that arise during ontogenesis and age-related changes can be found in the comprehensive review articles cited here (Wen et al. 2011; Kaufman et al. 1994; Czechowicz et al. 2007; Kondo et al. 2000; Yamamoto et al. 2013; Rankin et al. 2012; Rodriguez-Fraticelli et al. 2018; Lu et al. 2011; Brewer et al. 2016; Nguyen et al. 2018; Fugier-Vivier et al. 2004; Leventhal et al. 2012). The focus of this review is on the role of the stromal components within HSC niches. This discussion is focused on the hematopoiesis sustaining function of a variety of stromal niche components from multipotent MSCs and lineage-specific stromal progenitor cells to mature osteocytes.

3.4.2 MSPCs and Their Role in HSC Niches

First, it is necessary to introduce the terminology of different cellular components of stromal tissue, as it is not consistent throughout the literature. The term "mesenchymal stem cells" often used to refer to a heterogeneous population of several subsets of stem cells with different phenotypes and functions. They were originally isolated and characterized based on their plastic adherence properties (Wagner et al. 2006). The International Society for Cellular Therapy encourages the use of the term "MSCs" only for cells that meet specified stem cell criteria. Accordingly, the heterogenic population of immature stromal cell populations is to be referred to as "multipotent mesenchymal stromal cells" (MMSCs), and a mixed population of stem and progenitor cells is to be referred to as MSPCs (Horwitz et al. 2005).

The two distinct multipotent somatic stem cell types known as HSCs and MSCs interact closely both physically and functionally. These heterotypic stem-cell pairs are a defining structural feature within the BM HSC niches. MSC–HSC pairings are tightly regulated by the surrounding cellu-

lar and extracellular microenvironments, hormones, and the autonomic nervous system (Mendez-Ferrer et al. 2010, 2008).

MSCs were first identified by the pioneering work by Alexander Friedenstein, which led to the discovery of adult non-hematopoietic or MSCs in the BM over 50 years ago. He demonstrated that, in addition to containing multipotent HSC, BM harbors another population of multipotent stem cells of mesenchymal origin (Friedenstein et al. 1968, 1974). The following decades produced research addressing the MSCs differentiation into different lineages of tissue-specific precursors that has led to the development of a new branch of reparative and regenerative medicine (Li and Ikehara 2013b). The adaptive ability of MSCs induces tissue-specific regeneration and has the potential to be used in the treatment of numerous disorders (Li and Ikehara 2013a; Peired et al. 2016; Cheng et al. 2008).

Another potential clinical application of MSCs is their ability to play the role of immunomodulatory agents (Hugle and Daikeler 2010; Collins and Gilkeson 2013; Cras et al. 2015; Gharravi et al. 2018; Maria et al. 2017). Of particular importance is yet another, and no-less clinically significant, functional feature of MSCs: their role in sustaining autologous hematopoiesis and allogeneic HSC engraftment. Friedenstein has shown, in a mouse model, that MSCs and HSCs have the ability for intercellular cross-talk despite allogeneic disparity (Friedenstein and Kuralesova 1971). This finding was further explored and confirmed by other research studies (Sadovnikova et al. 1991; Chertkov et al. 1980; Kuznetsov et al. 1997; Krebsbach et al. 1997; Varas et al. 2000). The following discussion addresses the role of MSCs in regulating both predominantly quiescent and proliferative HSC subsets in the pathophysiologic settings. Additionally, it addresses the MSC response to cytoreductive regimens, including radiation, and the resulting outcomes affecting their supporting role of either autologous or allogeneic HSC subsets. Understanding how the MSCs support autologous hematopoiesis and allogeneic HSCs engraftment could provide a new direction in the search for a means to rectify their function under cytoreductive regimens and prevent the resulting co-morbidities.

Multipotent HSCs fate with respect to quiescence versus proliferation, self-renewal versus differentiation, migration, and engraftment, is influenced by the microenvironmental niches (Crane et al. 2017; Beerman et al. 2017; Morrison and Scadden 2014; Wilson et al. 2007; Schofield 1978). HSCs are highly dynamic (Voog and Jones 2010); they migrate between niches and enter or exit the peripheral circulation upon stimulation (Wright et al. 2001). They can also switch from dormancy to proliferation during homeostasis and repair (Wilson et al. 2007). The two major spatial allocations of HSCs are endosteal niches (also termed periarteriolar, as they are associated with arterioles located at the outer edge of the BM) and perivascular niches (also termed perisinusoidal, as they are localized around sinusoids located in the inner core of BM) (Kiel et al. 2005; Ding and Morrison 2013; Lambertsen and Weiss 1984; Lord et al. 1975). The minority of HSCs are quiescent (Ki-67$^-$) and reside in periendosteal BM, while the majority of HSCs, which represent a mixture of both dividing (Ki-67$^+$) and dormant (Ki-67$^-$) populations, are located in perivascular niches (Nombela-Arrieta et al. 2013; Acar et al. 2015; Kunisaki et al. 2013). A schematic illustration of the HSCs niche types in the BM and changes in the hematopoiesis induced by cytoreductive regimens is provided in Fig. 3.1.

Different HSC niches play unique and distinct roles in the regulation of hematopoiesis. Their structural and cellular compartments are clinically significant because they can change depending upon the environmental state with respect to their pathophysiologic and post-myeloablative treatment status (Yu and Scadden 2016).

Cells of stromal origin in HSC niches play a significant role in both hematologic homeostasis and pathology, including post-conditioning BM reconstitution (Maximow 1923; Friedenstein and Kuralesova 1971; Chertkov et al. 1980; Weiss 1976; Dexter et al. 1984a, b). Mutations in these non-hematopoietic cells can cause hematopoietic neoplasia (Yu and Scadden 2016). Stromal cells are capable of self-renewal and lineage-specific differentiation and aid in maintaining the integrity of both HSC and MSC niches (Prockop 1997; Mendez-Ferrer et al. 2010; Kolf et al. 2007). Numerous cell subsets with their unique phenotypes have been identified within the stromal compartments of HSC niches. Among them are cells positive for the following markers: SCF, CXCL12 (Mendez-Ferrer et al. 2010), PDGFRα, Nestin (Mendez-Ferrer et al. 2010), Sca-1 and CD51 (Pinho et al. 2013), Prx-1-Cre, LepR (Ding et al. 2012; Zhou et al. 2014), CD146 (Sacchetti et al. 2007), NG2 (Kozanoglu et al. 2009), and CD271 (Matsuoka et al. 2015). SCF and CXCL12 are expressed and synthesized, though at different levels, by osteoblasts (Sugiyama et al. 2006; Dar et al. 2005; Asada et al. 2017). CXCL12-abundant reticular (CAR) cells, with the highest expression of CXCL12 and SCF molecules among other stromal cells, (Sugiyama et al. 2006) and other types of cells surround bone arterioles (Asada et al. 2017; Ding and Morrison 2013).

Some mature stromal and non-stromal cells play a role in maintaining HSC niches as well. It was observed long ago that, with aging, "red" BM is gradually replaced by "yellow", which is a fatty tissue of mesenchymal origin. Navieras and colleagues have shown that BM adipocytes not only replace, but also negatively influence hematopoiesis (Naveiras et al. 2009). Macrophages also influence hematopoiesis, with recent studies revealing that they play a role in mobilizing HSCs into the peripheral blood circulation in response to granulocyte colony-stimulating factor (G-CSF)

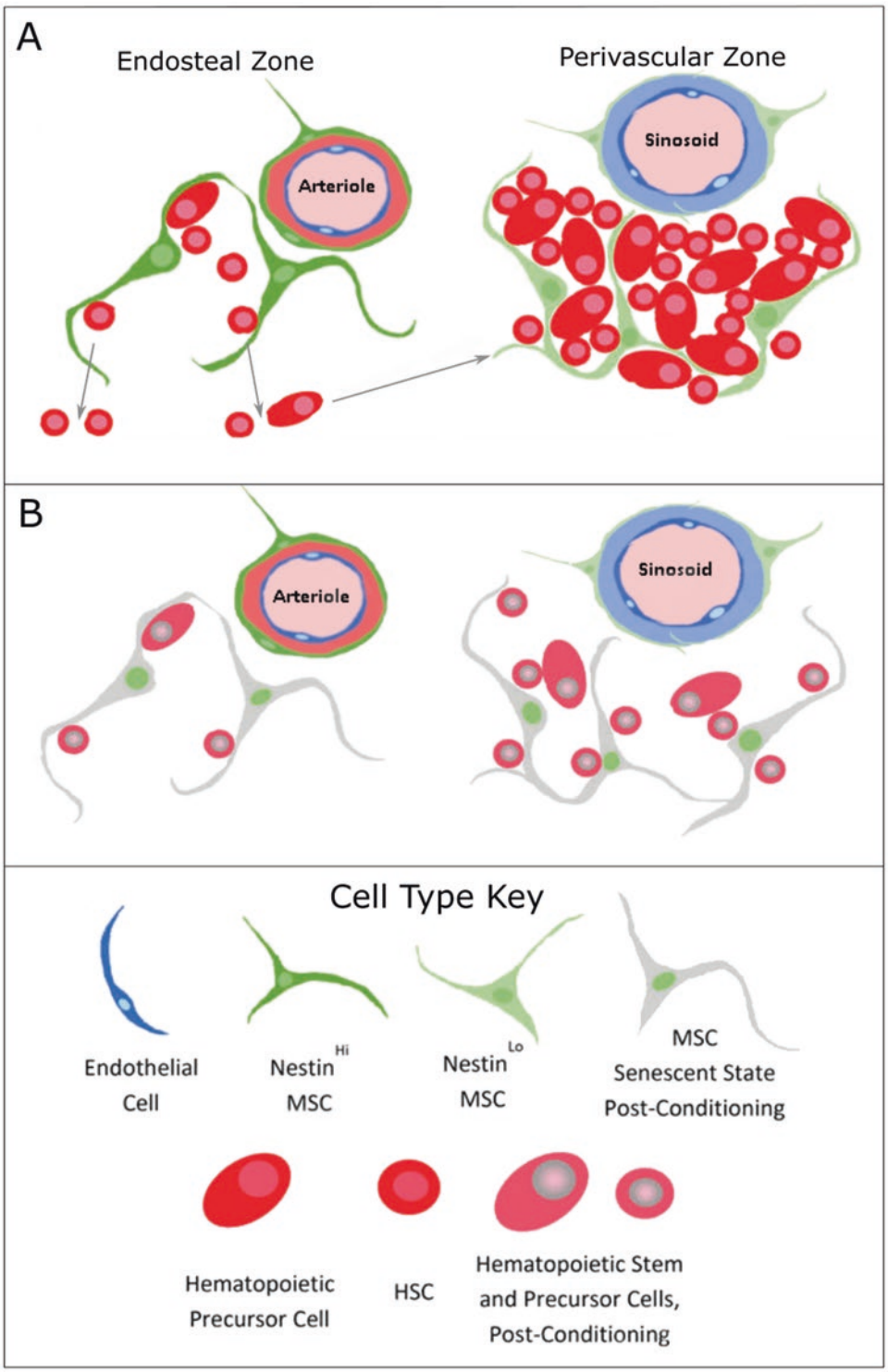

Fig. 3.1 Bone marrow aplasia resulting from impaired hemopoiesis supportive function of the mesenchymal cells. Mesenchymal stem cells (MSC) represent one of the major cellular components sustaining hemopoietic homeostasis in both long-term HSC (LT-HSC) niches surrounding arterioles located within the endosteal zone (Left) and short-term HSC (ST-HSC) niches in the vicinity of the sinusoids located in the perivascular zone (Right). (**a**) Hemopoiesis in physiologic condition. Left Panel: MSCs in the LT-HSC niches in the endosteal zone are characterized by the $Nestin^{Hi}/NG2^{+}/Sca1^{+}/LepR^{-}/SCF^{Lo}/CXCL12^{Lo}$ phenotype. These niches predominantly harbor (i) HSCs with long-term repopulating potential sustaining self-renewal (via symmetrical division) and (ii) population of the precursor hematopoietic cells initiating lineage-specific differentiation (via asymmetrical division) and thus losing the stem cell features. Right Panel: The latter cells migrate into ST-HSC niches where they undergo further differentiation, proliferation, and maturation into blood cells. The phenotype of MSCs in ST-HSC niches is $Nestin^{Lo}/NG2^{-}/Sca1^{-}/LepR^{+}/SCF^{Hi}/CXCL12^{Hi}$. (**b**) Hemopoiesis after cytoreductive regimens is reduced. All types of stem and progenitor cells of both hemopoietic and mesenchymal lineages are affected. Shown in gray: MSCs in a senescent state in response to cytoreductive regimens with impaired ability to sustain hemopoiesis in both LT-HSC and ST-HSC niches

(Winkler et al. 2010; Smith and Calvi 2013), in the retention of HSCs in endosteal MSC niches (Chow et al. 2011), and in the regulation of erythropoiesis (Sukhbaatar and Weichhart 2018). Activated α-smooth muscle actin positive macrophages preserve MSC pools from exhaustion under stress conditions (Ludin et al. 2012). Megakaryocytes and endothelial cells (ECs) are among other cells of non-stromal origin that also contribute to the function of HSC niches. Megakaryocytes regulate the quiescence state in HSCs via C-type lectin-like receptor-2 signaling (Nakamura-Ishizu et al. 2015). ECs lining blood vessels in the BM provide an input for the regulation of HSC niches via VE-cadherin, VEGFR2, and SCF and CXCL12 factors (Ding et al. 2012; Ding and Morrison 2013). Sympathetic nervous system fibers in the BM are associated with blood vessel and stromal cell network formation called the neuro-reticular complex. The neuro-reticular complex regulates the expression of CXCL12, one of the major factors regulating the maintenance of HSC niches (Yu and Scadden 2016; Yamazaki and Allen 1990; Mendez-Ferrer et al. 2008).

The regulatory impact of hematopoietic stroma on HSCs is mediated either by direct cell–cell interactions via different receptor/ligand axes, including cell adhesion molecules (Wilson et al. 2007), or by means of numerous cytokines and the extracellular matrix (Stier et al. 2005; Luo et al. 2011; Qian et al. 2007; Simons and Clevers 2011; Klamer et al. 2018; Sadovnikova et al. 1991; Discher et al. 2009). A few key regulatory molecules are particularly important for the recovery of hematopoiesis following myeloablation. Angiogenin is responsible for the recovery of hematopoiesis after myeloablation by regulating the quiescence state within HSC populations (Goncalves et al. 2016; Itkin et al. 2012). FGF2 promotes recovery of hematopoiesis after myeloablation by inducing proliferation and activation of stromal cells (Itkin et al. 2012). SLIT ligand sustains autologous and allogeneic HSC engraftment following transplantation (Smith-Berdan et al. 2011; Waterstrat et al. 2016).

Research efforts to classify the individual subsets of stromal cells in HSC niches and to identify their phenotype-specific profiles are still a work in progress. A detailed description of stromal and HSC niche components and structure is provided in these comprehensive reviews (Crane et al. 2017; Yu and Scadden 2016; Khlusov et al. 2018). A brief outline of stromal components identified in the endosteal and perivascular HSC niches relevant to the current discussion is provided below.

3.5 Endosteal/Osteoblastic HSC Niches

The stromal component of the endosteal HSC niches is composed of a large variety of cells from the immature stem and osteoprogenitor cells to mature osteocytes (Askmyr et al. 2009; Gong 1978; Askenasy et al. 2002). In multiple studies, osteolineage cells were shown to influence the progenies of either myeloid or lymphoid precursor cells depending upon the stage of their differentiation. The more mature osteolineage cells appear to have a greater influence on G-SCF-mediated mobilization of HSCs (Ferraro et al. 2011), while the less mature osteolineage cells impact maturation of T and B cells (Ding and Morrison 2013; Sato et al. 2013). Osteoblasts and osteoclasts play a critical role in regulating multipotent HSCs, specifically helping to sustain quiescent, LT-HSCs subsets, and preferentially locating them in the endosteal area (Calvi et al. 2003; Kunisaki et al. 2013). In crushed, enzymatically digested bones from which the marrow was flushed from the shaft, researchers isolated endosteum-adhered MSCs with high potential to support LT-HSCs (Haylock et al. 2007).

3.5.1 Osteoblasts

The role of endosteal osteoblasts in HSC maintenance and self-renewal was first proposed by Taichman and Emerson based on in vitro studies (Taichman and Emerson 1994; Taichman et al. 1996), which were later supported by in vivo evidence by others (Calvi et al. 2003; Zhang et al. 2003; Visnjic et al. 2004). An increased number of osteoblasts in the marrow cavity leads to an increased number of LT-HSCs, without affecting any other hematopoietic subpopulations in the BM (Calvi et al. 2003; Zhang et al. 2003). Furthermore, osteoblast ablation from the marrow cavity results in a loss of HSCs (Visnjic et al. 2004). Osteoblasts originate from MSCs and reside in the endosteum in tight connection with multipotent HSCs. This anatomic arrangement alone suggests that osteoblasts play a role in regulating HSCs (Khlusov et al. 2018; Taichman 2005). Osteoblasts can form bone when transplanted and recruit circulating host hematopoietic progenitors to re-establish a fully functional marrow of recipient origin (Krebsbach et al. 1997; Kuznetsov et al. 1997).

The term "osteoblasts" is often used to refer to cell populations, which include a range of cells from multipotent MSCs and osteoprogenitor cells to mature osteocytes (Riggs and Melton 1995; Mackie 2003). Subtypes of osteoblasts play different roles in regulating hematopoiesis (Yin and Li 2006). Some osteoblast products, such as thrombopoietin and Jagged-1, induce proliferative regulatory effects on HSC niches, while others including osteopontin (OPN) induce a quiescent state (Purton and Scadden 2008). An OPN-null microenvironment was shown to reduce multipotent hematopoietic HSC apoptosis (Stier et al. 2005).

Among "negative" regulators of HSCs are the spindle-shaped N-cadherin positive/CD45$^-$ osteoblastic cells, which were demonstrated to directly interact with HSCs (Zhang

et al. 2003) and provide "quiescence" signaling (Frisch et al. 2008; Taichman and Emerson 1994). However, whether direct cell–cell interactions mediate the influence of osteoblasts on MSCs remains unclear (Wilson et al. 2007; de Barros et al. 2010) because OPN is a substrate secreted by N-cadherin$^+$ cells (Nilsson et al. 2005; Stier et al. 2005). The ability of HSCs to migrate to the perisinusoidal area further confirms quiescent HSC niche localization in the periarteriolar zone (under direct or indirect regulatory impact from osteoblasts); this ability was demonstrated in an experimental setting using 5-fluorouracil (5FU) in vivo administration (Kunisaki et al. 2013).

3.5.2 Osteoclasts

Osteoclasts represent another cell population residing in the endosteum. Osteoclasts originate from CD34$^+$ hematopoietic cells of the monocyte/macrophage lineage and are necessary for mineralized-bone resorption (Martin and Sims 2005). Osteoblasts and osteoclasts interact closely to regulate HSC niches, and osteoclasts play both a positive and negative roles in colonizing osteoblasts. Their interaction is mediated via receptor activation by nuclear factor kappa B ligand, M-CSF, and osteoprotegerin expressed on osteoblasts with the corresponding axes counterparts on osteoclast precursor cells (Lacey et al. 1998). Osteoclasts promote post-transplantation hematopoietic recovery and mobilize hematopoietic progenitors into circulation (Frisch et al. 2008). However, the role of osteoclasts in HSC maintenance remains controversial and their functional interaction needs further investigation.

3.6 Nestin and Leptin

Nestin and Leptin were identified as markers that discriminate MSC populations supporting self-renewal long-term versus proliferative short-term HSC subsets. Nestin$^+$ cells are multipotent, self-renewing stem cells, primarily populating endosteal niches. They have BM colony-forming-unit fibroblastic (CFU-F) activity, the ability for multilineage differentiation toward mesenchymal lineages and show strong self-renewal potential in successive transplantations. They show distributions in regions adjacent to the endosteum or within the BM parenchyma. Immunostaining of the femoral bone sections demonstrated a close association between Nes-GFP$^+$ cells and HSCs. Nestin$^+$ cells represent MSCs that show a close physical association with HSCs. They have very high expression levels of core HSC maintenance genes. Nes-GFP$^+$ MSCs deletion significantly reduces BM HSCs (Mendez-Ferrer et al. 2010). Detailed 3D imaging and FACS analyses revealed two distinct subtypes of Nes-GFP$^+$ cells in mouse BM-based on their expression levels and cellular morphology. Nes-GFP^{bright} cells were much rarer (~0.002% of BM cells) than Nes-GFP^{dim} cells in both sternal and long bone BM. Nes-GFP^{bright} cells were found exclusively along arterioles and were termed Nesperi cells (from periarteriolar), while Nes-GFP^{dim} cells were reticular in shape and associated with sinusoids. Cells with these characteristics were termed as Nesretic cells. Both Nestin$^+$ cell subsets showed mesenchymal progenitor cell capacity, CFU-F activity in BM, with most of the CFU-F cells detected within the Nesperi cell compartment (Kunisaki et al. 2013). Also, Nesperi cells, but not Nesretic cells, were associated with sympathetic nerves and Schwann cells, which are involved in HSC maintenance (Katayama et al. 2006; Yamazaki et al. 2011). RNA-Seq analyses of Nesperi and Nesretic cells revealed differing cell cycle characteristics in these two cell subsets. Expression of genes in DNA replication and cell cycle pathways were significantly enriched in Nesretic cells. The proliferative activity of Nesretic and Nesperi cells were then compared by expression of the proliferation markers Ki-67 and proliferation cell nuclear antigen. Both markers were found significantly lower in Nesperi cells compared to Nesretic and Nes-cells. The population of Nesperi cells was more preserved compared to Nesretic cell in response to 5FU. This confirms that Nesperi cells predominantly reside in a quiescent state, which may provide some level of protection from myeloablation (Kunisaki et al. 2013).

3.6.1 Perivascular HSC Niches

Perivascular HSC niches are located near the BM sinusoids and harbor a mixture of HSCs, some dormant and other proliferating and differentiating toward lineage-committed progenitor cells. The majority of HSCs in perivascular niches are CD34$^+$/CD38$^+$/Ki-67$^+$ cells, which exhibit short-term repopulating activity and cannot reconstitute hematopoiesis in vivo (Hogan et al. 2002). Thus perivascular niches are regarded as ST-HSC niches, with mesenchymal cell summary phenotype described as LepR$^+$/SCF^{Hi}/CXCL12^{Hi}/NG2$^-$/Nestin^{Lo}/Sca1$^-$ cells (Kunisaki et al. 2013; Sugiyama et al. 2018; Mendez-Ferrer et al. 2010).

The CXCL12-abundant reticular (CAR) cells have a special role within perivascular HSC niches. They are vascular adhesion molecule 1 (VCAM1) positive cells which create a network within the BM. CAR cells have a high expression of CXCL12, which is significant because the CXCR4/CXCL12 axis is essential for homing and maintenance of HSCs (Greenbaum et al. 2013; Tzeng et al. 2011). CAR cells are also involved in the regulation of HSC differentiation and maintaining their undifferentiated state (Nagasawa et al. 1996; Omatsu et al. 2010; Sugiyama et al. 2006; Zou et al. 1998). The depletion of CAR cells results in a reduction of

HSCs in BM (Omatsu et al. 2010). Perivascular niches also contain endothelial cells with distinct characteristics compared to periarteriolar endothelial cells, which contribute to mechanisms regulating the maintenance of HSCs as well (Kiel et al. 2005; Ding et al. 2012; Ding and Morrison 2013; Chi et al. 2003).

3.6.2 Leptin Receptor (LepR)

Leptin receptor (LepR) is one of the defining markers in a subset of SCF-GFP^{+}/CXCL12^{Hi}/Nestin-GFP^{Lo} stromal cells that reside in perivascular niches and support ST-HSCs. LepR^{+} cells, developed in culture, form bone, cartilage, and adipocytes upon transplantation in vivo. They are quiescent in physiologic conditions but proliferate in response to bone injury and other stimuli. These cells are a major contributor to bone regeneration after irradiation or fracture (Zhou et al. 2014). Sections from wild-type mice exhibit perivascular LepR staining throughout the BM around both sinusoids and arterioles. Nearly all perisinusoidal LepR^{+} cells are SCF-GFP^{+}; however, the periarteriolar LepR^{+} cells, especially those densely surrounding larger arterioles, expressed less SCF-GFP (Zhou et al. 2014). LepR^{+}/CD45^{-}/Ter119^{-}/CD31^{-} BM stromal cells are uniformly positive for the MSC markers CD51 (Pinho et al. 2013) and PDGFRβ (Komada et al. 2012), and a majority are also positive for the MSC marker CD105 (Chan et al. 2009; Park et al. 2012). LepR^{+}/CD45^{-}/Ter119^{-} cells are heterogeneous for Sca-1 expression, a molecule important for MSC–HSC interaction (Morikawa et al. 2009; Omatsu et al. 2010). LepR^{+} perivascular cells have also been reported to represent an important source of soluble stem cell factor (SCF) which is required for the maintenance of HSCs in BM (Ding et al. 2012).

In addition to the listed above, NG2, Sca-1, SCF/c-Kit, and CXCL12/CXCR4 molecules are highly relevant to MSC-HSC interactions and expressed at different levels in endosteal and perivascular niches; these molecules are discussed below.

3.7 NG2

NG2 is a neural glial antigen which is a progenitor cell marker in the central nervous system. It is expressed in tissues originating from mesenchymal, but not hematopoietic cell lineages, and hence is used as an MSC marker for isolating and identifying MSCs within BM (Kozanoglu et al. 2009). NG2^{+}/Nes-GFP^{bright} peri-arteriolar niche cells promote HSC quiescence, and depletion of NG2^{+} cells was shown to switch HSCs to a non-quiescent state. Using the LepR marker, two cell phenotypes, NG2^{+}/LepR^{-} and NG2^{-}/LepR^{+}, were proposed to help differentiate stromal cells in HSC niches supporting either quiescent or proliferating HSC populations, respectively. NG2^{+}/Nes-GFP^{bright} (Nesperi) MSCs associate with the quiescent HSC population in the periarteriolar zone, while NG2^{-}/ Nes-GFP^{Lo} (Nesretic) cells are found in the peri-sinusoidal area, where the majority of HSCs express high levels of the proliferative marker Ki-67 (Kunisaki et al. 2013). When the HSC cell cycle is activated, HSCs re-distribute from periarteriolar niches to peri-sinusoidal niches. The depletion of NG2^{+} cells induces HSCs proliferative cycling and reduces functional long-term repopulating of HSCs in BM (Kunisaki et al. 2013).

3.8 Sca-1 (Ly-6 A/E)

Sca-1 (Ly-6 A/E) plays a role in HSC self-renewal, progenitor cell activation, and cell lineage fate (Bradfute et al. 2005). It is a cell surface protein found in numerous tissues, including hematopoietic (Spangrude et al. 1988) and mesenchymal (Baddoo et al. 2003) cells. Among mesenchymal cells, there are two cell subsets with a distinct difference in Sca-1 expression. Sca-1^{Hi} expression is found in endothelium lining all types of vasculature in BM, including the small arterioles, arterioles in endosteal niches, and sinusoids of perivascular niches. These Sca-1+ cells are also known as periarteriolar PaS cells with PDGFRα^{+}/Sca-1^{+}/CD45^{-}/Ter119^{-}/Nestin^{Hi} phenotype (Morikawa et al. 2009; Mendez-Ferrer et al. 2010; Kunisaki et al. 2013; Sugiyama et al. 2018). On the contrary, CAR (LepR^{+}) cells display a uniform lack of the Sca-1 expression independent of their location (in both periarteriolar and perivascular niches) (Omatsu et al. 2010; Ding et al. 2012; Seike et al. 2018). According to Ito et al., the Sca-1$^{-/-}$ HSCs have reduced self-renewal potential, and as a result, have a significantly decreased serial transplantation capacity (Ito et al. 2003).

3.9 SCF/c-Kit

SCF/c-Kit pathway plays a crucial role in controlling HSC renewal (Zhang et al. 2017; Galli et al. 1994; Lev et al. 1984; Driessen et al. 2003; Russell et al. 1959). Mutations in either SCF or c-Kit locus result in hematopoietic deficiency and anemia (Lacombe et al. 2013). Loss-of-function mutations in c-Kit impair the self-renewal of HSCs (Miller et al. 1997). Conditional deletion of c-Kit causes hematopoietic failure and BM ablation (Kimura et al. 2011). HSCs with low levels of c-Kit expression have the ability for self-renewal and long-term reconstitution potential, whereas HSCs with high levels of c-Kit show restricted self-renewal capacity (Shin et al. 2014). SCF has high expression in reticular stromal cells (Balduino et al. 2012; Han et al. 1993) specifically in CAR (LepR^{+}) cells (Driessen et al. 2003).

3.10 CXCL12

Stromal cell-derived factor-1 (SDF1), also known as CXCL12, is produced by osteoblasts and endothelial cells in the BM (Ponomaryov et al. 2000). The binding of CXCL12 to CXCR4 is important in mediating the homing and retention of HSCs in the BM (Mendez-Ferrer et al. 2008; Peled et al. 1999; Sugiyama et al. 2006). CXCL12 plays a role in regulating both HSC quiescence and proliferation. The difference in the outcomes correlates strongly with the molecule's expression level on stromal cells in perivascular compared to endosteal niches. The expression of CXCL12 is about 10 times higher in perivascular/reticular CAR cells compared to osteoblasts in the endosteal niches (Frisch et al. 2008; Balduino et al. 2012).

Table 3.1 provides summary of the above cited studies on the different subsets of stromal cells residing in HSC niches, supporting either long-term or short-term repopulating HSC. MSCs in the LT-HSC and ST-HSC niches have different phenotypes representing a distinct expression of the markers supporting the quiescent versus proliferative state, respectively.

In summary, high expression of Sca-1, Nestin, and NG2 in stromal cells characterizes MSCs supporting quiescent, long-term repopulating subsets of HSCs while low or no expression of these molecules characterizes MSCs in short-term HSC niches. Based on the physiologic or pathologic environment, the phenotypic profile of these cells adjusts constantly and dynamically. For example, G-CSF has the ability to either activate HSCs or to make them quiescent, it mobilizes HSCs from bone into peripheral circulation (Greenbaum and Link 2011) and induces their proliferation (Liu et al. 2008); however, it can also down-regulate CXCL12 in Nestin^{+} niche cells (Mendez-Ferrer et al. 2010). Periarteriolar and sinusoid HSC niches represent distinct sites harboring preferentially either quiescent or proliferating HSCs. However, this condition is not regarded as status quo. HSCs can change both their proliferative and dormancy status and their specific niche type location. This flexibility is the major factor regulating balance of the HSC pools, maintaining homeostasis through niche-specific stem cell dynamic repopulation, and mediating response to physiologic and pathologic stimuli (Kunisaki et al. 2013).

Table 3.1 Profiles of the MSCs in the LT-HSC and ST-HSC niches

Resident MSCs by HSC niche	Quiescent state markers	Proliferative state markers
LT-HSC	NG2^{+}/Nestin^{Hi}/Sca1^{+}	LepR^{-}/SCF^{Lo}/CXCL12^{Lo}
ST-HSC	NG2^{-}/Nestin^{Lo}/Sca1^{-}	LepR^{+}/SCF^{Hi}/CXCL12^{Hi}

Markers are divided into two phenotypes supporting quiescent versus proliferative state of HSCs, characteristic for MSCs residing in the LT-HSC and ST-HSC niches, respectively

3.11 Challenging the Dogma of Necessity to Clear Space in HSC Niches for Engraftment of the Donor-Derived Hematopoiesis

A long-held perception was that achieving a clinically successful allogeneic BMT, one which would sustain donor-derived HSC self-renewal and differentiation into mature hemopoietic cells, requires clearing/ablation of the autologous hematopoiesis within the recipient's BM (Voog and Jones 2010; Spangrude et al. 1988; Lu et al. 2019; Tomita et al. 1994). Conditioning approaches for allogeneic BMT have shifted, as outlined in Sect. 3.3, from myeloablative to non-myeloablative and reduced intensity conditioning regimens in order to minimize the adverse effects associated with the original myeloablative protocols. These new approaches have been shown to suppress graft rejection with efficiency comparable to myeloablative regimens, while reducing adverse side-effects. These new approaches have significantly improved patients' quality of life and prognosis; however, the associated side-effects remain significant.

In the case of hematopoietic malignancies, the positive outcome of myeloablation performed as a part of conditioning for BMT is a decrease of the malignant clones of hematopoietic cells. However, the primary objective of this regimen is to clear autologous hematopoiesis in the HSC niches to allow engraftment of the donor-derived HSCs and precursor cells. This step, often referred to as creating HSC niches, is currently a standard component for BMT protocols. This review presents evidence to challenge its necessity, especially in treatment for non-malignant disorders.

Numerous studies have shown that all conditioning regimens, to various degrees, affect the integrity of HSC niches (Dominici et al. 2009; Pietras et al. 2015; Lu et al. 2019). Figure 3.1a depicts the relative locations of ST- and LT-HSC niche types within the BM and the changes induced in these niches in response to myeloablation. In particular, different levels of pancytopenia result from chemotherapy regimens used to treat malignancies, immunosuppressive regimens used in transplantation medicine and amelioration of autoimmunity, and conditioning regimens used for BMT (Song et al. 2010; Sutton 2014; Diaconescu et al. 2004; Mehta et al. 2017). Figure 3.1b shows the functional status of ST- and LT- niches during physiologic hematopoiesis and in the post-conditioning aplasia, respectively. In the case of malignancies, this complication of inhibited hematopoiesis results

from a combination of local tumor-induced effects, the mechanisms of generalized immunosuppression induced by both cancer-related processes, and the iatrogenic outcomes of the therapeutic regimens (Diaconescu et al. 2004; Sutton 2014; Buckley et al. 2014). A question is then raised: what is the role that MSC impairment plays in autologous HSC self-renewal in patients with pancytopenia related to cytoreductive therapies? Along the same line of speculation, another question is also raised: do conditioning regimens prior to BMT damage the stromal compartments within the niches, rather than create HSC niches as intended, and thus weaken allogeneic HSC engraftment?

Numerous studies have been performed to investigate whether conditioning regimens are necessary for HSC engraftment and chimerism induction. Here, we provide an update on new perceptions and emerging concepts on the enigma of achieving side-effect-free hemopoietic chimerism induced by allogeneic HSC engraftment without GVHD. Some relatively old studies will be part of this discussion along with new perspectives from the latest developments in BMT. The major challenges in this respect can be presented as three intertwined efforts to: (i) understand the mechanisms of myeloablation and its impact on multipotent subsets of HSCs; (ii) re-evaluate myeloablation as a means for establishing niche-space for allogeneic HSC engraftment; (iii) advance efforts to establish a clinically efficient protocol for the prevention of GVHD by elimination of donor-derived effector cells and supplementing donor HSC populations with cell subsets aiding their engraftment.

3.11.1 Myeloablation and Its Impact on Multipotent HSCs

A recent publication from Irving Weissman's group provides a comprehensive analysis of the hierarchy of cell subsets among early hematopoietic precursors with their unique phenotypes and functional properties (Lu et al. 2019). Relevant to the current discussion is the basic scheme of these very complicated events involving different cells and their interactions. Hematopoietic homeostasis is sustained through the balance of symmetrical and asymmetrical types of HSC propagation. The first population provides self-renewal of the "stock" population of HSCs (Yamamoto et al. 2013). These cells cycle very infrequently and under homeostatic conditions primarily remain in the G0 phase of the cell cycle (Seita and Weissman 2010). The second population, upon each division, produces one daughter HSC with its original properties and another with newly acquired features which lead to proliferation and differentiation toward lineage specific committed progenitor cells (asymmetrical cell division) (Kondo et al. 2000; Rankin et al. 2012). The second cell population is heterogeneous (Sun et al. 2014; Rodriguez-Fraticelli et al. 2018) and has a much higher proliferation rate. Elucidation of mechanisms regulating intrinsic relationships between early HSCs, including their clonal lineage commitment, is still in progress (Lu et al. 2019). Factors regulating two major directions in the fate of HSCs, which include a self-renewal and commitment into lineage-specific differentiation paths, are numerous. Among them are cytokines, the extracellular matrix (Sadovnikova et al. 1991; Klamer et al. 2018; Stier et al. 2005; Luo et al. 2011; Qian et al. 2007; Simons and Clevers 2011), and a variety of cell populations (Smith and Calvi 2013).

A more advanced evaluation of the distinct HSC subsets recently became possible by a genetic barcode-based technology (Lu et al. 2011; Brewer et al. 2016; Nguyen et al. 2018). DNA barcodes allow tracking and quantitative assessment of labeled cell populations. Using this technology, Lu and colleagues demonstrated that after unconditioned transplantation, donor HSCs injected into the peripheral blood home and engraft into recipient niche spaces. Donor engraftment was shown to be possible due to migration of the autologous HSCs. The engrafted HSCs were shown to sustain physiologic hematopoiesis despite the recipient not having myeloablative conditioning. In irradiated mice, a small fraction of engrafted HSC clones expanded substantially faster than other clones during differentiation. This clonal behavior was termed "dominant differentiation" and clones exhibiting this behavior were termed "dominant" clones. Unlike post-conditioned HSC transplantations, homogeneous differentiation for all engrafted HSC clones was observed in unconditioned mice. This phenomenon was shown to be true for self-renewal of the HSC populations as well. Interestingly, the pattern of dominant expansion of different HSC subclones was shown in irradiated but not in unconditioned mice. In addition, lineage bias was also found in conditioned recipients, while absent in unconditioned recipients. Of high importance, only a small subset of engrafted HSC clones were involved in differentiation after conditioned transplantation while on the contrary, all engrafted HSCs underwent uniformed differentiation and self-renewal after unconditioned transplantation (Lu et al. 2019).

These findings indicate that integrity of the HSC regulatory mechanisms may be compromised by conditioning regimens (Beachy et al. 2004). In numerous studies, all conditioning regimens affected the physiologic function of HSCs niches, although to various degrees (Dominici et al. 2009; Pietras et al. 2015). These novel insights into mechanisms of outcomes of the conditioning regimens provide a new direction in the search for BMT protocols without side effects associated with cytoreductive regimens.

3.11.2 Myeloablation as a Means to Establish Niche Space for Allogeneic HSC Engraftment

The term "HSC niche" proposed by Raymond Schofield (Schofield 1978) alludes to the idea that there is a particular place in the BM occupied by the host's hemopoietic stem cells which needs to be ablated to create a space for donor-derived BM to engraft (Tomita et al. 1994; Bhattacharya et al. 2009). This doctrine regarding the necessity for conditioning prior to allogeneic BMT has been challenged by numerous studies (Cronkite et al. 1985; Migliaccio 2016; Czechowicz et al. 2007; Bhattacharya et al. 2009). Cronkite et al. demonstrated the possibility of inducing hematopoietic chimerism without the host's myeloablative conditioning over 30 years ago (Cronkite et al. 1985; Brecher et al. 1982). In these in vivo mouse studies, full hematopoietic chimerism was achieved by transplantation of mega doses of syngeneic male BM into female recipients, and the authors concluded that "special proliferative sites do not appear to be required" (Brecher et al. 1982). A chronicle of the following research performed in this direction is provided in the comprehensive review by Anna Migliaccio (Nilsson et al. 1999; Migliaccio 2016). In a confirmation of the findings by Cronkite, Quesenberry's group reported that non-ablated mice effectively secured long-term HSC engraftments and distribution patterns (Nilsson et al. 1997; Stewart et al. 1993).

By the late 1980s, BMT had become firmly established in clinical practice for the treatment of leukemias (Thomas et al. 1957). However, the clinical adaptation of BMT for the treatment of non-malignant diseases, which often are part of pediatric medicine has been hampered by the morbidity and mortality associated with conditioning regimens, a required component of BMT protocols. Many hematopoietic diseases arise from either an abnormal hyper- or hypo-proliferative states of BM (Thota and Gerds 2018; Corey and Oyarbide 2017; Andres et al. 2017). During the initial latent stages of these diseases, a patient's healthy HSCs compensate the diseased hematopoiesis. The effectiveness of BMT used as a treatment for these disorders after their initial clinical manifestation has been understood as based on the ability of donor-derived HSCs to adapt to the host's microenvironment and contribute to the host's deficient hematopoiesis (Thomas et al. 1957). What is the current view on the major requirements for the success of this process in a non-ablated recipient? Three of them are outlined below:

(i) Dose-number of transplanted cells. Administration of mega doses for transplantation of unmanipulated BM cells used has been shown by Cronkite, Quesenberry, and others to improve engraftment (Cronkite et al. 1985, 1987; Nilsson et al. 1997). A later study using limiting dilution assays for HSC transplantation revealed a dose-effect correlation between the number of transplanted donor HSCs and recipient's peripheral blood reconstitution (Purton and Scadden 2007). Additionally, another study showed that HSC lineage differentiation influenced by the amount of donor HSCs (Brewer et al. 2016).

(ii) Stem and precursor cell subsets composition. It takes the innate compensation capacity of a stem cell network to sustain a balance in blood subsets recovery. Different HSC clones expand their cell numbers at specific differentiation stages to compensate for the deficiencies of other HSCs (Nguyen et al. 2018). Harrison et al. developed competitive repopulation assays in the mouse model to identify HSCs in the recipient's BM (Harrison 1993; Harrison et al. 1993). The competitive assay showed that low numbers of HSCs can compete with and replace the far greater cell numbers of co-transplanted short-term precursor cells in a syngeneic setting. This finding suggests that an appropriate advantage would also allow donor HSCs to compete with the endogenous HSCs in non-myeloablated hosts (Migliaccio 2016). Later research showed that healthy donor HSCs indeed have an advantage over the diseased hematopoiesis in repopulating the recipient's HSC niches (Nguyen et al. 2018).

(iii) Genetic composition of transplanted HSCs. More recent studies introduced new insight into the processes of HSC differentiation and showed it to be heterogeneous at the clonal level (Nguyen et al. 2018). Using a co-transplantation experimental model and high throughput genetic barcode tracking technology, Nguyen et al. showed in the mouse model that wild-type (healthy) HSCs compete with and compensate for genetically defective or mutated HSCs from BM of the recipient (Nguyen et al. 2018).

Recently, Weissman group reported successful HSC transplantation in immunocompetent hosts without radiation or chemotherapy, achieving robust chimerism in the mouse model by a combination of anti-c-Kit antibodies (Ab) and CD47 antagonists. The authors suggested that this protocol holds promise for the treatment of non-malignant disorders in combination with the monoclonal antibody HU5F9G4, and speculated that cytopenia, developed as a result of an "intended" objective of this therapy to clear the host's hemopoietic niches, could be managed by supplementary treatment or by the administration of a mega dose of donor's $CD34^+$ cells (Chhabra et al. 2018).

We would like to point out that there is some inconsistency in terminology with respect to conditioning. A common notion is that the term conditioning implies (as in the study cited above) the use of radiation or chemotherapy. However, the elimination of HSCs and precursor cell subsets by other means, such as antibodies, also ultimately results in myeloablation and cytopenia. It may be time to reconsider

the definition of BM recipient conditioning, at least for some of the current therapeutic regimens.

Is there indeed always a need for ablation of endogenous HSCs to clear a space for donor-derived BM to engraft? Is it possible to avoid pancytopenia associated with the majority of the currently employed conditioning regimens? It is now recognized that the HSC niches are not permanent, but rather dynamic structures (Cronkite et al. 1985; Voog and Jones 2010). The Nagasawa group demonstrated the existence of empty HSC niches available for engraftment of donor-derived BM (Shimoto et al. 2017). This finding suggests the possibility for donor-derived HSCs to compete with host's HSCs or simply to occupy empty HSC niches. Further, studies have shown that HSCs can be transplanted without the use of conditioning (Czechowicz et al. 2007; Bhattacharya et al. 2006) and unconditioned transplantation minimally perturbs natural hematopoiesis (Lu et al. 2019). These findings warrant further exploration into unconditioned HSC-based therapies (Rio et al. 2018).

Whole BMT was shown to be successful without myeloablation in the early era of attempts to induce allogeneic chimerism (Brecher et al. 1982; Nilsson et al. 1997). However, whole BMT without depletion of effector cells from donor BM inoculum may result in GVHD, one of the most dangerous and potentially fatal outcomes of BMT. GVHD is directly responsible for fatalities in up to 30% of allogeneic BMT patients and up to 50% of transplant recipients (Blazar et al. 2012; Pasquini et al. 2010; Kernan et al. 1993; Laurence et al. 2012).

3.11.3 Prevention of GVHD by Eliminating Donor-Derived Effector Cells and Supplementing Donor HSC Populations with Cell Subsets Aiding/Facilitating Their Engraftment

A potential remedy to GVHD was thought to be the administration of BM inoculum that was free of effector immune cells. Flow cytometry-based live cell sorting allows isolation of purified HSC populations that do not contain effector immune cells. However, purification of the BM graft inoculum from the effector immune cells significantly reduced the efficiency of allogeneic HSC engraftment and reduced reproducible consistency in the induction of allogeneic chimerism (Bhattacharya et al. 2006).

The Ildstad group explored a promising approach to induce allogeneic chimerism and aid the HSC engraftment by using, in the mouse model, a combination of HSCs purified from the effector cells to preclude GVHD with facilitating cells (FCs) of hemopoietic origin. This protocol was successful in some cases; however, the results were not consistently reproducible. Further studies of FC populations have led to advancements in the understanding of their nature. As FC research continues, their phenotype description is becoming better resolved. Originally, the FC phenotype was proposed as $CD8^+/CD3^+/CD45R^+/Thy\ l^+/Class\ II^{dim/intermediate}/TCR^-$ (Kaufman et al. 1994). In later attempts to define FC populations, Fugier-Vivier et al. proposed the term plasmacytoid precursor dendritic cells (DCs) (p-pre-DC) with phenotype $CD8^+/B220^+/CD11c^{dim}/CD11b^-$ defining this cell population (Fugier-Vivier et al. 2004). The understanding of FC was further modified and proposed to be comprised by two $CD8^+/TCR^-$ subsets: (i) $CD56^{dim/-}/CD3^+/HLA\text{-}DR^+/CD11c^-/CD123^-$ and (ii) $CD56^{bright}/CD19^+/CD11c^+/CD11b^+/CD3^-$ (Leventhal et al. 2012). In more follow-up research by Ildstad's group, a change in the definition of FC populations resulted in two subtypes with distinct phenotypes of: (i) $CD8^+/TCR^-/CD56^-$ and (ii) $CD8^+/TCR^-/CD56^{bright}$ with the majority of both subtypes of FCs expressing CXCR4 (Huang et al. 2016). While the research to identify the precise FC phenotype is still ongoing, these cells are currently being used in clinical studies as part of a regimen aiming at improving kidney transplantation outcomes (Leventhal et al. 2015). However, the protocols utilized in these studies still use RIC regimens, and the results of this approach are far from desirable with respect to the patient's length and quality-of-life.

Numerous other groups have dedicated years of research to identify which cells hold the ability to facilitate/support the engraftment of the allogeneic HSCs. In addition to attempts to delineate such cells among the hemopoietic cell subsets, they have studied a large cohort of non-hemopoietic cells, including osteoblasts, Schwann cells, and other cell populations with different phenotypes, including early mesenchymal precursor cells (Calvi et al. 2003; Itkin et al. 2016; Acar et al. 2015; Kunisaki et al. 2013; Ludin et al. 2012; Greenbaum et al. 2013; Yamazaki et al. 2011), sinusoidal endothelial cells, and CAR cells (Kiel et al. 2005; Ding et al. 2012; Sugiyama et al. 2006; Omatsu et al. 2010, 2014).

3.12 Potential Benefits of Clinical Adaptation of the MSPCs into Cytoreductive Therapies for Malignancies and Protocols for Induction of Allogeneic Hematopoietic Chimerism

3.12.1 MSPCs to Support Reconstitution of the Autologous Hematopoiesis Under Cytoreductive Therapies for Malignancies

MSPCs play a critical role in sustaining hematopoiesis in the HSC niches (Kfoury and Scadden 2015; Ding and Morrison 2013; Kunisaki et al. 2013; Acar et al. 2015). Their damage

by cytoreductive regimens includes induction of the senescent state, which contributes to impairing their ability to sustain hematopoiesis in the HSC niches and the resulting pancytopenia (Carbonneau et al. 2012; Rana et al. 2012). Thus, it may be possible to prevent such iatrogenic outcome of cancer therapies by supplementing the cytoreductive protocols with exogenous MSPCs. The ability of MSPC and HSC to cross-talk despite of the MHC disparity (Chertkov et al. 1980; Najar et al. 2019; Varas et al. 2000) allows utilization of MSPCs from unrelated donors and commercial sources, overcoming concerns about the availability of sufficient quantities of MSPCs for such cellular therapies (Brewer et al. 2016).

3.12.2 MSPCs for Promoting Induction of Allogeneic Hematopoietic Chimerism

Mesenchymal cells have a threefold advantage for their adaptation into BMT protocols. Their role in the induction of allogeneic hematopoietic chimerism comprises: (i) compensation of the impaired function of their autologous MSPC counterparts in the HSC niches in supporting hematopoiesis, (ii) suppression of the development of (GVHD), and (iii) potential for elimination of the conditioning regimens. Ability of these cells to interact with the MHC-disparate HSCs provides an additional benefit because, as described above for their utilization in the anti-cancer therapy, due to this feature, cell quantity is practically unlimited as they can be obtained from the nonmatching donors and commercial sources.

Data generated in the NOD mouse model of T1D provide an encouraging example of potential clinical adaptation of BMT for the therapy of autoimmune disease. Restoration of glycemic homeostasis after onset of hyperglycemia was achieved in the *antea*-diabetic NOD model as a result of physiologically sufficient regeneration of β cells after induction of allogeneic hematopoietic chimerism. The euglycemia restored in these animals lasted for life (Zorina et al. 2003). Whether these data can be successfully extrapolated into clinical setting remains to be explored. For example, would the regenerative potential for full physiologically sufficient β cell regeneration in human endocrine pancreas be comparable to that observed in the mouse model? And if so, how long after clinical diagnosis of T1D amelioration of autoimmunity would still allow β cell regeneration to occur? There is an important distinction between the detection of the hyperglycemia in mice and clinical diagnosis of T1D in humans, as usually T1D goes undiagnosed for quite some time.

Intensive research is ongoing on the therapeutic potential of the immunomodulatory approaches, and via induction of hematopoietic chimerism particularly for the treatment of many autoimmune, degenerative, and other non-malignant disorders. Development of a clinically safe and effective MSPCs-based conditioning-free protocol for BMT would significantly advance these treatments.

3.13 Summary and Conclusions

The evolving understanding of the dynamic structure of BM and the role of mesenchymal stroma in supporting hematopoiesis introduces new directions for therapeutic exploration. This review covers two independent venues for adaptation of the donor-derived MSPCs for therapeutic applications targeting: (i) improvement of reconstitution of the autologous hematopoiesis under cytoreductive therapies for the treatment of malignancies and (ii) induction of allogeneic hematopoietic chimerism without conditioning for the treatment of non-malignant disorders.

Cytoreductive therapies broadly used for the therapy of malignant disorders damage the HSC niche stromal component and impair its supportive role in reconstituting the autologous hematopoiesis (Abbuehl et al. 2017; Beachy et al. 2004; Lu et al. 2019). The resulting pancytopenia inflicts numerous co-morbidities in the cancer patients as iatrogenic outcomes of the therapeutic intervention (Tzeng et al. 2011; Mehta et al. 2017; Buckley et al. 2014). MSPCs are shown to play a vital role in hematopoietic homeostasis. This makes them a valid candidate for their supplementation into cytoreductive therapies for malignancies. Donor-derived MSPCs can compensate for the deficient capacity of the autologous stromal cell populations damaged in the BM of patients subjected to myeloablative therapies, an ability that is supported by their capability to cross-talk with HSCs despite the MHC disparity (Friedenstein and Kuralesova 1971; Chertkov et al. 1980; Varas et al. 2000). This feature provides an additional benefit for the clinical utilization of these cells, as the unlimited quantity of MSPCs could be obtained from the nonmatched donors and commercial sources. These data advocate the exploration of adaptation of MSPCs into chemo, radiation, and immunosuppressive therapies for their ability to sustain autologous hematopoietic reconstitution and alleviation of cytopenia.

This review also discusses the therapeutic potential of adapting MSPCs into BMT protocol for induction of allogeneic chimerism. BMT has become a routine therapeutic modality for different cancers, most commonly hematopoietic malignancies. In addition, induction of allogeneic chimerism holds potential to treat numerous non-malignant disorders as well. Ongoing' research and clinical trials are targeting the clinical adeptness of BMT for treating autoimmunity and for sustaining regeneration and reparative processes in the affected organs and tissues. However, accumulating experimental evidence indicates that the cyto-

reductive myeloablative regimens, which represent a standard component of the currently employed conditioning protocols for BMT, damage MSPCs in the HSC niches and impair their ability to sustain hematopoiesis. This leads to two questions: (i) Should conditioning, performed to aid donor-derived HSCs to engraft but producing BM aplasia and pancytopenia, be eliminated from the BMT protocols? (ii) Could induction of allogeneic hematopoietic chimerism be successful without such conditioning? The data from numerous studies, reviewed in this chapter, offer new insight into the BM functional and structural dynamics. They include finding that LT- and ST-HSC niches are not static structures and that HSC can migrate between them in response to physiologic or pathologic stimuli (Mendez-Ferrer et al. 2010; Crane et al. 2017; Morrison and Scadden 2014; Wilson et al. 2007; Voog and Jones 2010; Wright et al. 2001; Kiel et al. 2005; Yu and Scadden 2016; Kolf et al. 2007). Additionally, BM was shown to have empty unoccupied niches (Shimoto et al. 2017; Nguyen et al. 2018). These findings suggest the possibility for donor-derived HSCs to compete with the autologous HSC in occupying the niches. They even have an advantage in this respect, as it was demonstrated that healthy donor HSCs have the ability to out-compete diseased host HSCs for niche space during engraftment (Nguyen et al. 2018; Migliaccio 2016). In addition, more efficient- and lineage-balanced reconstitution of hematopoietic homeostasis was observed in hosts that did not receive conditioning prior to BMT compared to recipients that did (Czechowicz et al. 2007; Lu et al. 2019; Bhattacharya et al. 2009). These findings, in response to the first question, support the notion that the necessity of the conditioning step for BMT might be challenged. The proposed answer to the second question, about securing clinically successful BMT without conditioning, is that MSPCs represent a strong candidate for supporting BM engraftment without conditioning (Black and Zorina 2020). The rational for this suggestion is many-fold. MSPCs play a vital role in sustaining hematopoiesis in both LT- and ST-HSC niches (Kfoury and Scadden 2015; Acar et al. 2015; Kunisaki et al. 2013; Ding and Morrison 2013). MSPCs also have been shown to have immunomodulatory properties and the capacity to suppress GVHD (Le Blanc et al. 2008; Ball et al. 2013; Kebriaei et al. 2009; Dander et al. 2012; Introna et al. 2014). The ability of MSPCs to cross-talk with HSCs despite the MHC disparity (Friedenstein and Kuralesova 1971; Chertkov et al. 1980; Varas et al. 2000) allows availability of these cells in unlimited quantity from non-matched donors or commercial sources (Cronkite et al. 1985; Brewer et al. 2016; Nilsson et al. 1999; Purton and Scadden 2007).

Many questions still remain to be addressed. What specific subset within mesenchymal cell population, or their combination, would be optimal for their adaptation into proposed therapeutic applications? What phenotype and genetic profile would define such population(s) of MSPCs? If propagated in culture, what stage of maturation would be preferable? As we do not know yet all distinctions of the functional diversity of the mesenchymal cells isolated from the BM, umbilical cord, adipose tissue, or other sources, another question is what source of these cells will be used? The dose/cell numbers, as well as the MSPC/HSC ratio, for supplementation into cytoreductive regimens, have yet to be identified as well.

However, based on emerging new insights on the role of the MSC–HSC axis in hematopoietic homeostasis and in response to cytoreductive regimens, it is safe to conclude that exploration of allogeneic MSPCs' potential to sustain reconstitution of hematopoiesis in patients subjected to cytoreductive therapies for malignancies holds a promise to alleviate pancytopenia and associated comorbidities. Furthermore, development of a MSPCs-based conditioning-free protocol for BMT can provide a new venue for treating numerous non-malignant disorders.

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4 Mesenchymal Stem Cell-Derived Secretome: A New Remedy for the Treatment of Autoimmune and Inflammatory Diseases

Carl Randall Harrell and Vladislav Volarevic

Abbreviations

AF	Amniotic fluid
Ang-1	Angiopoietin-1
AT	Adipose tissue
CTLs	Cytotoxic T-lymphocytes
bFGF	Basic fibroblast growth factor
BNDF	Brain derived neurotrophic factor
DCs	Dendritic cells
DP	Dental pulp
ESCs	Embryonic stem cells
EVs	Extracellular vesicles
Exos	Exosomes
Exo-d-MAPPS	Exosome-derived Multiple Allogeneic Protein Paracrine Signaling
FasL	First apoptosis signal ligand
HGF	Hepatic growth factor
HO-1	Hemeoxygenase-1 (HO-1)
IDO	Indolamine 2,3-dioxygenase
IL	Interleukin
IL-R	IL-1 receptor
IL-Ra	IL-1 receptor antagonist
LECs	Lung epithelial cells
LPS	Lipopolysaccharides
MHC	Major histocompatibility complex
MicroRNAs	miRNAs
MIF	Migration inhibitory factor
M-CSF	Monocyte colony-stimulating factor
MSCs	Mesenchymal stem cells
MSC-CM	MSC-derived conditioned medium
MSC-Exos	MSC-derived exosomes
MSC-EVs	MSC-derived extracellular vesicles
NK	Natural killer
NKT	Natural killer T-cells
NO	Nitric oxide
OPN	Osteopontin
PAX5	Paired box 5
PGF	Placental growth factor
ST	Synovial tissue
TGF-β	Transforming growth factor-β
UC	Umbilical cord
PEDF	Pigment epithelium-derived factor
PGE2	Prostaglandin E2
PTECs	Proximal tubular epithelial cells
TSG-6	TNF-α stimulated gene/protein 6
VEFG	Vascular endothelial growth factor
TIMP1	Tissue inhibitor of metalloproteinase-1
TRAIL	TNF-related apoptosis-inducing ligand

C. R. Harrell
Regenerative Processing Plant, LLC, Palm Harbor, FL, USA

V. Volarevic (✉)
Department of Microbiology and immunology, Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

4.1 Introduction

During the last three decades, immunosuppressive drugs have been frequently used in clinical practice due to the increase of autoimmune and inflammatory diseases (Ji et al. 2016; Schein 2020). However, long-term use of immunosuppressive agents may result in the development of severe infections due to the inhibition of anti-microbial immune response (McCaughan 2004). Therefore, generation and clinical use of new immunoregulatory drugs which would suppress detrimental immune response without causing life-threatening immunosuppression is urgently needed (Holt 2017).

Mesenchymal stem cells (MSCs) are self-renewable, rapidly proliferating cells which reside in almost all post-natal tissues (Friedenstein et al. 1970; Volarevic et al. 2018; Najar et al. 2020), MSCs which are used in clinical settings are usually derived from bone marrow (BM), adipose tissue (AT), dental pulp (DP), amniotic fluid (AF), umbilical cord (UC) (Álvarez-Viejo 2020; Um et al. 2020; Xie et al. 2020; Zhang et al. 2020). MSCs possess huge potential for differentiation and, under specific culture conditions, may generate cells of mesodermal, neuroectodermal, or endodermal origin (Fan et al. 2020). Additionally, MSCs are immunoregulatory and angiomodulatory cells (Juárez-Navarro et al. 2020; Ryu et al. 2020; Volarevic et al. 2017; Xu et al. 2020).

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MSC-sourced immunomodulatory factors (hepatic growth factor [HGF], nitric oxide [NO], indolamine 2,3-dioxygenase [IDO], interleukin [IL]-10, transforming growth factor-β [TGF-β], IL-1 receptor antagonist [IL-1Ra], hemeoxygenase-1 [HO-1], prostaglandin E2 [PGE2], TNF-α stimulated gene/protein 6 [TSG-6]) and pro-angiogenic factors (vascular endothelial growth factor [VEGF], placental growth factor [PGF], HGF, basic fibroblast growth factor [bFGF], TGF-β, platelet-derived growth factor [PDGF]), regulate phenotype and function of immune cells and induce proliferation of endothelial cells, enabling repair and regeneration of injured and inflamed tissues (Álvaro-Afonso et al. 2020; Nazari-Shafti et al. 2020; Volarevic et al. 2017).

4.2 Therapeutic Potential of MSCs

Due to the potential for differentiation in the cells of all germ layers *in vitro*, a powerful capacity for immuno- and angiomodulation, MSCs have been explored as potentially new therapeutic agents for the treatment of inflammatory and degenerative diseases in a large number of experimental studies and clinical trials (Abedi et al. 2020; Harrell et al. 2019a, b; Master et al. 2020). Easy acquisition, abundant and suitable tissue sources for their isolation, rapid growth rate, maintenance of differentiation potential after repeated passages *in vitro*, homing to the sites of injury, and efficient engraftment in inflamed tissues after systemic administration, are the main characteristics of MSCs that enable their clinical use (Gundestrup et al. 2020; Hague et al. 2020; Song et al. 2020). Results obtained in animal models of acute and chronic organ-specific and systemic inflammatory disorders demonstrated that MSCs were able to efficiently attenuate T, NKT, neutrophil, and macrophage-driven inflammation in the lungs, liver, brain, skin, gut, cartilage, and bone (Badyra et al. 2020; Bozorgmehr et al. 2020; Song et al. 2020; Wang et al. 2020).

However, despite these promising results, it should be noted that several safety concerns limit the clinical use of MSCs (Volarevic et al. 2018). Transplanted MSCs can differentiate into the cells of mesodermal origin under the influence of local growth factors. Encapsulated structures containing calcifications and ossifications were found in ischemic lesions of MSC-treated mouse hearts. Although human MSCs were considered genetically stable, chromosomal aberrations in long-term cultures of human MSCs have recently been demonstrated, suggesting the need for rigorous genetic analysis prior to their clinical use (Volarevic et al. 2018).

In addition, patients suffering from chronic inflammatory disease and treated with a combination of immunosuppressive drugs and MSCs have developed respiratory and gastrointestinal infections due to severe immunosuppression, suggesting that MSCs should not be given together with immunosuppressants (Volarevic et al. 2018). As nucleated cells, MSCs express major class I histocompatibility complex (MHC) molecules and, therefore, can elicit strong allogeneic immune responses in the MHC class I mismatched recipients, resulting in tissue injury and inflammation (Gazdic et al. 2015).

Importantly, MSC-dependent side effects were not seen in animals and patients that received MSC-sourced secretome which contains MSC-derived immunoregulatory molecules, trophic and growth factors. MSC-sourced bioactive compounds are either dissolved in MSC-conditioned medium (MSC-CM) or enveloped within encapsulated extracellular vesicles (MSC-EVs): apoptotic bodies, microvesicles, and exosomes (Exos) (Harrell et al. 2020a, b). Due to their nano-sized dimension and lipid bilayers, MSC-Exos easily penetrate through all biological barriers and deliver their content (immunomodulatory cytokines, chemokines, microRNAs [miRNAs]) directly into the cytosol of target cells (Volarevic et al. 2017; Harrell et al. 2020a, b; Manchikanti et al. 2020).

Given the fact that the vast majority of MSC-based beneficial effects relied on the activity of MSC-sourced immunoregulatory, vasoactive and trophic factors, the therapeutic use of MSC-derived secretome is currently considered as a potential replacement for MSC-cell-based therapy (Harrell et al. 2020a, b; Pu et al. 2020; Massa et al. 2020). Therefore, in this chapter, we have summarized current knowledge about molecular mechanisms responsible for the beneficial effects of MSC-derived secretome in the treatment of autoimmune and inflammatory diseases.

4.3 Modulation of Immune Cell Phenotype and Function by MSC-Derived Secretome

Proliferation, migratory properties, phenotype and effector function of neutrophils, macrophages, dendritic cells (DCs), natural killer (NK), natural killer T (NKT) cells, T and B lymphocytes are regulated by MSC-derived molecules (Asgarpour et al. 2020; Maumus et al. 2020; Volarevic et al. 2017; Liu et al. 2020). MSC-sourced IL-8 and macrophage migration inhibitory factor (MIF) enhance the phagocytic ability of neutrophils and macrophages, contributing to the elimination of microbial pathogens (Bazzoni et al. 2020; Volarevic et al. 2017; Zhao et al. 2020). MSC-derived PGE2, TSG-6, and IL-6 induce polarization of inflammatory (M1) macrophages into immunosuppressive (M2) cells. M2, alternatively activated macrophages, have reduced capacity for the production of inflammatory cytokines (tumor necrosis factor-alpha [TNF-α], IL-1, and IL-12), and secrete a large amount of IL-10 and TGF-β that suppress ongoing inflammation (Volarevic et al. 2017).

The antigen-presenting capacity of DCs was also altered by MSC-derived secretome (Nauta et al. 2006). MSC-sourced IL-6 and monocyte colony-stimulating factor (M-CSF) suppress the proliferation of activated DCs by inducing G0/G1 cell cycle arrest through the inhibition of cyclin D2 (Nauta et al. 2006). In addition, MSC-derived IL-6 induced conversion of inflammatory, IL-12, and TNF-α-producing DCs into tolerogenic, IL-10-producing cells which promote generation and expansion of immunosuppressive regulatory T cells (Tregs) (Spaggiari et al. 2009). MSC-derived PGE2 and Galectin 3 down-regulate the expression of co-stimulatory molecules (CD40, CD80, and CD86) and MHC class II proteins on activated DCs (Nikolic et al. 2018), attenuating their capacity for the activation of naïve T cells (Nauta et al. 2006).

MSC-derived secretome may directly act on T lymphocytes to suppress their proliferation (Aggarwal and Pittenger 2005; Glennie et al. 2005). MSC-sourced TGF-β and HO-1 inhibit IL-2-dependent activation of Janus kinase (JAK)-STAT and ERK/MAPK kinase pathways, while MSC-derived PGE2 activates cyclin-dependent kinase inhibitor p27kip1 in T cells, preventing their proliferation (Aggarwal and Pittenger 2005; Glennie et al. 2005).

An interplay between MSC-derived NO and Kynurenine (the final products of IDO activity) was responsible for MSC-dependent inhibition of inflammatory T and NKT cells (Ren et al. 2009; Gazdic et al. 2018a, b). MSC-sourced NO suppressed proliferation of T cells by preventing phosphorylation of STAT5, while MSC-derived IDO and Kynurenine induced generation of regulatory phenotype inflammatory T and NKT cells (Gazdic et al. 2017).

NO and IDO-dependent attenuation of acute liver injury in MSC-CM-treated mice resulted in a reduced presence of liver infiltrated inflammatory (IFN-γ and IL-17 producing) NKT cells and with an increased presence of immunosuppressive IL-10 producing CD4+CD25+FoxP3+ Tregs and FoxP3+ regulatory NKT cells (NKTregs) in the injured livers (Gazdic et al. 2018a, b; Milosavljevic et al. 2017), suggesting that MSC-derived secretome altered phenotype and function of NKT cells, as well. MSC-sourced secretome down-regulated the expression of apoptosis-inducing ligands (First apoptosis signal ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL) on NKT cells and polarized them into immunosuppressive, IL-10-producing cells (Gazdic et al. 2018a, b). The addition of 1-methyl-DL-tryptophan (specific IDO inhibitor) or L-NG-monomethyl arginine citrate (specific iNOS inhibitor) in MSC-CM completely abrogated immunoregulatory capacity of MSC-sourced secretome and restored inflammatory characteristics of MSC-CM-treated NKT cells, suggesting that interplay between NO and IDO/Kynurenine pathways was mainly responsible for MSC-CM-based suppression of NKT cells (Gazdic et al. 2018a, b; Milosavljevic et al. 2017). Additionally, MSC-derived Kynurenine induced conversion of inflammatory, IL-17-producing Th17 lymphocytes, and NKT17 cells in immunosuppressive Tregs and NK Tregs (Gazdic et al. 2018a, b; Milosavljevic et al. 2017). Furthermore, MSC-sourced NO and Kynurenine significantly increased the expression of CD62L and CCR7 and also increased the production of immunosuppressive IL-10 and TGF-β in Tregs and NK Tregs enhancing their migratory and immunosuppressive capacities (Gazdic et al. 2018a, b).

MSC-sourced secretome efficiently modulated proliferation, activation, and cytotoxicity of NK and cytotoxic T lymphocytes (CTLs). A summary of the mechanism of treatment benefits with MSCs-derived secretome is shown in Table 4.1. MSC-derived IDO, PGE-2, and TGF-β reduced the expression of activating receptors (NKp30, NKp44, and NKG2D) and attenuated cytotoxicity of CTLs and NK cells (Gazdic et al. 2017). In a similar manner, as it was observed in T and NKT cells, MSC-derived secretome attenuated proliferation, and effector function of B cells (Gazdic et al. 2017). MSC-sourced NO and Kynurenine suppressed influx of IL-6 and TNF-α-producing, inflammatory B cells in the liver and increased presence of liver-infiltrated immunosuppressive, (IL-10- and TGF-β-producing) marginal zone (MZ)-like regulatory B cells (CD23-CD21+IgM+), resulting in the significant attenuation of acute liver inflammation (Gazdic et al. 2018a, b). MSC-derived Kynurenine induced suppressed ERK1/2 phosphorylation which led to the cell cycle arrest of activated B cells (Gazdic et al.). MSC-derived chemokine (C–C motif) ligand 2 (CCL2) and IDO modulate expression of the paired box (PAX)5 and STAT3 in plasma cells and inhibit the production of antibodies (Gazdic et al. 2017).

4.4 MSC-Derived Secretome as a New Therapeutic Agent: Evidence Provided by Animal Studies

Both systemic and local administration of MSC-CM and MSC-Exos had beneficial effects in the treatment of inflammatory and/or ischemic lung, liver, kidney, brain, and eye diseases (Xunian and Kalluri 2020; Monsel et al. 2016; Matthay et al. 2019; Casiraghi et al. 2020; Forsberg et al. 2020; Maqsood et al. 2020) (Table 4.2).

Intra-tracheal injection of MSC-CM significantly reduced pulmonary edema and inflammation in an experimental animal model of Lipopolysaccharides (LPS)-induced acute lung injury (Monsel et al. 2016). MSC-CM improved the lung endothelial barrier and restored alveolar fluid clearance in an *ex vivo* perfused human lungs injured by LPS (Monsel et al. 2016). MSC-derived keratinocyte growth factor (KGF) and insulin growth factor (IGF)-1 induced the generation of immunosuppressive M2 phenotype in alveolar macrophages, contributing to the enhanced repair and regeneration of

Table 4.1 Molecular mechanisms responsible for the therapeutic effects of mesenchymal stem cell-derived secretome

MSC-derived factor	Target cell(s)	Biological effect
IL-8, MIF	Neutrophils; macrophages	Enhanced phagocytic ability; increased removal of microbial pathogens
PGE2, TSG-6, IL-6	Macrophages	Generation of anti-inflammatory phenotype; enhanced production of immunosuppressive cytokines (IL-10 and TGF-β); attenuated production of inflammatory cytokines (TNF-α, IL-1β, and IL-12)
KGF; IGF-1	Macrophage	Induction of immunosuppressive phenotype
IL-6, M-CSF	DCs	G0/G1 cell cycle arrest' suppressed proliferation; inhibition of inflammatory phenotype and generation of tolerogenic phenotype; reduced production of inflammatory cytokines (TNF-α and IL-12); and increased production of anti-inflammatory IL-10
PGE2, Galectin 3	DCs	Down-regulated expression of co-stimulatory molecules (CD40, CD80, and CD86) and MHC class II proteins; reduced capacity for antigen-presentation and activation of naive T cells
TGF-β, HO-1	T cells	Inhibition of IL-2-dependent signaling pathways; suppressed proliferation
PGE2	T cells	Activation of cyclin-dependent kinase inhibitor p27kip1; suppressed proliferation
NO, IDO, Kynurenine	T cells, NKT cells	Suppressed proliferation; down-regulated expression of apoptosis-inducing ligands; generation of regulatory phenotype; increased production of immunosuppressive cytokines (IL-10 and TGF-β)
IDO, PGE-2, TGF-β	NK cells, CTLs	Down-regulated expression of activating receptors (NKp30, NKp44, and NKG2D); attenuated cytotoxicity
NO, Kynurenine	B cells	Reduced production of inflammatory cytokines (IL-6 and TNF-α); generation of regulatory phenotype; increased production of immunosuppressive cytokines (IL-10 and TGF-β); cell cycle arrest and reduced proliferation
CCL2, IDO	Plasma cells	Attenuated production of antibodies

Table 4.2 Beneficial effects of MSC-derived secretome in animal models of inflammatory and ischemic diseases

MSC-sourced secretome	MSC-sourced factor	Animal model	Beneficial effect(s)
MSC-CM	KGF, IGF-1	LPS-induced acute lung injury	Reduced pulmonary edema and inflammation
MSC-CM	M-CSF, OPN	BPD model	Enhanced regeneration of injured lung epithelial cells; reduced influx of neutrophils and macrophages in the injured lungs
MSC-CM	Adiponectin, IL-10	Acute and chronic asthma	Reduced airway hyper-responsiveness, peribronchial inflammation, and airway remodeling
MSC-Exos	IL-1Ra	Cigarette smoke induced chronic airway inflammation	Attenuated airway inflammation
MSC-Exos	miR-21-5p	I/R-induced lung injury	Reduced apoptosis of LECs; attenuated lung injury
MSC-CM	IDO, Kynurenine	CCL-induced liver fibrosis	Reduced hepatotoxicity; attenuated activation of stellate cells
MSC-CM	IL-6, fibrinogen-like protein 1	CCL-induced liver fibrosis	Increased proliferation of hepatocytes; reduced apoptosis of hepatocytes
MSC-Exos	miR-233	Autoimmune hepatitis	Increased proliferation of hepatocytes; reduced apoptosis of hepatocytes
MSC-CM; MSC-Exos	NO, let-7 miRs	Cisplatin-induced AKI	Reduced apoptosis of tubular cells, improved renal function
MSC-CM	TIMP-1, IGF-1; BDNF;	Ischemic stroke	Reduced ischemic lesion; reduced brain edema; improved cognitive and motor skills
MSC-Exos	PEDF	I/R-induced brain injury	Reduced neural loss
MSC-Exos	miR-17-92 & 21	EAU	Enhanced survival and neuritogenesis of injured RGCs

(continued)

Table 4.2 (continued)

MSC-sourced secretome	MSC-sourced factor	Animal model	Beneficial effect(s)
MSC-Exos	miR-19, miR-21	Myocardial infarction	Reduced cardiomyocyte apoptosis; high VEGF expression; neovascularization in ischemic hearts; improved myocardial recovery

injured lung parenchyma in MSC-CM-treated animals. MSC-CM showed beneficial effects in an experimental murine model of bronchopulmonary dysplasia (BPD). MSC-CM prevented blood vessel remodeling and alveolar injury and significantly reduced the influx of neutrophils and macrophages in the lungs of hyperoxia-exposed mice (Monsel et al. 2016). Beneficial effects of MSC-CM were relied on immunomodulatory and trophic effects of M-CSF and osteopontin (OPN) which suppressed IL-6 and IL-1β-driven lung inflammation and enhanced regeneration of injured lung epithelial cells. Remarkable improvement in lung function of MSC-CM-treated hyperoxic pups was associated with increase of M-CSF and OPN, and stanniocalcin-1 and down-regulation of IL-6 and IL-1β (Monsel et al. 2016).

MSC-CM efficiently attenuated acute and chronic asthma (Monsel et al. 2016). MSC-CM inhibited the production of IL-4 and IL-13 in lung-infiltrated CD4+Th2 cells, promoted expansion of immunosuppressive Tregs and increased the production of anti-inflammatory IL-10 in alveolar macrophages. MSC-sourced adiponectin was considered as the most important MSC-derived bioactive factor which prevented airway hyperresponsiveness, peri-bronchial inflammation and airway remodeling in asthmatic lungs of MSC-CM-treated experimental animals (Monsel et al. 2016). In a similar manner as MSC-CM, MSC-Exos efficiently attenuated airway inflammation, enhanced proliferation and immunosuppressive properties of Tregs, and also enhanced the production of anti-inflammatory cytokines (IL-10 and TGF-β) in lung-infiltrated immune cells (Monsel et al. 2016). MSC-Exo-based product ("Exosome-derived Multiple Allogeneic Protein Paracrine Signaling [Exo-d-MAPPS]") significantly improved respiratory function, down-regulated serum levels of inflammatory cytokines (TNF-α, IL-1β, IL-12, and IFN-γ), increased the serum concentration of immunosuppressive IL-10 and attenuated chronic airway inflammation in cigarette smoke-exposed mice (Harrell et al. 2019a, b, 2020a, b). Exo-d-MAPPS suppressed the production of inflammatory cytokines in lung-infiltrated macrophages, neutrophils, NK and NKT cells and alleviated the antigen-presenting properties of lung-infiltrated macrophages and DCs (Harrell et al. 2020a, b). Additionally, Exo-d-MAPPS induced the expansion of immunosuppressive IL-10-producing alternatively activated macrophages, regulatory DCs, and Tregs in inflamed lungs which resulted in the attenuation of chronic airway inflammation (Harrell et al. 2020a, b). MSC-sourced IL-1Ra was mainly responsible for beneficial effects of Exo-d-MAPPS (Harrell et al. 2019a, b, 2020a, b). By binding to the IL-1 receptor (IL-1R) on lung epithelial cells, MSC-derived IL-1Ra prevented pro-inflammatory events, initiated by IL-1:IL-1R axis, including the synthesis of inflammatory cytokines and chemokines (Harrell et al. 2020a, b).

In addition to their anti-inflammatory properties, MSC-Exos interfered with cell death/survival pathways in lung epithelial cells (LECs) as well (Li et al. 2019). Through the delivery of miRNAs, which regulate apoptosis/autophagy-related signaling, MSC-Exos promoted the survival of un-injured LECs during the progression of lung injury and inflammation. Through the delivery of miR-21-5p, which inhibited the activity of pro-apoptotic proteins Phosphatase and tensin homolog (PTEN) and Programmed cell death protein 4 (PDCD4), MSC-Exos prevented the apoptosis of LECs and enabled enhanced regeneration of injured lungs. Pre-treatment of MSCs with miR-21-5p antagomir significantly abrogated capacity of MSC-Exos to suppress PTEN and PDCD4-induced apoptosis of LECs and completely diminished MSC-Exo-mediated therapeutic effects in the alleviation of Ischemia/Reperfusion (I/R)-induced lung injury (Li et al. 2019).

In a similar manner, as it was observed in inflamed lungs, MSC-CM and MSC-Exos managed to alleviate detrimental immune response in fibrotic liver as well (Gazdic et al. 2018a, b). Significantly improved survival of MSC-CM-treated mice was a consequence of MSC-CM-dependent increased proliferation and reduced the apoptosis of hepatocytes as well as MSC-CM-induced suppression of hepatotoxicity of liver-infiltrated immune cells (Gazdic et al. 2018a, b). MSC-derived IL-6 and fibrinogen-like protein 1 increased expression of anti-apoptotic proteins in hepatocytes, while MSC-derived IDO was mainly responsible for MSC-CM-mediated attenuation of a detrimental immune response in the inflamed and fibrotic livers (Gazdic et al. 2018a, b; Milosavljevic et al. 2018). MSC-CM, in Kynurenine dependent manner, prevented Th17 cell-driven activation of pro-fibrogenic stellate cells by inducing conversion of CD4+Th17 cells into protective CD4+FoxP3+IL-10+ Tregs (Milosavljevic et al. 2018).

Systemic administration of MSC-Exos managed to prevent hepatocyte cell death in animal models of fulminant and autoimmune hepatitis (Gazdic et al. 2018a, b). Hepatoprotective effects of MSC-Exos were relied on miR-233-dependent suppression of caspase-3-driven apoptosis

and on inhibition of caspase-1-induced pyroptosis of hepatocytes. Additionally, MSC-Exos enhanced hepatocyte proliferation in injured livers by inducing sphingosine kinase (SK1)-dependent activation of sphingosine-1-phosphate (S1P) which regulated hepatocyte growth and survival (Nojima et al. 2016).

MSC-CM and MSC-Exos significantly reduced apoptosis of tubular cells, improved renal function, and increased survival of mice suffering from acute kidney injury (AKI) and renal fibrosis (Liu et al. 2018). MSC-CM suppressed generation and expansion of inflammatory DCs, Th1, and Th17 cells (Simovic et al. 2017). MSC-derived NO was mainly responsible for MSC-CM-mediated reno-protective effects since lack of NO in MSC-CM completely restored inflammatory phenotype of renal-infiltrating DCs and T cells in cisplatin+MSC-CM treated mice (Simovic et al. 2017). MSC-Exo-based reno-protection was completely diminished by RNase pre-treatment, confirming the hypothesis that beneficial effects of MSCs-Exos have mainly relied on the activity of the MSC-Exo-delivered mRNA. Among various renoprotective and immunomodulatory miRNAs, members of let-7 miR-family most effectively down-regulated the expression of apoptosis-related genes in PTECs and prevented progression of AKI in experimental animals (Gatti et al. 2011).

MSC-CM and MSC-Exos efficiently protected the brain tissue of experimental mice and rats from ischemic injury (Jiang et al. 2019). Intra-cerebroventricular administration of MSC-CM markedly reduced ischemic lesion and brain edema. MSC-derived tissue inhibitor of metalloproteinase-1 (TIMP-1) and progranulin were responsible for these beneficial effects of MSC-CM (Jiang et al. 2019). Additionally, MSC-sourced IGF-1 and brain-derived neurotrophic factor (BDNF) significantly improved cognitive and motor skills in experimental animals crucially contributing to their functional recovery from the stroke. MSC-CM improved motor functions in MSC-CM-treated experimental animals by reducing neuronal loss through the suppression of caspase-3-driven apoptosis of neural cells in the motor cortex (Jiang et al. 2019). MSC-Exos, through the delivery of pigment epithelium-derived factor (PEDF), increased the expression of autophagy-related protein LC3 and suppressed caspase-3-driven apoptosis in neurons, significantly reducing I/R--induced brain injury (Huang et al. 2018). These MSC-Exo-mediated beneficial effects were completely abrogated by autophagy inhibitor, 3-methyladenine, confirming a crucially important role of autophagy induction for anti-apoptotic effects of MSC-Exos in the brain (Huang et al. 2018).

In a similar manner, as it was observed in the brain, MSC-sourced secretome alleviated eye inflammation (Harrell et al. 2018). Intraocular injection of MSC-Exos prevented T cell-dependent injury of retinal cells. MSC-Exos efficiently ameliorated experimental autoimmune uveitis by attenuating production of inflammatory cytokines (TNF-α, IFN-γ, and IL-17) in effector T cells. In addition, MSC-Exos successfully delivered trophic, vasoactive, and immunoregulatory factors to the inner retina and efficiently promoted survival and neuritogenesis of injured retinal ganglion cells (RGCs) (Harrell et al. 2018). MSC-Exos, through the delivery of miR-17-92 and miR21, down-regulated expression of PTEN (well-known suppressor of RGC axonal growth), promoted axonal regeneration and survival of RGCs (Harrell et al. 2018).

In line with these findings are results obtained in an experimental animal model of myocardial infarction (Wang et al. 2017). MSC-Exos, in miR-21 and miR-19-dependent manner, suppressed PTEN-driven apoptosis of cardiomyocytes and improved myocardial recovery after ischemic injury. Through the delivery of miR-19, MSC-Exos down-regulated the activation of PTEN and induced phosphorylation and activation of Akt kinase which, in turn, up-regulated anti-apoptotic Bcl-2 protein and prevented apoptotic loss of injured cardiomyocytes (Wang et al. 2017). Additionally, MSC-Exos in a miR-21-dependent manner improved cardiac function by inducing neo-angiogenesis in ischemic hearts through the enhanced expression of VEGF (Wang et al. 2017).

4.5 Clinical use of MSC-Derived Secretome

Despite the fact that the results obtained in animal studies indicated beneficial effects of MSC-sourced secretome in the therapy of inflammatory diseases and suggested its superiority to cell-based therapy in terms of safety, MSC-CM, and MSC-Exos had been explored in only a few ongoing or already conducted clinical trials (Harrell et al. 2020a, b) (Table 4.3).

Results obtained in two already completed and published clinical studies provided evidence that multiple, local, and systemic applications of MSC-CM and MSC-Exos were well-tolerated and safe therapeutic approaches which led to the efficient attenuation of ongoing inflammation in injured tissues, enabling their enhanced repair and regeneration (Prakoeswa et al. 2018; Nassar et al. 2016). Topical administration of amniotic membrane-mesenchymal stem cell-conditioned medium (AMMSC-CM) was efficiently healed the chronic plantar ulcers in patients suffering from leprosy (Prakoeswa et al. 2018). AMMSC-CM was applied every 3 days for up to 8 weeks and the percentage of healed ulcers had been continuously increasing with the AMMSC-CM-treatment. Importantly, there were no side effects or complications related to AMMSC-CM-based therapy. The beneficial effects of AMMSC-CM were significantly enhanced by vita-

Table 4.3 Clinical use of mesenchymal stem cell-derived secretome

MSC-sourced secretome	Route of administration	Clinical condition	Reference/clinical trial identification number
AMMSC-CM	Local/topical	Chronic plantar ulcers	Prakoeswa et al. (2018)
WJMSC-CM	Local/topical	Chronic ulcers	NCT04134676
MSC-Exos	Local/topical	Ulcers of EB patients	NCT04173650
MSC-Exos	Intravenous	Chronic renal inflammation	Nassar et al. (2016)
miR-124-overexpressing MSC-Exos	Intracerebral	Ischemic brain injury	NCT03384433
MSC-Exos	Intraocular	DED	Harrell et al. (2018)
MSC-Exos	Intraocular	DED-related symptoms in patients with GvHD	NCT04213248
MSC-Exos	Intraocular	Macular holes	NCT03437759
MSC-Exos	Intravenous	SARS-CoV-2-induced ARDS	Sengupta et al. (2020)
MSC-Exos	Intravenous	ATAAD-related MODS	NCT04356300

min E (α-tocopherol) which, due to antioxidant and anti-inflammatory properties, promoted AMMSC-CM-induced repair and regeneration of chronic plantar ulcers (Prakoeswa et al. 2018). In line with these promising results, Sukmawati and colleagues designed a clinical trial to investigate the therapeutic potential of WJ-CM (NCT04134676). WJ-MSCCM was applied to the wound and closed by a transparent dressing. WJ-CM-based therapy was given 2 weeks and 38 patients were enrolled in this trial. This study was completed in June 2020 and results are expected in the following months (Harrell et al. 2020a, b).

The safety and efficacy of MSC-Exos in the treatment of Epidermolysis Bullosa (EB) lesions will be examined in the upcoming clinical trial that will recruit 10 patients (NCT04173650). MSC-Exos will be applied topically, once a day for 2 months. The level of healing and scaring of MSC-Exo-treated lesions as well as the change in itching and pain will be biweekly evaluated during the 60 days of follow-up. It is expected that this clinical study will start in September 2020 and the first results are expecting in November 2021 (Harrell et al. 2020a, b).

Results obtained in a clinical study conducted by Nassar and colleagues demonstrated beneficial effects of MSC-Exos in the alleviation of chronic renal inflammation (Nassar et al. 2016). Remarkably, improved renal function was observed in 20 patients with chronic kidney disease (grade III–IV) who received two doses (1 week apart) of MSC-Exos (100μg/kg/dose). The first dose of MSC-Exos was infused intravenously, while the second dose of MSC-Exos was injected via the renal artery (Nassar et al. 2016). Renal function was significantly improved in MSC-Exo-treated patients, as evidenced by an estimated glomerular filtration rate (eGFR) and urinary albumin creatinine ratio. The cytokine profile of MSC-Exo-treated patients showed that MSC-Exos suppressed TNF-α-driven inflammation in the kidneys. Additionally, elevated serum levels of immunosuppressive cytokines (TGF-β and IL-10) were noticed in MSC-Exo-treated patients, indicating that MSC-Exos induced generation of immunosuppressive phenotype in immune cells resulting in the generation of an anti-inflammatory immune response in the kidneys. Importantly, administration of MSC-Exos did not provoke any unwanted side event during the 12 months of follow-up, indicating that MSC-Exos could be considered as potential new therapeutic agents in the treatment of renal inflammatory diseases (Nassar et al. 2016).

Following these promising results, many other research groups explored the potential of MSC-Exos for the treatment of a wide variety of clinical conditions (Harrell et al. 2020a, b). The therapeutic efficacy of MSC-Exos in the repair of ischemic brain lesions will be evaluated in the clinical trial which will be conducted in Iran (NCT03384433). Since miR-124-overexpressing MSC-Exos showed therapeutic effects in an experimental animal model of ischemic stroke, these genetically engineered MSC-sourced EVs (200 mg of total protein) will be injected directly into the ischemic brains of patients, 1 month after the stroke. According to the protocol, an incidence of side effects (deteriorating stroke, brain edema, and seizures) and degree of disability will be evaluated in five MSC-Exo-treated patients during the 12 months of follow-up.

Intraocular administration of MSC-derived secretome has been explored as a new therapeutic approach for the treatment of inflammatory eye diseases (Harrell et al. 2018). The capacity of MSC-derived Kynurenine to suppress Th17 cell-driven inflammation in the eye was crucially responsible for the beneficial effects of MSC-Exos in the treatment of dry eye disease (DED) (Harrell et al. 2018). MSC-Exo-based alleviation of DED-related symptoms in patients with Graft-versus-host disease (GvHD) is the main aim of the clinical trial which is currently recruiting patients in China (NCT04213248). According to the study protocol, GvHD patients will receive combined therapy of artificial tears (14 days) and MSC-Exos (10 ug/drop, 14 days). Therapeutic efficacy of MSC-Exos in the alleviation of eye inflammation

and attenuation of DED-related symptoms will be determined by Ocular Surface Disease Index (OSDI), Tear break time, Best Corrected Visual Acuity (BCVA), Conjunctiva Redness Score (CRS) (Harrell et al. 2020a, b). MSC-Exos-based healing of large and refractory macular holes (MH) in the eyes was the main objective of the clinical trial initiated in March 2017 (NCT03437759). According to the study protocol, 44 patients will receive 50 or 20μg MSC-Exos (dissolved in 10μl of phosphate-buffered saline). After the air-liquid exchange, MSC-Exos will be administered in the vitreous cavity around MH. BCVA, fundoscopy, optical coherence tomography, and physical examination will be used to analyze the efficacy and potential side effects of MSC-Exo-based therapy during 6 months of follow-up (Harrell et al. 2020a, b).

From December 2019, the world population has been faced with a new coronavirus disease (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus (SARS-CoV-2), which penetrates the depths of the lungs and cause severe pneumonia and life-threatening acute respiratory distress syndrome (ARDS) (Li and Ma 2020). Single intravenous infusion of MSC-Exos significantly improved lung function, alleviated systemic inflammation, and increased the total number of circulating neutrophils and lymphocytes in the majority of COVID-19 patients (Sengupta et al. 2020). MSC-Exos significantly improved oxygenation and efficiently attenuated SARS-CoV-2-related ARDS in 71% of MSC-Exo-treated patients (17/24) who completely recovered within a week after the administration of MSC-Exos. Despite of these promising results, it should be noted that MSC-Exo-based therapy was not effective in 29% (7/24) of COVID-19 patients. Four patients passed away for reasons unrelated to the MSC-Exos administration, while three patients remained critically ill and required mechanical ventilation and intensive care (Sengupta et al. 2020).

Administration of MSC-derived secretome has been explored as possible new therapeutic approach for the treatment of life-treating diseases. Multiple organ dysfunction syndrome (MODS) is one of the main causes of post-operative death for patients who underwent acute type A aortic dissection (ATAAD). Researchers from Fujian Medical University are currently recruiting patients to participate in the clinical trial which will examine therapeutic effects of MSC-Exos in the prevention and therapy of ATAAD-related MODS (NCT04356300). According to the study protocol, ATAAD patients will intravenously receive MSC-Exos (150 mg) immediately after the onset of MODS. MSC-Exos will be injected daily for two weeks. Therapeutic effects of MSC-Exos on cardiovascular, gastrointestinal, urogenital, and neural system of patients suffering from ATAAD-related MODS will be evaluated for 6 months.

4.6 Conclusion and Future Directions

A large amount of evidence suggested that the therapeutic use of MSC-derived secretome had multiple advantages over MSC-based therapy (Volarevic et al. 2018; Harrell et al. 2020a, b). Application of MSC-CM and MSC-Exos, which had similar biological effects as their parental cells, avoided safety issues raised by local or systemic injection of MSCs, such as activation of an allogeneic immune response, unwanted differentiation of transplanted MSCs and intravascular obstruction by intravenously infused MSCs (Volarevic et al. 2018).

However, it should be emphasized that there are still some challenges that should be addressed before MSC-CM or MSC-Exos could be worldwide offered as universal remedy for the treatment of autoimmune and inflammatory diseases. MSCs are a heterogeneous population of cells with variable growth potential, distinct morphologic and functional characteristics, including different migratory, immunosuppressive and angio-modulatory properties (Song et al. 2020). Concentrations of immunomodulatory and trophic factors in MSC-derived secretome are heterogeneous and depend on the tissue origin of MSCs from which secretome was derived (Volarevic et al. 2017). Therefore, the pre-selection of the most effective tissue source for a particular disease is of crucial importance for successful clinical use of MSC-sourced secretome. The optimal therapeutic dose of MSC-Exos has to be defined for each clinical condition according to the treatment plan, route of administration, and longevity of MSC-Exos in the target tissue (Mead et al. 2018). Additionally, precise MSC-derived factor(s) responsible for beneficial effects of MSC-CM and MSC-Exos should be determined for each inflammatory disease. In this way, defined therapeutic factor(s) could be overexpressed in MSCs, and, consequently, would be present in high concentration in MSC-derived secretome. Therefore, up-coming experimental and clinical studies have to optimize protocols for isolation and therapeutic application (route of injection and dose) of MSC-CM and MSC-Exos to maximize their therapeutic potential and efficacy.

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Cardiac Regenerative Therapy in Diabetes: Challenges and Potential Therapeutics

5

Paras Kumar Mishra

Abbreviations

CPCs	Cardiac progenitor cells
DM	Diabetes mellitus
ESCs	Embryonic stem cells
hCSCs	Human cardiac stem cells
iPSCs	Induced pluripotent stem cells
MI	Myocardial infarction
miRNA	microRNA
MMP	Matrix metalloproteinase

5.1 Introduction

Diabetes mellitus (DM) is caused due to deficiency of insulin production (type 1 DM or T1DM) or impaired insulin function (T2DM) (Chavali et al. 2013). DM promotes vascular disease and inflammation that lead to atherosclerosis and myocardial infarction (MI) (Fuller et al. 1983; Matheus et al. 2013). MI causes deprivation of oxygen and nutrients to myocardial cells, which result in their death. Myocardial cell death instigates cardiac remodeling where cardiomyocytes are hypertrophied to meet the excess workload and fibrosis is increased via extracellular matrix turnover to fill the vacuum created due to myocardial cell death (Kar et al. 2019; Mishra et al. 2013). Although these changes in the heart are initially adaptive, if not prevented, they could further the remodeling process resulting in the transition from compensatory cardiac remodeling to decompensatory heart failure.

P. K. Mishra (✉)
Department of Cellular and Integrative Physiology, University of Nebraska Medical Center, Omaha, NE, USA
e-mail: paraskumar.mishra@unmc.edu

The heart is a terminally differentiated organ with a limited regeneration capacity (Laflamme and Murry 2011). Thus, loss of myocardial cells is a permanent damage that impairs cardiac function. If untreated, increased workload on the remaining cardiac cells augments adverse cardiac remodeling and increases the risk of heart failure. Thus, supplementing the MI heart with new cardiac cells is a promising approach to replenish the dead myocardium and mitigate cardiac remodeling (Tzahor and Poss 2017). Several preclinical studies have demonstrated successful myocardial tissue regeneration after MI, and also improvement in cardiac functions by stem cells (Park et al. 2019; Liu et al. 2018). There are several approaches for cardiac regeneration (Hashimoto et al. 2018). An update on the cellular therapy is nicely reviewed by Peng and Abdel-Latif (Peng and Abdel-Latif 2019). However, these approaches are focused on the non-DM MI heart. The effect of DM on these processes remains unclear. Here, we focus on the challenges of cardiac regeneration in DM MI heart and provide new avenues to alleviate them.

5.2 Regenerative Therapy Approaches

The concept of cardiac regeneration was based on the initial idea that the heart has resident stem cells, which help in cardiac repair and take care of regular wear and tear in the heart by generating new myocardial cells (Bearzi et al. 2014). MI causes a massive loss of myocardial cells, which is beyond the capacity of resident cardiac stem cells (CSCs) to repair. Thus, exogenous stem cells are needed to replenish dead myocardial tissue following MI. The area of regenerative therapy is continuously progressing, and new approaches for cardiac regeneration are developing with the advancement of knowledge in this field (Cahill et al. 2017).

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5.2.1 Presence of Resident CSCs

Although several studies have supported the concept of resident CSCs in myocardial tissue regeneration, current findings revealed that the adult heart has a very small population of CSCs, which could not be adequate for cardiac repair via differentiation (van Berlo et al. 2014). There is consensus on the limited capacity of cardiomyocyte regeneration in the adult heart (Eschenhagen et al. 2017). It is posited that stem-cell therapy was able to regenerate myocardium in preclinical studies by paracrine factors of stem cells (factors released from the stem cells). The paracrine factors influence the microenvironment of the myocardium and induce cardiac regeneration (Li et al. 2019). These factors include transforming growth factor-beta superfamily, fibroblast growth factors, Notch and hedgehog signaling, and several other transcription factors such as GATA4, NKX2.5, etc. (Witman et al. 2020). Also, several microRNAs (miRNAs), the tiny regulatory RNAs, are involved in inducing cardiac regeneration (Eulalio et al. 2012; Srivastava and Heidersbach 2013; Bartel 2004). Thus, the current knowledge in regenerative therapy suggests that providing a combination of paracrine factors is a promising approach for myocardial regeneration in the MI heart.

5.2.2 Mechanical Scaffold and Cardiac Regeneration

In addition to paracrine factors, there is a crucial role of mechanical scaffold of the heart in cardiac regeneration (Engler et al. 2008). The role of extracellular matrix and mechanical structures of the heart on cardiac repair has been extensively reviewed by Mishra et al. (2013). Developing a stem-cell-derived cell sheet is another promising area for myocardial tissue repair following MI (Guo et al. 2020).

5.2.3 Stem Cells and Cardiac Regeneration

In all above-mentioned approaches, stem cells are required. However, obtaining human stem cells from the same individual is a major limitation. Using stem cells obtained from other individuals has a limitation of increased immunogenic reaction. Induced pluripotent stem cells (iPSC) provide a new approach for cardiac regeneration and circumvent the above-mentioned limitations of stem cells. In iPSC, stem cells are developed from the same individual by de-differentiating cells from a tissue, such as skin tissue, then re-differentiating them into cardiac lineage (Kishino et al. 2020). Although this is a powerful approach, the rejection rate of iPSC after transplantation could be a potential limitation (Apostolou and Hochedlinger 2011).

5.2.4 Altering Resident Cells to Improve Cardiac Function

To avoid the challenges associated with the injection of exogenous materials such as cells and paracrine factors, a novel approach is to alter the resident cells in the heart for cardiac repair. The number of fibroblast cells is high and cardiomyocytes is low in the MI heart due to increase myocardial cell death. A new approach is to convert fibroblast cells into cardiomyocytes. Using several miRNAs and transcription factors, cardiac fibroblasts have been transdifferentiated into cardiomyocytes both in vivo and in vitro, suggesting that transdifferentiation of fibroblasts could be a viable approach for cardiac regeneration in the MI heart (Nam et al. 2013; Jayawardena et al. 2012).

Although regenerative therapy, including different types of stem cells, cardiosphere-derived cells, engineered heart tissue, and paracrine factors, have demonstrated success in preclinical models, there are several leaps in strategies needed to translate them into humans, to make new myocardium following MI (Song et al. 2010; Nam et al. 2013; Makkar et al. 2012; Ashur and Frishman 2018; Witman et al. 2020).

These approaches are further challenged by DM, which makes an entirely different microenvironment than the non-DM MI heart (Fig. 5.1).

5.3 Differences in the Diabetic and nondiabetic Heart

DM is an independent cause of heart failure (Rubler et al. 1972). Also, it increases the risk of heart failure (Nichols et al. 2004). Thus, the DM heart differs from the non-DM heart. Few major differences are outlined in the following sections.

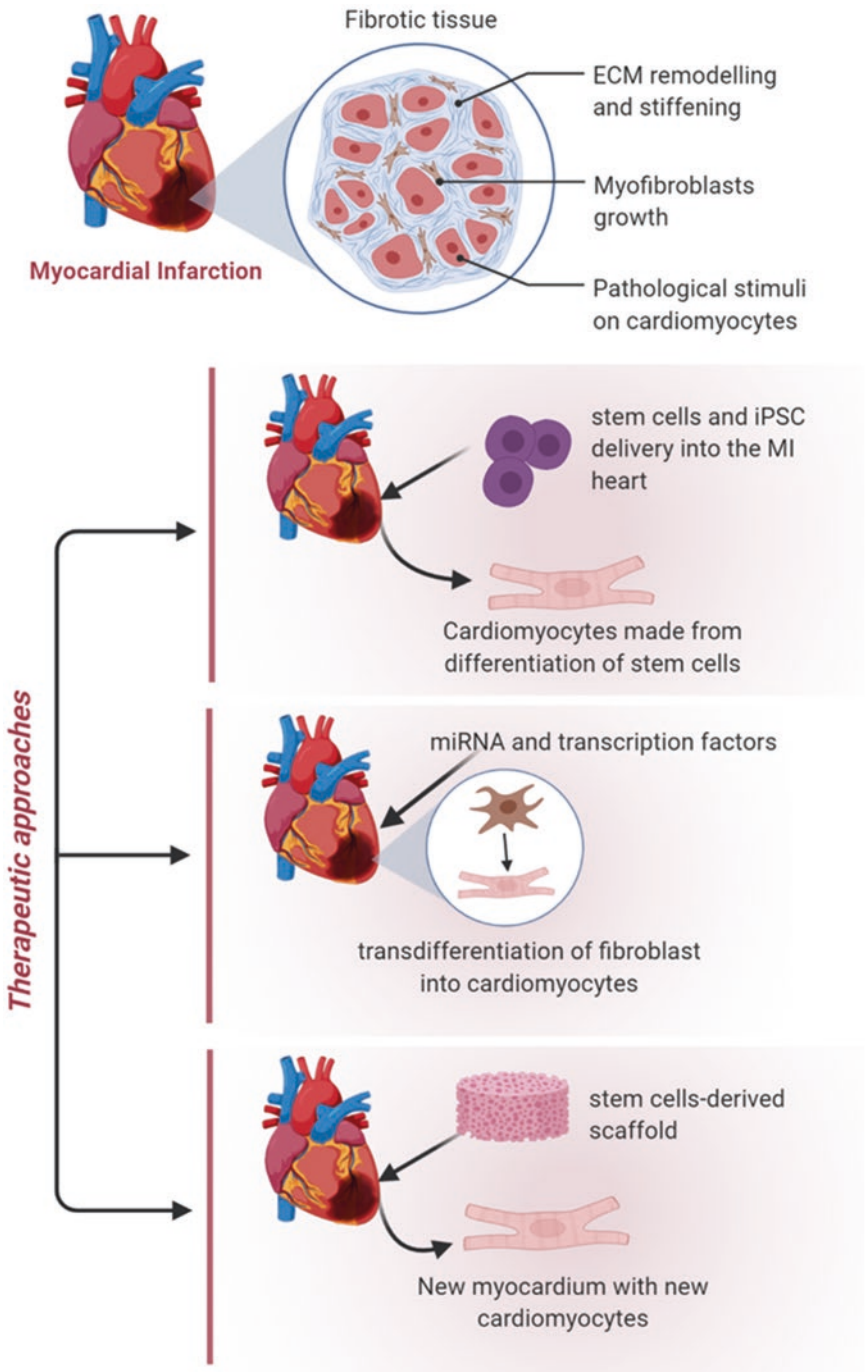

Fig. 5.1 Regenerative approaches for cardiac repair following myocardial infarction (MI). MI causes extracellular matrix (ECM) remodeling by increasing its degradation and turnover. Fibroblast cells become myofibroblasts for ECM deposition and ultimately scar formation. The myocardial cells including cardiomyocytes die due to pathological stimuli and the remaining cardiomyocytes become bigger in size (hypertrophy) to meet the demand of increased workload for the contractility of the heart. Three major therapeutic approaches for cardiac regeneration are: (1) delivery of stem cells (different types of stem cells, or induced pluripotent stem cells/iPSCs) into the area adjacent to the infarcted zone. Stem cells are directly delivered into the myocardium. Alternatively, they are delivered into the bloodstream from which they reach to the heart via circulation. (2) Injection of few miRNAs and transcription factors into the adjacent area of infarction, which will transdifferentiate fibroblasts into cardiomyocytes. Thus, new cardiomyocytes could improve function of the MI heart. (3) Treating the MI heart with stem-cell-derived scaffold, which will form new myocardium with cardiomyocytes and improve cardiac function of the MI heart. Figures created by BioRender

5.3.1 The Heart Is a Highly Metabolically Active Organ

The heart is metabolically highly active organ where a large number of mitochondria are continuously working to produce energy for cardiac contractility. The heart uses a combination of metabolic substrates: glucose (30–40%), fatty acids (60–70%), and a significantly less amount of ketone bodies (Lopaschuk et al. 1994). Lack of glucose substrate in the DM heart causes a metabolic shift towards fatty acids. Glucose takes less time to metabolize and thus is essential substrate to the heart during exercise, where energy is required in short time. In the DM heart, lack of glucose oxidation causes metabolic remodeling, and mitochondria

are under tremendous stress to generate energy from fatty acids oxidation, which takes comparatively more extended time and more oxygen molecules than glucose oxidation. Mitochondrial stress, damage, and dysfunction are hallmarks of the DM heart (Mishra et al. 2017). Thus, the DM heart is in different metabolic state than the non-DM heart.

5.3.2 DM also Alters the Microenvironment of Myocardium

DM also alters the microenvironment of myocardium. Increased inflammation, oxidative stress, and cell death signals render a toxic microenvironment to resident cells in the heart (Mishra et al. 2017). The microenvironment is crucial for stem-cell survival and differentiation. In the hyperglycemic microenvironment of the DM heart, myocardial regeneration by transplanted stem cells has several issues with survival and differentiation into cardiac lineages (Yadav et al. 2020; Yadav and Mishra 2021).

5.3.3 Diabetes and Heart Function Control

The function of the heart is controlled by neurons in the brain centers that constitute the central mechanism of regulation of heart function (Dampney 2016). DM is a systemic problem and the control via central mechanisms differ from the non-DM heart. Impaired neuronal function due to neuropathy in the DM heart alters several molecular signaling, including signaling to decrease contractility of cardiomyocytes (Malone 2016). Thus, the DM heart differs from the non-DM heart at metabolic, microenvironmental, and functional levels.

5.4 Challenges and Potential Therapeutic Strategies for Cardiac Regeneration in the Diabetic MI Heart

Although cardiac regenerative therapy has its own limitations for myocardial tissue regeneration in the human MI heart, DM adds further limitations. The potential impediments are associated with the unique physiological status of the DM heart as described above. To improve regenerative therapy in diabetic MI heart, it is critical to supplement the stem cells with other factors that help to adapt to the DM microenvironment. These factors should improve survival, migration, and differentiation of stem cells and maintain the metabolic and functional status of cardiac lineages similar to that of the DM heart. The major challenges and potential remedies for myocardial tissue regeneration in the DM MI heart are described below.

5.4.1 The Metabolic Status of the Transplanted Cells

The metabolic status of the transplanted cells derived from healthy individuals differs from myocardial cells of the DM heart. Thus, the transplanted cells do not maintain their normal survival and differentiation process, which depends on metabolism, in the DM MI heart. It is found that a healthy heart becomes pathological when transplanted into a DM patient (Marfella et al. 2020). Thus, it is likely that normal stem cells will change their survival and differentiation potential in the DM MI heart. A potential remedy is to culture the stem cells in a hyperglycemic medium before transplanting them into the DM MI heart. This prior exposure to hyperglycemic environment will allow these cells to adapt to the microenvironment of the DM MI heart, which will in turn help them to adapt to the physiological conditions of the DM MI heart.

5.4.2 The Toxic Microenvironment in the Diabetic Heart and Cell Survival

The toxic microenvironment is a significant problem for the survival and differentiation of the transplanted stem cells in the DM MI heart. This microenvironment increases mortality and impairs the differentiation of transplanted cells into cardiomyocyte lineage. It may also impair the migration of the transplanted cells into the border zone area of the MI heart, which is important for the stem cells that are delivered through blood circulation.

To support the survival of stem cells after transplantation into the DM MI heart, supplementing paracrine factors that will enhance the survival and differentiation of stem cells could be a potential strategy. For example, we found that matrix metalloproteinase-9 (MMP9) is upregulated in human cardiac stem cells (hCSCs) in hyperglycemic environment. Notably, inhibition of MMP9 improves survival of hCSCs by suppressing cell death via apoptosis and pyroptosis (Yadav et al. 2020). Moreover, MMP9 inhibition modulates epigenetic modifications and autophagy to regulate the differentiation of hCSCs (Yadav and Mishra 2021). Thus, targeting MMP9 in the stem cells or providing an inhibitor of MMP9 with stem cells during transplantation could be a strategy to improve the survival and differentiation of stem cells.

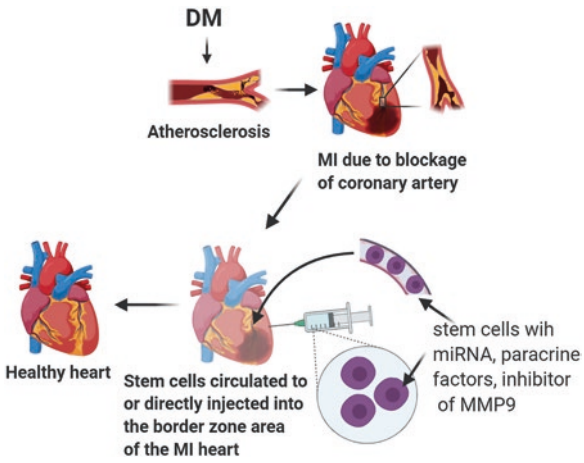

Fig. 5.2 Potential regenerative approaches for cardiac repair in the diabetic myocardial infarction (MI) heart. Diabetes mellitus (DM) promotes atherosclerosis to increase the risk of MI. The DM heart is metabolically and structurally different from the non-DM heart and its microenvironment is more toxic than the non-DM heart. Thus, factors that increase survival and differentiation of stem cells and improve myocardial performance should be supplemented with stem cells to increase the efficiency of cardiac regeneration in the DM MI heart. Based on our previous studies and published reports, we proposed that addition of miR-133a mimic and/or matrix metalloproteinase-9 (MMP9) inhibitor could improve cardiac regeneration and function in the DM MI heart. However, these approaches are yet to be tested and validated in the preclinical models. Figures created by BioRender

Similarly, transcription factors and miRNAs involved in cardiogenesis are potential factors that can be added to stem cells for enhancing cardiac regeneration in the DM MI heart (Takaya et al. 2009; Wilson et al. 2010; Srivastava and Heidersbach 2013; Witman et al. 2020).

5.4.3 DM Reduces Contractility of Cardiomyocytes

DM impairs the contractility of cardiomyocytes. Thus, the contractility of transplanted cells-derived cardiomyocytes could not align in beating with the resident cardiomyocytes. Synchronizing the beating of newly differentiated cardiomyocytes with the resident cardiomyocytes poses a major challenge in regenerative therapy. The contractility of the heart depends on neuronal control via central mechanisms. DM causes neuropathy and induces oxidative stress in neurons of the brain centers that regulate the contractility of cardiomyocytes. Thus, DM poses a double challenge in cardiac regeneration at the level of neuronal control and cardiomyocytes contractility.

A potential approach to circumvent this issue is to add miR-133a mimic to the transplanted stem cells. We found that miR-133a overexpression has protective effects on cardiac neurons where it improves norepinephrine release at the cardio-neuronal junctions that controls beta-adrenergic receptors function and improves the contractility in the DM heart (Nandi et al. 2016). Moreover, miR-133a is anti-hypertrophic and anti-fibrotic (Care et al. 2007; Matkovich et al. 2010). Thus, it will alter the microenvironment of the DM heart to improve the survival and differentiation of the transplanted stem cells. In the DM heart, miR-133a overexpression also mitigates cardiac hypertrophy, fibrosis, and dysfunction, which will further improve myocardial function in the DM MI heart (Nandi et al. 2016, 2018).

Supplementation of other paracrine factors, such as antioxidants, that restores the microenvironment, alleviates neuronal damage, and induces stem-cell survival and differentiation, would improve cardiac regeneration in the DM MI heart (Fig. 5.2).

5.5 Future Directions

5.5.1 Limitations of Current Approaches

Despite progress in the medical science, heart disease remains a leading cause of death (Mc Namara et al. 2019). Although several medications are available to regulate the contractility of the heart, no drug treatment can produce new myocardial cells in the MI heart (Dorn and Molkentin 2004; Cahill et al. 2017). The only option to get a new myocardial tissue in the MI heart is through regenerative therapy. Thus, regenerative therapy has high significance for developing a treatment option for the MI heart.

Empirical evidence demonstrates the efficacy of different regenerative approaches, which we have discussed, in preclinical studies. Based on encouraging results, several of these approaches and a variety of stem cells have been used in clinical trials (Muller et al. 2018; Rajabzadeh et al. 2019;

Rikhtegar et al. 2019; Yu et al. 2017; Mazzola and Di Pasquale 2020). However, due to various limitations, none of them has been translated into a clinical set up to treat a failing human heart (Liew et al. 2020). Thus, there is no regenerative therapy available in clinics to repair human myocardium following MI.

5.5.2 Pitfalls and Alternatives of Current Regenerative Approaches

Several reasons are posited for the failure of stem-cell therapy: (1) The type of stem cell: it is assumed that cardiac progenitor cells (CPCs) could be a better option for myocardial regeneration due to its cardiac origin. However, the lack of a large population of resident cardiac stem cells is a limitation (He et al. 2020; Garbern and Lee 2013). An alternative is differentiating nonmyocardial cells, such as embryonic stem cells (ESCs), into cardiac progenitor cells (CPCs) for cardiac regeneration (Liu et al. 2018). It is germane to mention here that direct transplantation of ESCs or iPSC may lead to teratoma (formation of undesired tissue) in the heart (Hubscher et al. 2017; Riegler et al. 2016). Moreover, the benefit of cardiac stem cells (CSCs) therapy is due to immune response and not due to differentiation of CSCs into cardiac lineages (Vagnozzi et al. 2020; Li et al. 2019). (2) Heterogeneity of stem-cell population: if CPCs are at different stages of maturation during transplantation, it is plausible that they could not differentiate into cardiomyocytes at the same time in a particular chamber of the heart (Kane and Terracciano 2017). Thus, the rate of contractility of differentiated cardiomyocytes would vary, which may lead to arrhythmia (Almeida et al. 2015; Nakanishi et al. 2019). (3) Endocrine versus paracrine effects: growing evidence suggests that paracrine factors have significant roles in cardiac regeneration (Konstandin et al. 2013; Sid-Otmane et al. 2020). Several experiments were conducted where Y-chromosome was probed to determine whether the transplanted stem cells, per se, contribute significantly to the formation of new myocardium (Laflamme et al. 2002; Kikuchi et al. 2010; Hsieh et al. 2007). To determine if extra-cardiac stem cells contribute to cardiomyocytes formation, human female allograft hearts (Y-chromosome absent) were transplanted into male patients (Y-chromosome present) and were evaluated. The transplanted hearts have revealed detectable Y-chromosome-positive cardiomyocytes, suggesting the potential of extra-cardiac cells to generate new cardiomyocytes. However, the percentage of Y-chromosome-positive cardiomyocytes was very small (0.04%), whereas host-derived cardiomyocytes (Y-chromosome negative) were high (29%), suggesting that extra-cardiac cells have less contribution to myocardial regeneration (Laflamme et al. 2002). These findings lead to developing a new idea that paracrine factors released from the stem cells could have a major role in cardiac regeneration and cardiomyocytes turnover. To determine if the heart has the inherent regenerative capacity, a new mouse strain was created with cardiomyocytes-specific transgenic GFP (green fluorescent protein) expression. In the time course of 1 year, the GFP expression was examined in the heart. There was no change in the number of GFP-positive cardiomyocytes, suggesting that new cardiomyocytes are not formed during normal aging (Hsieh et al. 2007).

These findings stimulated new thoughts and ideas. Innovative approaches have been developed, such as transdifferentiation of resident fibroblasts into cardiomyocytes using some factors and miRNAs, and using stem-cell-derived scaffold to regenerate new myocardium (Nam et al. 2013; Jayawardena et al. 2012; Guo et al. 2020). Although these approaches are promising, they warrant further investigations and validations.

5.5.3 New Aspects to Consider Improving Regenerative Therapy

It is important to note that the above-mentioned potential approaches require standardization. It is assumed that a combination of cells and paracrine factors could be a better approach for cardiac regeneration. However, these strategies require further investigation. Few new aspects for regenerative therapy are discussed below.

5.5.3.1 The Timing of Treatment

The early-stage and late-stage MI have different microenvironments, including the status of inflammation, extracellular matrix deposition, and overall cardiac remodeling (Zimmer et al. 2019). Thus, the strategy for regenerative therapy needs adjustment according to the timing of treatment (Jugdutt 1993). The paracrine factors, including miRNAs, alter at the early- and late-stage of MI (Chistiakov et al. 2016; Zhai et al. 2020). Thus, approaches to determine the paracrine factors at different MI stages are crucial for cardiac repair (Sebastiao et al. 2020).

5.5.3.2 DM Causes Several Comorbidities

DM causes several comorbidities influencing cardiac regeneration (Hanefeld et al. 2020; Cai and Keller 2014). The drug treatment to control the comorbidity and glycemic state of DM patients are critical when deciding the paracrine factors for cardiac regeneration in the DM MI heart (Smani et al. 2019). These paracrine factors may have adverse off-target effects. Thus, preclinical studies focusing on these aspects need further investigation in the DM MI heart.

Preclinical to clinical trial transition: we have discussed potential paracrine factors and miRNAs to improve cardiac

regeneration in the DM MI heart. The choice of delivery approach for these factors is also important. Different drug delivery approaches are available and they need further investigation depending on the type of cells/paracrine factors/combination factors, pathological condition of the DM, and the stage of MI (Hastings et al. 2015). These promising targets warrant preclinical studies in rodents and large animals before testing them into clinical trials.

5.5.3.3 Key Aspects For Regenerating Diabetic Myocardium

The DM MI myocardium has different microenvironments than the non-DM MI heart. Accordingly, regenerative approaches need adjustment. Few approaches specifically targeted to the DM MI heart are described below:

(A) Targeting key paracrine factors that are involved in DM-induced adverse cardiac remodeling, such as metabolism, mitochondrial damage, and dysfunction (Gollmer et al. 2020; Mishra et al. 2017). Factors that improve myocardial energy resources such as ketone bodies and inhibit mitochondrial damage and dysfunction such as improving mitophagy - degrades damaged mitochondria and increases mitochondrial quality control -are potential avenues to target for improving the microenvironment for regenerative therapy in the DM MI heart (Dabek et al. 2020; Laffel 1999; Tong et al. 2019; Morales et al. 2020).

(B) Synergistic approach where factors involved in transdifferentiation, improving microenvironment, fine-tuning the regulatory network, and stem-cell survival and differentiation would be another potential avenue (Mishra et al. 2010, 2013; Tyagi et al. 2011). Investigating a combination of miRNA such as miR-133a and paracrine factors that are highly efficient for cardiac regeneration in the DM MI heart is another potential avenue. These combinations suppress apoptosis, mitigate fibrosis, and improve transdifferentiation and stem-cell differentiation (Izarra et al. 2017; Matkovich et al. 2010; Nandi et al. 2016; Nam et al. 2013; Yang et al. 2020).

In summary, a new regenerative approach that target specifically to the diabetic myocardium is necessary for myocardial tissue repair in the DM MI heart. The currently used regenerative approaches that are used in the non-DM MI heart may not be applicable to cardiac repair in the DM MI heart due to different pathophysiological conditions and microenvironment.

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Macrophage Response to Biomaterials in Cardiovascular Applications

6

Sushmita Roy, Eric G. Schmuck, and Amish N. Raval

Abbreviations

BMDM Bone-marrow-derived macrophages
CCL7 C-C Motif Chemokine Ligand 7
CCL-8 C-C Motif Chemokine Ligand 8
CABG Coronary artery bypass grafting
DAMPs Damage-associated molecular patterns
2DE Two-dimensional electrophoresis
dECM Decellularized extracellular matrix
DHT Dehydrothermal
EDAC 1-(3-(Dimethylamino) propyl)-3-ethylcarbodimide hydrochloride
ECM Extracellular matrix
ePTFE Expanded polytetrafluoroethylene
HF Heart failure
HSCs Hematopoietic stem cells
IL-1β Interleukin 1 beta
LV Left ventricle
LVAD Left ventricular assist device
MALDI-ToF Matrix-assisted laser desorption ionization–time of flight
MMP Matrix metalloproteinases
PAAm Polyacrylamide
PAANa poly(acrylic acid)
PCNU Polycarbonate urethane
PEG Polyethylene glycol
PET Polyethylene terephthalate
PLA Poly(lactic acid)
PU Polyurethane
PU NPs Polyurethane nanoparticles
TNFα Tumor necrosis factor alpha
SIS Small intestinal submucosa
TGF-β1 Transforming growth factor-beta1
THP-1 Transformed human mononuclear cell line
UBM Urinary bladder matrix
VEGF Vascular endothelial growth factor

S. Roy · E. G. Schmuck · A. N. Raval (✉)
University of Wisconsin School of Medicine and Public Health, Madison, WI, USA
e-mail: anr@medicine.wisc.edu

6.1 Introduction

Macrophages and their circulating parent cell, monocytes, are a population of immune cells that play a key role in tissue repair, regeneration, and fibrosis. Tissue repair is a critical biological process for preserving tissue integrity, organ function, and survival in all organisms and is initiated with rapid recruitment, proliferation, and activation of cells from hematopoietic and non-hematopoietic compartments. The outcome of repair can range from cellular regeneration, where tissue renewal facilitates functional recovery, to replacement of damaged tissue with fibrosis, which is an early pro-survival adaptive response that may lead to later loss of function (Bouchery and Harris 2019).

Macrophages reside in all tissues from early development to phagocytize necrotic debris and senescent cells, while also supporting growth and tissue repair. Macrophages and their precursor cells, monocytes, respond to stimuli from the local microenvironment and differentiate into unique phenotypes via a process known as macrophage polarization. Based on observations in mice, macrophages are classically divided into two general populations: pro-inflammatory (M1) and anti-inflammatory/pro-resolving (M2). M1 macrophages secret pro-inflammatory cytokines, activate endothelial cells, and recruit immune cells into the inflamed tissue (Pace et al. 1983). Conversely, M2 macrophages resolve inflammation. These immune cells promote phagocytosis of apoptotic cells, increase collagen deposition, and coordinate tissue integrity while releasing anti-inflammatory mediators (Viola et al. 2019). While this M1/M2 categorization is convenient for research models, in humans, macrophages express several heterogeneous phenotypes with subpopulations that may have cardio-reparative properties (Mariani et al. 2019). This chapter provides an overview of biomaterials with known macrophage interactions in the heart.

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6.2 Role of Monocyte/Macrophages in Cardiac Repair

Early studies discovered that macrophages differentiated from circulating blood monocytes that originated from marrow-derived hematopoietic stem cells (Van Furth and Cohn 1968). The M1 and M2 classification, derived from mouse models, describe two subsets of circulating monocytes: Ly6C^{hi} monocytes and Ly6C^{low} monocytes. Ly6C^{hi} are CD11b^{+}CD115^{+}CCR2^{high}CX3CR1^{low} and Ly6C^{low} are CD11b^{+}CD115^{+}CCR2^{low}CX3CR1^{high} (Si et al. 2010). Experiments using global transcriptional profiling suggest that the human equivalent to mouse Ly6C^{hi} monocytes is CD14^{+}CD16^{-} monocytes, while the human equivalent to mouse Ly6C^{low} monocytes is CD14^{int}CD16^{+} monocytes, although these cells may have different roles in vivo (Ingersoll et al. 2010).

In recent years, a population of resident-tissue macrophages established during embryonic development and maintained by self-renewal has been described (Ginhoux et al. 2010; Schulz et al. 2012; Guilliams et al. 2013; Jakubzick et al. 2013; Epelman et al. 2014). Resident macrophages maintain homeostasis and tissue repair following injury independent of monocyte-derived macrophage recruitment (Stahl et al. 2018).

Following myocardial injury, inflammation and resolution are achieved through differential recruitment of macrophages. Following myocardial infarction (MI), for example, resident cardiac macrophages are rapidly depleted and replaced by infiltrating monocyte-derived macrophages (Heidt et al. 2014). In mice, infiltrating macrophages initially exhibit a pro-inflammatory M1-like phenotype at 1–3 days post-MI to drive acute inflammation and clear necrotic cells. By 5–7 days post-MI, these macrophages adopt an M2-like phenotype to initiate the resolution of the inflammation and restore cardiac tissue (Di Franco et al. 2018; Ma et al. 2018). Therefore, a time-dependent switch from M1 to M2 macrophages is necessary to clear necrotic cells and promote tissue repair (O'Rourke et al. 2019). These events are summarized in Fig. 6.1.

6.3 Biomaterials–Monocyte Interaction and Monocyte/Macrophage Differentiation

Cellular and extracellular matrix (ECM) interactions are closely regulated by integrin adhesion receptors (Lee et al. 2015). Integrins are transmembrane receptors that bind their globular head domain with ECM, which provides anchorage, and trigger signals that direct cell survival, migration, cell-cycle progression, and expression of differentiated cell phenotypes (Fig. 6.2).

Integrins have a central role in the development, organization, maintenance, and repair of various tissues. It has been previously shown that integrin interaction with biomaterials governs macrophage polarization and α2β1 integrin blocks the induction of M2 macrophages (Cha et al. 2017). Differentiation of monocytes to macrophages is influenced by the cell adherence properties of the biomaterial, the biomaterial stiffness, the absence of cell proliferation, and the expression of certain macrophage-specific surface markers (Ramprasad et al. 1996; Sandor et al. 2016).

For example, macrophage differentiation was previously shown to depend on the chemical crosslinking of the biomaterial. To modulate scaffold stiffness, Sridharan et al. used collagen scaffolds cross-linked with two chemical cross-linking agents; ethyldimethylaminopropyl carbodiimide (EDAC) and genipin, to independently modulate the stiffness of scaffolds (Sridharan et al. 2019). The investigators found that genipin cross-linking suppressed both pro-inflammatory and anti-inflammatory responses from macrophages, while EDAC cross-linking increased pro-inflammatory M1 macrophage phenotype and anti-inflammatory M2 phenotype (Sridharan et al. 2019).

Bai et al. showed that macrophage adhesion is supported by CCN1, which is an angiogenic matricellular protein that is expressed at sites of inflammation and wound repair, via integrin $\alpha_M\beta_2$ and syndecan-4 as coreceptors (Bai et al. 2010). CCN1 activates NFκB-mediated transcription and induces pro-inflammatory M1 macrophages to participate in Th1 responses (Lau 2011). Matrix fibril orientation and density also play a role in macrophage polarization. According to Sapudom et al., THP-1 monocytes cultured on three-dimensional (3D) collagen matrices with varying fibril densities differentiated into uncommitted macrophages. Interestingly, the secretion of cytokines was enhanced with increased fibril density (Sapudom et al. 2020). In summary, macrophages respond to both the chemical and physical properties of scaffolds to promote favorable remodeling outcomes after biomaterial implantation (Sridharan et al. 2019).

Herein, we will discuss the different biomaterials that have been explored in cardiac applications and the known macrophage response to those tissues and relevant outcomes.

6.4 Biomaterials in Cardiac Repair and Regeneration

Biocompatibility, biodegradation, and inflammatory (or anti-inflammatory) properties must be considered when using biomaterials for cardiac repair. When combined with therapeutic cells, biomaterials should facilitate cell engraftment

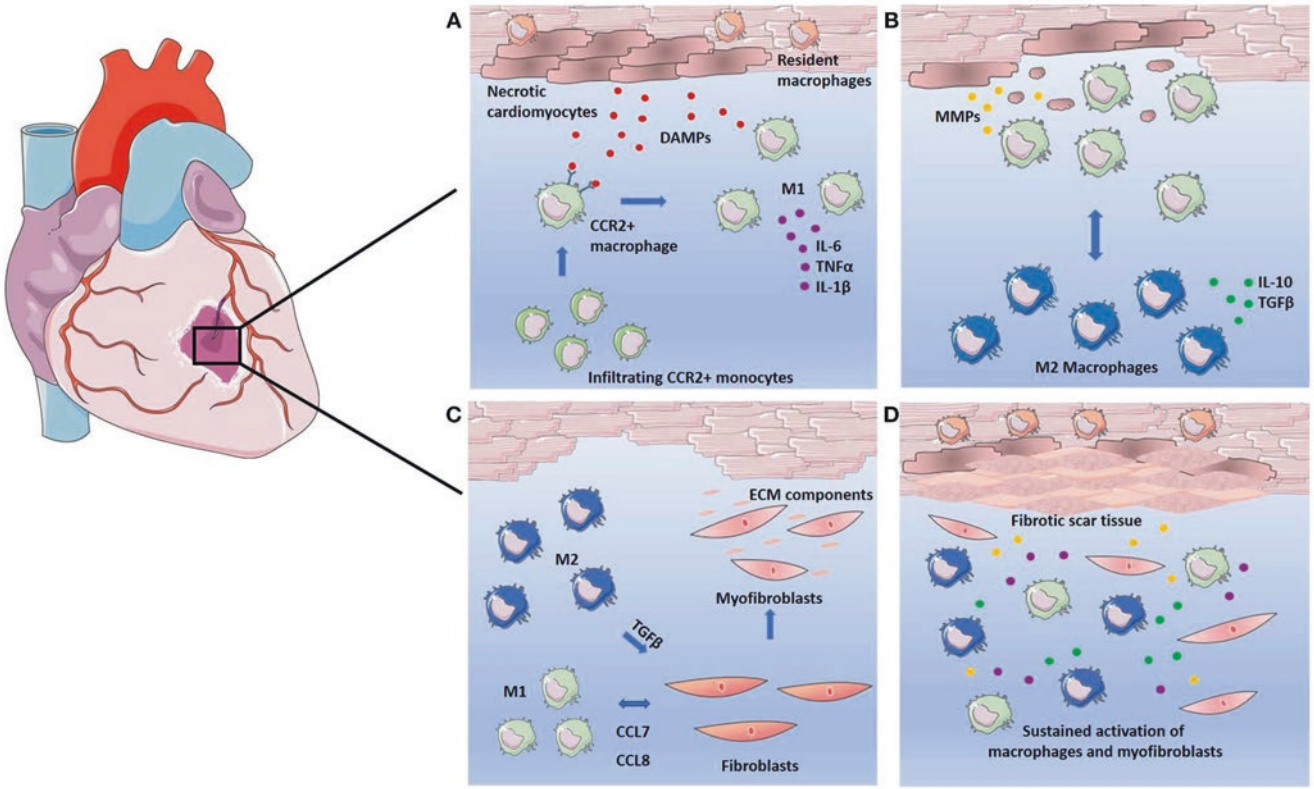

Fig. 6.1 Macrophages in response to infarction. (**a**) Cardiomyocytes undergo necrosis, release damage-associated molecular patterns (DAMPs), and attract chemokine receptor CCR2$^+$-circulating monocytes. CCR2$^+$ monocytes differentiate into pro-inflammatory M1 macrophages that replace resident macrophages and secrete pro-inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and Interleukin 1 beta (IL-1β). (**b**) M1 macrophages clear necrotic cell debris through phagocytosis and induce the breakdown of extracellular matrix (ECM) via secretion of matrix metalloproteinase (MMP). M2 macrophages secrete the anti-inflammatory cytokine IL-10 and growth factor TGFβ. (**c**) Both M1 and M2 macrophages facilitate a fibrotic response. M1 macrophages recruit cardiac fibroblasts via C-C Motif Chemokine Ligand 7 (CCL7) and C-C Motif Chemokine Ligand 8 (CCL8)-mediated signaling. M2 macrophages induce fibroblast differentiation into myofibroblasts, which in turn secrete extracellular matrix (ECM) components to facilitate tissue repair. (**d**) Sustained activation of macrophages leads to the continuous secretion of growth factors, pro-inflammatory cytokines, and matrix metalloproteinases (MMP). Continued breakdown of ECM, as well as overproduction of ECM components, leads to adverse remodeling and fibrosis (Adapted from O'Rourke et al., Front Cardiovasc. Med. 2019)

and integration with host tissue (Pascual-Gil et al. 2015; Zammaretti and Jaconi 2004). Both synthetic and natural biomaterials are discussed as candidates for cardiac repair (Zammaretti and Jaconi 2004). Figure 6.3 shows various biomaterials categorized as synthetic or natural for cardiac repair.

6.4.1 Synthetic Biomaterials

Synthetic biomaterials are polymers, metals, or a combination of both. They have excellent strength and durability; however, biocompatibility and toxicity can be an issue. Synthetic biomaterials are typically used for controlled drug delivery, whereas natural biomaterials are often used to assist with cell/tissue engraftment and integration. Synthetic biomaterials can be further classified as degradable and nondegradable polymers.

6.4.1.1 Degradable Polymer

Poly(Lactic Acid) (PLA) Nanofiber

These are synthetic biomaterials (pore size 12–30 μm) generated by freeze-drying and electro-spinning. Madden et al. showed a shift in transplanted macrophage phenotype to an M2 state with increased angiogenesis, reduced fibrosis, and favorable ventricular remodeling up to 28 days posttreatment in a rat MI model (Madden et al. 2010). This suggests that macrophages polarized by PLA exhibit an anti-inflammatory and cardio-reparative response.

Polyurethane Patches

This bi-layered polyurethane (PU) biomaterial is manufactured using an electrospinning process (D'Amore et al. 2016). In a rat cardiac ischemia model, this degradable patch increased LV anterior wall thickness and prevented adverse ischemic ventricular wall remodeling. This biomaterial facil-

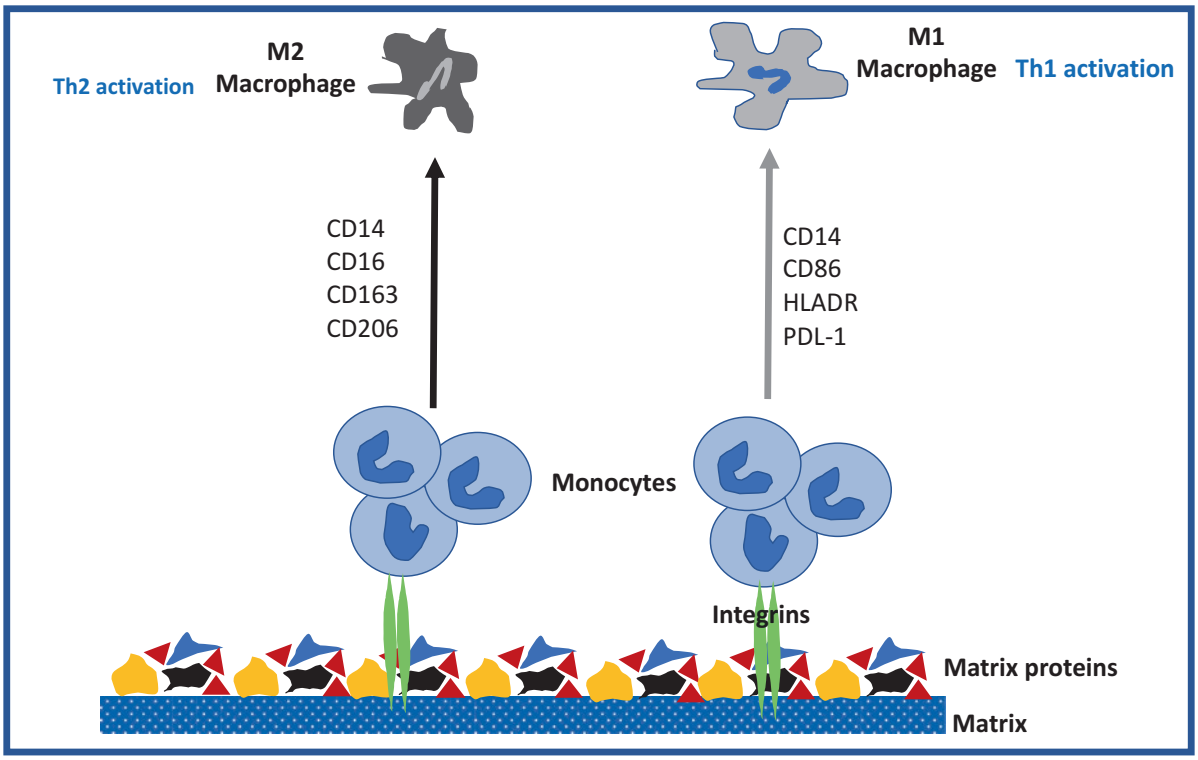

Fig. 6.2 Macrophage and biomaterial interaction via integrin

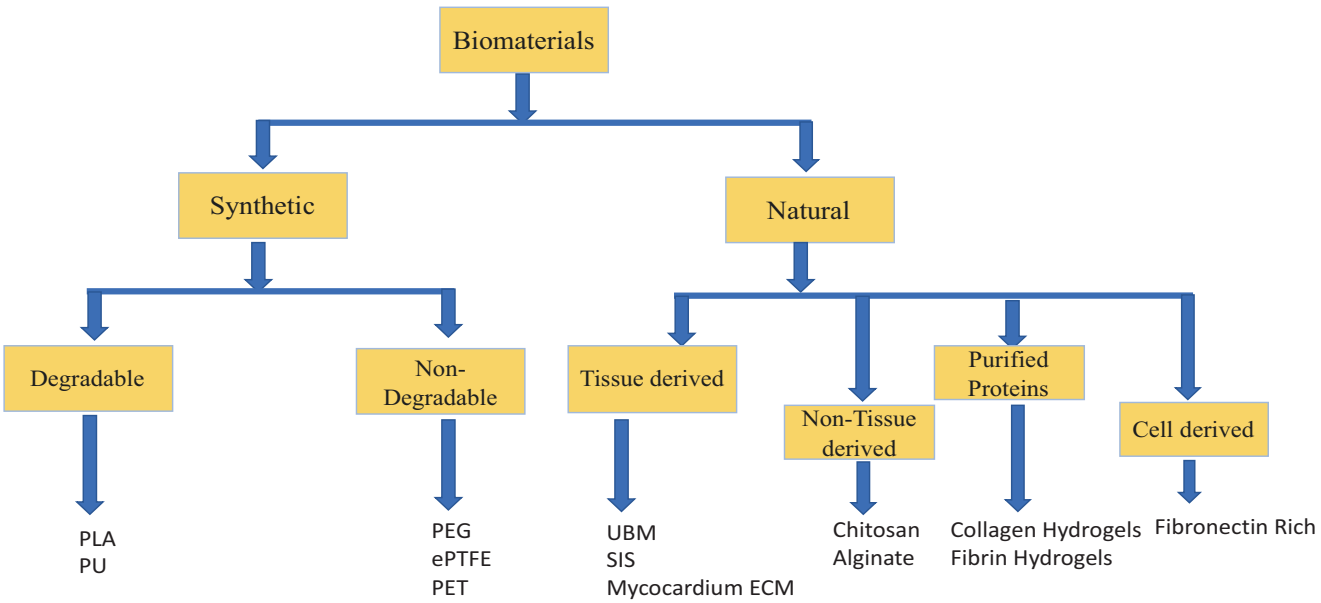

Fig. 6.3 Flow chart showing candidate biomaterials for cardiac repair and their derivation. *PLA* poly(lactic acid), *PU* polyurethane, *PEG* polyethylene glycol, *ePTFE* expanded polytetrafluoroethylene, *PET* polyethylene terephthalate, *UBM* urinary bladder matrix, *SIS* small intestinal submucosa, *myocardium ECM* myocardial extracellular matrix

itated endogenous tissue growth within the patch, increased cell recruitment, decreased inflammation, and stimulated the formation of new capillary beds that perfused networks inside and underneath the patch regions (Di Franco et al. 2018). Further, polyurethane (PU) nanoparticles decreased secretion of pro-inflammatory cytokines (TNF-α and IL-1β) from M1 macrophages, suggesting that these biomaterials inhibit macrophage polarization toward M1 expression but not M2 expression. On the other hand, M2 macrophages cultured on PU films showed less expression of IL-10 than those

on tissue culture polystyrene (Huang et al. 2018). The PU patch or polycarbonate-urethane (PCNU) patch is shown to influence macrophage function, cell size, changes in the actin cytoskeleton, and multinucleation. Dinnes et al. observed differences in macrophage protein expression influenced by PCNU by two-dimensional electrophoresis combined with matrix-assisted laser desorption ionization–time of flight (MALDI-ToF) mass spectrometry. In this study, macrophages responded to PU by modulation of structural proteins (i.e., actin, vimentin, and tubulin) suggesting that a proteomics approach could be used to detect protein expression profile changes in macrophages cultured on different material surfaces (Dinnes et al. 2007; Shrestha et al. 2020).

6.4.1.2 Non-Degradable Polymer

Polyethylene Glycol (PEG)

Macrophages cultured on polyethylene glycol (PEG) results in low TNF-α, IL-1β, IL-6, and IL-8 expression. Recently, an injectable hydrogel consisting of polyethylene glycol and fibrinogen loaded with vascular endothelial growth factor (VEGF) was administered by intramyocardial injection. This composite resulted in increased arteriogenesis and improved cardiac function in a subacute rat MI model, although the direct influence of this therapy on macrophages was not investigated (Rufaihah et al. 2013). In other work, PEG did not appear to influence macrophage polarization (Bartneck et al. 2014; Su et al. 2015; Reichel et al. 2019).

Clinically, Coseal (Baxter Healthcare, Hayward, CA) is a commercially available sprayable polymeric matrix composed of two synthetic PEG polymers that form a strong hydrogel that adheres to tissue (Konertz et al. 2003; Zhibo and Miaobo 2009). This biomaterial has been used as a sealant to control bleeding during cardiac surgery, such as heart transplantation in left ventricular assist device patients (Cannata et al. 2009). Currently, it is unclear if or how Coseal affects macrophage expression.

Expanded Polytetrafluoroethylene

Macrophages cultured on polytetrafluoroethylene (ePTFE) produced more IL-1α, IL-1β, IL-6, and TNF-α pro-inflammatory cytokines and MCP-1, MIP1-β, and MCP-3 (chemokines), and these effects were linked to increasing pore size of the material (Bhardwaj et al. 2001; Schachtrupp et al. 2003). Commercially, ePTFE is a three-layer polymer with a middle microporous, elastic layer surrounded by two layers of polymer fibrils (Aumsuwan et al. 2011). ePTFE has a high resistance to allergic reaction and inflammation (Verbelen et al. 2010), and therefore, it is an excellent option for shunts (Doble et al. 2008), tissue reconstruction (Miyazaki et al. 2011), and valve repair (Ando and Takahashi 2009). Bota et al. showed that macrophages cultured on ePTFE increased their expression of IL-1β, interleukin 6, TNF-α, monocyte chemotactic protein-1, and macrophage inflammatory protein 1-β after 4–24 h (Bota et al. 2010). This suggests that macrophages polarized by ePTFE may lead to a greater inflammatory response.

Polyethylene Terephthalate

Polyethylene terephthalate (PET) is a thermoplastic polymer used for constructing vascular grafts heart valves and sutures. They are also available with collagen or albumin coating, to prevent graft infection (Kudo et al. 2002). Macrophages cultured on PET produce pro-inflammatory cytokines MCP-3, TNF-α, IL-6, IL-1β, MIP-1α (Brodbeck et al. 2002; Jones et al. 2007; Grotenhuis et al. 2013), and the pro-inflammatory chemokine IL-8 (Jones et al. 2007). Macrophages cultured on PET + poly(benzyl *N*,*N*-diethyldithiocarbamate-*co*-styrene) (BDEDTC) + polyacrylamide and PET + BDEDTC + sodium salt of poly(acrylic acid) surfaces have higher IL-10 (anti-inflammatory cytokine) production and lower IL-8 (inflammatory cytokine) production compared to macrophages cultured on PET matrix cultured for 3–10 days (Brodbeck et al. 2002; Jones et al. 2007). This suggests that macrophages polarized by PET may exhibit either inflammatory or anti-inflammatory responses depending on pore-size and specific biomaterial components.

6.4.2 Natural Biomaterials

Natural biomaterials are derived from native tissues from autologous (same individual), allogeneic (same-species donor), or xenogeneic (animal) sources (Lam and Wu 2012). Examples of common natural biomaterials developed for research purposes and/or clinical care are discussed below.

6.4.2.1 Tissue-Derived (Natural) Biomaterials

Decellularization is performed through chemical, physical, or combination methods to remove cells (and genetic material) while maintaining structural proteins. Removing cells from adult heart tissue, urinary bladder, small intestinal submucosa, etc. leads to a decellularized extracellular matrix (dECM). dECM can also be manufactured from cultured cardiac fibroblasts. "Soluble" dECM follow an additional step to break down the ECM structure into a liquid form. After decellularization, cardiac dECM provides a complex combination of biochemical and mechanical cues that favor cell attachment, proliferation, and cardiovascular differentiation (Gilbert et al. 2006; Iop et al. 2017). dECM can have additional chemical cross-links to alter the degradation speed (Almeida et al. 2014). Examples of dECM manufactured for investigational or clinical use are described below.

Urinary Bladder Matrix-Derived Biomaterial

Commercially available, decellularized urinary bladder matrix (UBM) has been used in several clinical applications including cardiac repair (Kochupura et al. 2005; Wainwright et al. 2012; Remlinger et al. 2013). UBM is most frequently manufactured from porcine bladders but has also been manufactured from human bladders. Decellularized UBM is composed of 98% collagen(s), 1% ECM glycoproteins, and 1% proteoglycans (Sadtler et al. 2017; Wainwright et al. 2012). Badylak et al. showed that decellularized urinary bladder matrix allografts promote anti-inflammatory macrophage polarization, as measured by a lower C-C chemokine receptor type 2:CD163 (CCR7:CD163) ratio, and a reduced fibrotic response to abdominal wall implants in rats, compared to cellular autografts (Badylak et. al., 2008). They also observed that the decellularization process is a major factor in the immune response (Brown et al. 2009; Sicari et al. 2014). UBM directs macrophage toward an M2-like, anti-inflammatory phenotype. The solubilized urinary bladder biomaterial induced macrophages to secrete PGE2, a potent modulator of the immune response that suppresses classic inflammatory factors such as TNFα, NO, and phagocytosis. It has been observed that hyaluronic acid, contained in UBM, has an important role in mediating the M2-like phenotype.

Small Intestinal Submucosa-Derived Biomaterial

Small intestinal submucosa (SIS) is a popular device that is used in cardiovascular applications. It is derived from the submucosa of the small intestine, and most commonly from porcine sources. SIS was first used as a large vascular autograft in a dog in 1989 (Badylak et al. 1989). SIS has been used widely in patients for a variety of reconstructions at various sites including the skin (MacLeod et al. 2004), urinary tract (Alpert et al. 2005), and intestine (Sardeli et al. 2005). SIS is decellularized into a four-ply sheet, which contains structural proteins like collagens, adhesion molecules, and matricellular proteins to promote "constructive" remodeling. Small intestinal submucosa extracellular matrix (SIS-ECM) is mostly composed of collagen (predominantly collagen type I), with minor amounts of collagen type III, IV, V, and VI, elastin, fibronectin, and laminin (Badylak et al. 2009). Additionally, it has a collagen fiber configuration, which is highly suited for cardiovascular tissue engineering that requires strength and stiffness (Badylak 2007). Further, Huleihel et al. also showed that macrophages from two different sources, murine bone marrow-derived macrophages (BMDM) and a transformed human mononuclear cell line (THP-1 cells) cultured on urinary bladder matrix (UBM-ECM) or small intestinal submucosa (SIS-ECM) responded differently to the same source of macrophages cultured on them (Huleihel et al. 2017). Therefore, the protein composition of the biomaterial plays a key role in influencing macrophage function and phenotype.

Myocardium ECM-Derived Biomaterial

Injectable hydrogel derived from the porcine myocardial ECM has been tested as a biomaterial for cardiac repair post-MI (Singelyn et al. 2009). Since biomaterial components are tissue-specific, using ECM from noncardiac tissues fails to provide adequate or appropriate signaling to cells in the myocardium (Leor et al. 2005; Singelyn et al. 2009) Characteristically, mammalian myocardial ECM is composed primarily of collagen type I (~80%) with lesser amounts of collagen type III (~10%), collagen type V (<5%), and small amounts of fibronectin, laminin, and elastin. Cardiac ECM also contains glycoproteins, proteoglycans, and glycosaminoglycans. In animal models, this hydrogel has been associated with decreased inflammation, decreased cardiomyocyte apoptosis, increased neovascularization, and reduced fibrosis (Singelyn et al. 2009). Becker et al. developed a novel composite biomaterial by processing human cardiac ECM into a hydrogel and combining it with a cell-free amniotic membrane via a dry-coating procedure. In this process, macrophages secreted less pro-inflammatory cytokines, with no T-cell proliferation and macrophage polarization (Becker et al. 2018).

In an open-label trial, decellularized porcine myocardium ("Ventrigel," Ventrix, San Diego, CA) was delivered by transendocardial catheter injection into 15 post-MI patients. Safety and feasibility of the approach were observed (Traverse et al. 2019), thus setting the stage for future randomized-controlled trials.

6.4.2.2 Nontissue-Derived (Natural) Biomaterials

Chitosan

Chitosan or chitin is a natural linear polymer obtained by chitin deacetylation and has been widely used for tissue replacement including in cardiac applications (Liu et al. 2012; Martins et al. 2014). This natural material has high biocompatibility and biodegradability and can combine with other conductive materials to enable electrical transmission (Ceccaldi et al. 2014; Martins et al. 2014). Chitosan induces an M2-like phenotype with low TNF-α and high IL-10 and TGF-β1 levels cytokines (Oliveira et al. 2012). Macrophages cultured on chitosan for 10 days resulted in significant downregulation of the pro-inflammatory markers, CD86 and MHCII. Further, the production of pro-inflammatory cytokines, such as TNF-α, decreased with time in culture on chitosan, while anti-inflammatory IL-10 and TGF-β1 significantly increased. In contrast, another study showed that chitosan was associated with an M1-like response with high production of TNF-α and low expression of IL-6 (Almeida et al. 2014). On balance, the evidence suggests that chitosan surfaces drive polarization of human macrophages toward an anti-inflammatory phenotype.

Alginate

Alginates are anionic linear polysaccharides, which form hydrogels through ionic crosslinking via divalent cations (Wee and Gombotz 1998; Bidarra et al. 2014). This biomaterial also enables cell and protein retention within the hydrogel ex vivo and then control-release the therapeutic agents when administered in vivo (Wang et al. 2012; Moshaverinia et al. 2013). Alginates delivered into animal models of MI have shown improved cardiac function and increased scar thickness (Landa et al. 2008) without introducing ventricular arrhythmias (Landa et al. 2008; Leor et al. 2009). Delcassian et al. showed that alginates may intrinsically stimulate M2 macrophages to acquire a unique polarization state that is characterized by enhanced expression of CD86 and IL1β, and low expression of IL12 and high IL10 (Delcassian et al. 2019). Another study evaluated the efficacy of alginate hydrogel implants in dogs with heart failure (HF) induced by repetitive coronary microembolization (Sabbah et al. 2013). Four months posttreatment, alginate implantation significantly increased ejection fraction, wall thickness, improved left ventricular sphericity, reduced left ventricular end-diastolic pressure as well as end-diastolic and end-systolic volumes compared to controls. Ludwinski et al. developed a method for placing artery-stimulating macrophages inside alginate capsules measuring 300 μm in diameter. The alginate coating did not effect on the macrophage viability in culture. Intramuscular injection of alginate encapsulated macrophages into a mouse hindlimb ischemia model resulted in improved retention and improved limb perfusion compared to unencapsulated macrophages (Ludwinski et al. 2019).

The AUGMENT-Heart Failure trial investigated the feasibility and safety of alginate-based intervention in a trial enrolling 78 patients with advanced ischemic and nonischemic HF (Wee and Gombotz 1998; Bidarra et al. 2014). Subjects were randomized (1:1) to an injectable calcium alginate hydrogel "Algisyl" (LoneStar Heart, Inc. Laguna Hills, CA) combined with standard medical therapy (SMT) or SMT alone. At 1 year, Algisyl plus SMT improved exercise capacity, symptoms, and clinical HF status compared to SMT alone, although there were no differences observed in left ventricular ejection fraction or chamber volumes (Mann et al. 2016). In a separate trial, Lee et al. measured the effects of Algisyl in combination with coronary artery bypass grafting (CABG) on left ventricular function and wall stress in HF patients. Combined Algisyl+ CABG treatment resulted in reduced myofiber stress, restored LV geometry, and improved function (Lee et al. 2013). IK-5001 (BioLineRx, Jerusalem, Israel), composed of 1% sodium alginate plus and 0.3% calcium gluconate (Leor et al. 2009), was tested in a feasibility trial (n = 27) that delivered this biomaterial by intracoronary infusion into the infarct-related coronary artery of patients with recent MI (Frey et al. 2014). IK-5001 was well tolerated and the left ventricular dimension and function were preserved in these patients. Taken together, alginate biomaterials delivered in HF patients appear safe; however, larger randomized controlled trials are required to confirm its effectiveness.

6.4.2.3 Purified Proteins

Collagen Hydrogels

Collagen has been investigated extensively for cardiac repair applications. Although the results are mixed, in general, it appears that collagen biomaterials are either inert to or polarize macrophages to an M1-like pro-inflammatory phenotype. Wesley et al. demonstrated that collagen type-I matrix activated macrophages (Wesley et al. 1998) into a pro-inflammatory phenotype. Macrophages cultured on native collagen (decellularized bovine pericardium) for 14 days released more matrix metalloproteinase-2 (Ariganello et al. 2011). In this study, decellularized bovine pericardium did not appear to activate pro-inflammatory macrophages (Ariganello et al. 2011). Macrophages cultured on acellular porcine-derived dermis meshes for 7 days showed a high pro-inflammatory response with an increase in IL-1β, IL-6, IL-8, and VEGF expression (Orenstein et al. 2010). Macrophages cultured on collagen coatings expressed mostly M1 surface markers (CD86+) (Fearing and Van Dyke 2014; Kajahn et al. 2012). These macrophages produced increased levels of pro-inflammatory cytokines. In a separate study, collagen sponge did not produce IL-1β and IL-6 production showing that the response of the macrophages was not pro-inflammatory (Bhattacharjee et al. 2013). The effect of a collagen patch as a slow-release reservoir of VEGF-165 was studied by Miyagi et al. This biomaterial supported in vivo vascularization onto the patch, in a right ventricle defect rat model (Miyagi et al. 2011). A collagen patch alone was also shown to preserve infarcted heart contractility, attenuate adverse remodeling, and improve heart function (Serpooshan et al. 2013). Thus, in preclinical models of MI, collagen hydrogels appear to preserve heart contractility and produce increased levels of pro-inflammatory cytokines.

The MAGNUM feasibility and safety trial tested a scaffold of type-I collagen coupled with cells grafted onto infarcted left ventricles. The trial was done on 20 patients with left ventricular postischemic myocardial scars with an indication for coronary artery bypass graft surgery. In the last 10 patients, the collagen matrix was seeded with bone marrow cells, placed onto the scar. This approach was safe, increased the infarct scar thickness, decreased cardiac wall stress, prevented adverse ventricular remodeling, and improved diastolic function (Chachques et al. 2008).

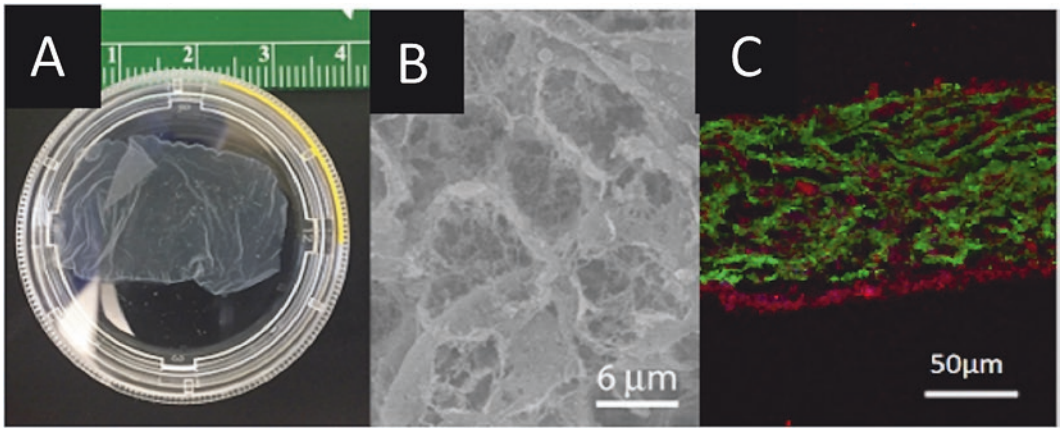

Fig. 6.4 Cardiac fibroblast extracellular matrix (F-ECM)) sheets are produced following isolation and high-density culture of cardiac fibroblasts obtained from healthy hearts of cadaveric donors. A decellularization step is performed that does not require chemical crosslinking. The intact, 140 µm thin matrix sheet (**A**) has a honey-combed appearance on electron microscopy (**B**). Immunofluorescent imaging of a longitudinal section of the matrix shows abundant fibronectin (green) throughout, lesser type-I collagen (red), and no intact cells (DAPI, blue) (C). (Adapted from Schmuck et al., Cardiovasc Eng Technol., 2014)

Fibrin

Fibrin is a fibrous protein involved in blood clotting and is a major component of the ECM formed after tissue injury. This is another natural gel that has been broadly used for cardiac cell encapsulation. Black et al. in 2009 showed that myocardial constructs were created by entrapping neonatal rat cardiac cells in fibrin gel (Black et al. 2009). Aligned cardiomyocytes within 3D fibrin hydrogels maintained their synchronous beating behavior even after 2 months of in vitro culture (Huang et al. 2007). When macrophages were cultured on fibrin biomaterial, there was an enhanced secretion of TNF-α. In contrast, cells cultured on fibrin gels by combining fibrinogen with thrombin secreted much higher levels of IL-10 and lesser amounts of TNF-α (Hsieh et al. 2017). Macrophages maintained their anti-inflammatory behavior when cultured on fibrin gels in the presence of soluble fibrinogen. According to Hseih et al., fibrinogen is a key switch in regulating macrophage phenotype behavior, providing a valuable immunomodulatory strategy for tissue repair (Hsieh et al. 2017).

Fibrin sealant is a common topical hemostatic agent in patients undergoing cardiac surgery (Rousou et al. 1989). Furthermore, the "Transplantation of Human Embryonic Stem Cell-Derived Progenitors in Severe Heart Failure (ESCORT)" trial used fibrin as an intraoperative cell retention agent (Menasche et al. 2018). Six patients (57–81 years) were included in the trial and it demonstrated the safety and feasibility of this approach in patients with ischemic HF.

6.4.2.4 Cell-Derived Matrices

Cell-derived biomaterials, such as cardiac fibroblast-derived ECM, have emerged as a unique biomaterial for cardiac applications. Cardiac fibroblasts are cells of mesenchymal lineage origin that are primarily responsible for the production and maintenance of cardiac ECM. In addition, cardiac fibroblasts also secrete extracellular matrix proteins and are essential for organ development, wound healing, and immunomodulation (Krenning et al. 2010; Furtado et al. 2014; Lajiness and Conway 2014). Under high-density culture conditions, cardiac fibroblasts produce a fibronectin rich ECM sheet in vivo. The sheets were decellularized, using non-chemical crosslinking methods, resulting in a honeycombed matrix that is free of residual cellular debris (Fig. 6.4) (Schmuck et al. 2014). Interestingly, this matrix can be lyophilized for storage, rehydrated as a sheet for later use, or milled into a powder that can be resuspended in the solution for use as an injectable (Fig. 6.4). When monocytes are cultured on them, they educate monocytes to a distinct population of macrophages with anti-inflammatory potential secreting high levels of IL-6 and VEGF. They also recruit mesenchymal stem cells, which are cells with known anti-inflammatory properties (Roy et al. 2020).

6.5 Conclusions

Numerous natural and synthetic biomaterials are being investigated or are in current clinical use for the treatment of patients with HF and MI. Despite a wide assortment of available biomaterials, only a few have been tested in human cardiac disease trials worldwide. Most biomaterials demonstrate some ability to influence macrophage polarization to result in heterogeneous flow marker or cytokine expression in vitro, although a significant gap still exists in our understanding of their functional expression in vivo. Indeed, biomaterials that influence macrophages into performing anti-inflammatory, pro-angiogenic, and pro-regenerative functions could have

enormous therapeutic potential for patients who suffer from cardiovascular disease.

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Evolution of Stem Cells in Cardio-Regenerative Therapy

7

Adegbenro Omotuyi John Fakoya, Iziegbe Fenemigho, Chisom Valentine Asuzu, Ewaenosa Esohe Ukponmwan, Kingsley Chinonyerem Nnawuba, and Khawaja Husnain Haider

Abbreviations

ADSCs	Adipose tissue derived stem cells
AMI	Acute myocardial infarction
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MNCs	Bone marrow derived mononuclear cells
BMP-4	Bone morphogenetic protein 4
CDCs	Cardiosphere derived cells
CMs	Cardiomyocytes
CPCs	Cardiac progenitor cells
CSCs	Cardiac stem cells
CVD	Cardiovascular disease
DMD	Deuchene's muscular dytrophy
ECs	Endothelial cells
EDV	End diastolic volume
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
hESCs	Human ESCs
hiPSCS	Human induced pluripotent stem cells
HSCs	Hematopoietic stem cells
IGF-1	Insulin-like growth factor-1
iPSCs	Induced pluripotent stem cells
LV	Left ventricle
LVEDV	LV end-diastolic volume
LVEF	Left ventricular ejection fraction
LVESV	LV end-systolic volume
LVFS	Left ventricular fractional shortening
MenSCs	Menstrual blood-derived stem cells
mESCs	Mouse ESCs
MI	Myocardial infarction
MSCs	Mesenchymal stem cells
PDGFA	Platelet-derived growth factor alpha
PSCs	Pluripotent stem cells
SDF-1	Stromal cell derived factor
SkMs	Skeletal myoblasts
STW	Systolic wall thickness
TDGF1	Teratocarcinoma-derived growth factor 1
THBS1	Thrombospondin 1
VEGF	Vascular endothelial growth factor

A. O. J. Fakoya (✉)
Department of Anatomical Sciences, University of Medicine and Health Sciences, Basseterre, St. Kitts & Nevis

I. Fenemigho
Department of Family Medicine, BronxCare Health System, Bronx, NY, USA

C. V. Asuzu
Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

E. E. Ukponmwan
Department of Pediatrics, Lincoln medical center, Bronx, NY, USA

K. C. Nnawuba
Caribbean Medical University, School of Medicine, Willemstad, Curacao

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukayriyah, Saudi Arabia

7.1 Introduction

The discussion on stem cell use in cardiovascular disease would be incomplete without a conceptual understanding of the evolutional classification of these stem cell therapies into first, second, and next generations. First-generation stem cell therapy (see Fig. 7.1) uses the cells that are derived from adult tissues and include those that can be isolated from the bone marrow (BM), such as bone marrow mononuclear cells (BM-MNCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and mesenchymal stem cells (MSCs) (Zuk et al. 2001). In addition to being isolated from the BM, MSCs can also be harvested from a range of other tissues, including adipose tissue, dental pulp, placenta, umbilical cord blood, and Wharton's jelly (Mathiasen et al. 2009; Golpanian et al. 2016).

Second-generation stem cell therapy comprises pluripotent stem cells (PSCs) or cardiac lineage-guided or directed stem cells achieved by either gene or other modifications (Bearzi et al. 2007). These cells include c-kit+ cardiac stem

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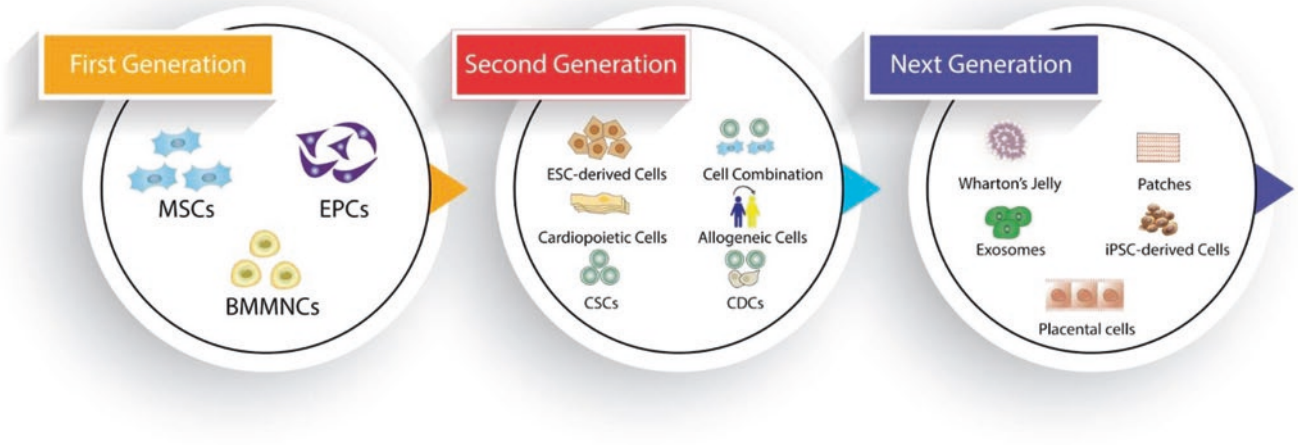

Fig. 7.1 Evolution of cardiac regenerative therapies: First-generation stem are a heterogeneous group comprised of BM-derived mononuclear cell and endothelial progenitor cells (EPCs). Second-generation stem cells include cardiac lineage directed cells like c-kit+ CSCs and cardiosphere-derived cells. Exosomes, patches, and placenta-derived cells make up third-generation cells

cells (CSCs) with clonogenic and multipotent properties and cardiosphere-derived cells (CDCs), which are a group of myocardial cells made up of an admixture of CSCs and their support cells (Messina et al. 2004; Li et al. 2012). Next-generation stem cell therapy involves molecular and engineered constructs with innovative clinical entities like exosomes, patches, and placental cells, induced pluripotent stem cells (iPSC), and Wharton's Jelly derivatives (Cambria et al. 2017).

7.2 First Generation

7.2.1 Bone Marrow Stem Cells

BM-derived stem cells are stem cells housed in the BM and offer the unique advantage of the ease of isolation, expansion, and self-renewal. These stem cells consist of heterogeneous subpopulations of hematopoietic (bone marrow-HSC [BM-HSCs]), mesenchymal (bone marrow MSCs [BM-MSCs]), endothelial progenitor (bone marrow endothelial progenitor cells [BM-EPCs]), and side population cells (Strauer and Steinhoff 2011). Both hematopoietic and mesenchymal subpopulations are multipotent and capable of induced differentiability into cardiomyocytes (CMs) (Zuk et al. 2001; Mathiasen et al. 2009). The significant difference between these cell lines is their surface receptors with BM-HSCs hosting CD133, CD34, or CD117 (c-kit) receptors and BM-MSCs characterized by CD105, CD73, and CD90 (Donndorf et al. 2013). The role of BM-EPCs lies in their angiogenic potential and the ability to enhance endothelial repair (Teng et al. 2012).

7.2.1.1 BM-Derived Stem Cells in Pre-Clinical Cardiovascular Research

Orlic et al. (2001) were the first to demonstrate the regenerative potential of transplanted BM-derived stem cells injected intramyocardially into the infarcted heart of a small mammal (Orlic et al. 2001). After documenting several subpopulations of cardiomyocytes, vascular elements, endothelial and smooth muscle cells, they concluded that BM-derived cells could engraft in vivo with myocardial repair capacity. Subsequently, multiple animal studies using acute myocardial infarction (AMI) models have successfully demonstrated favorable results, mostly reducing the infarct size, elevated left ventricular ejection fraction (LVEF), and neovascularization. Other benefits that have been reported include apoptosis and fibrosis reduction and increased the expression of vascular endothelial growth factor (VEGF) (Tang et al. 2004; Kanelidis et al. 2017).

Bone Marrow Mesenchymal Stem Cells

Toma et al. (2002) demonstrated the ability of BM-MSCs to transform into cardiomyocytes (CMs) after engraftment into the host myocardium of murine hearts (Toma et al. 2002). In addition to sarcomeric rearrangement of contractile elements, immunohistochemical analysis showed desmin expression, β-myosin heavy chain, α-actinin, cardiac troponin T, and phospholamban in quantities similar to those of the host cardiac cells. Kudo et al. demonstrated decreased fibrosis and high host engraftment of BM-MSCs following intramyocardial (IM) injection (Kudo et al. 2003). However, other studies were challenged by low engraftment rates of approximately 6–12% (Barbash et al. 2003). These data led to the invention of an epicardial patch embedded with human BM-MSCs for the transplantation onto the host AMI model.

The patch-based delivery resulted in a phenomenal 23% engraftment rate 1 week later, in addition to a reduction in LV dilation compared to controls (Simpson et al. 2007).

During the last two decades, large mammalian acute and chronic MI models have contributed enormously to the wealth of knowledge regarding BM-MSCs applicability in cardiac regeneration. Armando et al. injected BM-Di-I and DAPI-labeled MSCs in the swine model of AMI. They highlighted vasculogenesis and myogenesist by increased VEGF expression and specific CM proteins (Amado et al. 2005, 2006). There was also a concomitant decrease in infarct size and an improvement in LEVF. Hatzistergos et al. (2010) showed engraftment and, most importantly, differentiation into three lineages, that is, CMs, endothelium, and smooth muscle cells (Hatzistergos et al. 2010). The underlying theory demonstrated by most of these studies is that cardiac regeneration by BM-MSCs is mainly due to the paracrine effects of secreted growth factors, exosomes, and microvesicles. The proliferation of endogenous CSCs reinforced this theory following the injection of BM-MSCs in the infarcted myocardium.

In chronic ischemia, BM-MSCs have also shown beneficial effects following injection or transplantation. Silva et al. showed an increased vascularization and elevated cardiac physiologic functions in chronic ischemia using canine heart models (Silva et al. 2005). Meanwhile, Schuleri et al. showed that BM-MSCs could reverse remodeling and decrease fibrotic tissue following the IM injection (Schuleri et al. 2009). They also observed a dose-dependent increase in the contractility in the infarcted area. Quevedo et al. performed a transendocardial injection of allogenic BM-MSCs in a swine model with chronic ischemic cardiomyopathy and reported an improvement in LEVF, contractility, vasculogenesis, and blood flow after 12 weeks compared to the comparison group (Quevedo et al. 2009). Leveraging the immunosuppressive attributes of BM-MSCs has seen enthusiasm develop in their potential for non-ischemic cardiac disorders such as acute myocarditis. Ohnishi et al. demonstrated the ability of BM-MSCs to ameliorate inflammation following induced myocarditis in Lewis rats (Ohnishi et al. 2007). When transplanted, these cells decreased the influx of CD68+ inflammatory cells and the expression of monocyte chemoattractant protein-1 (MCP-1) in addition to an increased cardiac function.

Bone Marrow Progenitor Cells

BM progenitor cells (MPCs/Stro-1^{+}/CD34^{-}) are immature BM-MSCs with distinct self-renewal capacity. A 40 and 50% reduction in scar size and vascularity, respectively, was noted by Houtgraaf et al. following the injection of MPCs into a sheep model of MI (Houtgraaf et al. 2013). Cheng et al. demonstrated elevation of LEVF, increased wall thickness, and vascularity (Cheng et al. 2013). In another study involving non-ischemic animal heart pathology, a transendocardial injection of these cells caused a reduction in LV end-systolic volume (LVESV), decreased fibrosis development, and LEVF maintenance (Psaltis et al. 2010). Scientists who propose using these cells rather than MSCs argue that MPCs are favorable due to their superior ability to replicate and differentiate (Psaltis et al. 2008).

7.2.1.2 Bone Marrow Mononuclear Cells

BM mononuclear cells (BM-MNCs) comprise a heterogeneous group of cells to in which MSCs, HSCs, and EPCs are the integral constituent cell types (Lambers and Kume 2016). Kobayashi et al. demonstrated a rise in vasculogenesis 2 weeks after the IM injection into a rat MI model (Kobayashi et al. 2000). Another rat MI model with a fibrin matrix also shows increased vasculogenesis after transplantation (Ryu et al. 2005). Large animal studies involving BM-MNCs were also promising in their results. Transendocardial injection of BM-MNCs improved myocardial vasculogenesis, increased blood flow, and attenuated infarct size 4 weeks after the cell therapy (Fuchs et al. 2001). Kamihata et al. showed an increase in LEVF following BM-MNCs injection in the peri-infarct zone and a lower LV end-diastolic volume (LVEDV) to body weight ratio (Kamihata et al. 2001). Alestalo et al. also demonstrated an absolute increase in LVEF after BM-MNC injection in the experimentally infarcted heart (Alestalo et al. 2015a, b). They reported a direct relationship between LEVF and retained donor cells, implying that stem cell retention was crucial for success. Similarly, in their experiment involving canine MI models, Mathieu et al. showed increased end-systolic elastance, decreased infarct size, and N-terminal B-type natriuretic pro-peptide (NT-proBNP) levels (Mathieu et al. 2009).

7.2.1.3 Bone Marrow-Derived Stem Cells in Cardiovascular Clinical Research

The therapeutic potential of BM-derived stem cells in cardiovascular disease treatment has been shown by numerous clinical trials, with crucial landmark trials summarized in Table 7.1. A meta-analysis involving 50 studies conducted between 2003 and 2011 revealed that the transplantation of BM-derived stem cells improved LEVF by approximately 4% on average with an equivalent concomitant reduction in infarct size compared to the cell therapy group. It was also observed that cell therapy benefited patients with AMI and chronic ischemic heart disease (Jeevanantham et al. 2012). Landmark clinical studies involving BM-derived stem cells in cardiovascular research are summarized in Table 7.1.

Acute Myocardial Infarction

Among the earliest clinical trials demonstrating the therapeutic effectiveness of BM-derived stem cells is the Transplantation of Progenitor Cells and Regeneration

Table 7.1 Clinical trials involving bone marrow derived stem cells

Cardiovascular condition	Trial(Refs.)	Follow up	N	Cell type and dose	LEVF	LV volumes	Scar size
AMI	**TOPCARE-AMI** (Schächinger et al. 2004)	12 months	59	CPCs; BM-MNCs 7.3×10^6	↑	↓ ESV	↓
	BOOST (Meyer et al. 2009)	6 months	60	2.4×10^9 BM-MNCs vs control	↑	NS	NA
	REPAIR-AMI (Schächinger et al. 2006)	4 months	204	198×10^6 BM-MNCs vs placebo	↑	NS	NA
	TIME	6 months	120	150×10^6 BM-MNCs vs placebo	NS	NS	NS
	LateTime	6 months	87	150×10^6 BM-MNCs vs placebo	NS	NS	NS
	SWISS AMI (Sürder et al. 2010)	12 months	192	153×10^6 BMMNCs vs control	NS	NS	NS
	BOOST-2 (Wollert et al. 2017)	6 months	188	20.6×10^8 vs 7.0×10^8 BM-MNCs vs placebo	NS	NS	NS
	Pre-SERVE-AMI	12 months	161	14.9×10^6 CD34+ vs placebo	NS	NS	NS
Ischemic cardiomyopathy							
	Perin et al.	4 months	21	25.5 × 106 BM-MNCs vs control	↑	↓ESV	NA
	TOPCARE-CHD (Assmus et al. 2007)	3 months	75	22×10^6 CPCs vs BM-MNCs 205×10^6 BM-MNCs vs control	↑LVEF	NS	NS
	FOCUS CCTRN (Perin et al. 2012)	6 months	92	100×10^6 BM-MNCs vs placebo	NS	NS	NS
	MSC-HF (Mathiasen et al. 2020)	6 months	55	77×10^6 BM-MSC vs placebo	↑	↓ ESV	↓
	POSEIDON (Hare et al. 2012)	13 months	30	20, 100, or 200×10^6 BM-MSCs (Allo vs auto)	NS	Allo: ↓EDV	↓
	TAC-HFT (Heldman et al. 2014)	2 months	65	100×10^6 BM-MSCs vs 100×10^6 BM-MNCs vs placebo	NS	NS	MSC: ↓

Allo = Allogenic, AMI = Acute myocardial infarction; BM-MNC- Bone marrow mononuclear cells; EDV = End diastolic volume; EDD = End-diastolic diameter; FS = Fractional shortening; LV = Left ventricle; ESV = End-systolic volume; BM-MSCs = Bone marrow mesenchymal cells; NA = Not assessed; NS = Non-significant.

Enhancement in AMI (TOPCARE-AMI) (Table 7.1) (Schächinger et al. 2004). In this trial, BM-MNCs-given post-MI caused an increase in LEVF and decreased infarct size. Meanwhile, the BOOST (bone marrow transfer to enhance ST-elevation infarct regeneration) trial is a well-known study and one of the pioneer clinical trials in BM stem cell research and its intersection with cardiovascular regenerative therapy (Meyer et al. 2009). The BOOST trial was a randomized controlled trial by design. It showed similar results to the TOPCARE-AMI trial, while the only difference was that the BOOST trial involved a control group that received only MI-standard care protocol (Meyer et al. 2009).

Also, the LEUVEN-AMI trial team conducted a randomized, double-blind, placebo-controlled study in which 67 patients who received BM-derived stem cells as an adjunct to percutaneous coronary intervention for STEMI and reported a slight increase in global LEVF in the treatment group (Janssens et al. 2006; Schächinger et al. 2006). The largest known Phase III trial is the reinfusion of enriched progenitor cells and infarction remodeling in AMI (REPAIR-AMI) (Table 7.1). This double-blinded, placebo-controlled study involving the intracoronary injection of BM-derived stem cells into the reperfused hearts of 204 patients post-MI revealed a reduction in mortality, need for reperfusion, and re-infarction during the 1-year follow-up. Another large trial ASTAMI, (autologous stem-cell transplantation in AMI) showed disappointing results without a distinguishable difference in the LV function 3 years after cell infusion (Beitnes et al. 2009). Similarly, the multicenter, placebo-controlled TIME and LateTIME trials reported no difference in study endpoints of LEVF and wall-motion between the treatment and control arms (Traverse et al. 2010, 2018). Likewise, the SWISS-AMI (Swiss multicenter intracoronary stem cells study in AMI) trial, plagued by a high attrition rate, reported no improvement in LEVF or reduction in scar size 1 year after treatment (Sürder et al. 2010). With the BOOST-2 trial, an attempt at reproducing the same study endpoints of the initial BOOST trial resulted in a colossal failure with no significant improvement in LEVF in the treatment group than control group (Wollert et al. 2017).

Chronic Ischemic Cardiomyopathy

The earliest clinical study to examine the effectiveness of BM stem cells, especially BM-MNCs, on chronic ischemic cardiomyopathy was conducted by Perin et al. After 4 months of follow-up, they recorded an elevated LEVF, a reduction in LVESV, and improved mechanics of the segments of the heart that received transendocardial delivery of these (Perin et al. 2003). Another trial, the TOPCARE-CHD (transplantation of progenitor cells and regeneration enhancement in chronic post-infarction heart failure), demonstrated a decrease in LEVF and a proportional decrease in NT-proBNP (Assmus et al. 2007). Similarly, the FOCUS-CCTRN (first mononuclear cells injected in the USA conducted by the cardiovascular cell therapy research network) also showed promising results with a decrease in LEVF and infarct size after treatment compared to the comparator group (Perin et al. 2012).

The TAC-HFT (the transendocardial autologous cells hMSCs or hBMCs) has the pedigree of being the earliest to explore the effects of BM-MSCs on ischemic cardiomyopathy. Treated patients reported a better quality of life (QoL) as ascertained by better scores on Minnesota Living with the Heart Failure Questionnaire (MLHFQ). Other metrics, like the 6-minute walk distance (6MWD), were boosted while a reduction in infarct size was noticed (Heldman et al. 2014). The POSEIDON trial (percutaneous stem cell injection delivery effects on neomyogensis) only reinforced BM-derived stem cells' potential to reduce infarct size even though the study focused on a comparative analysis of the effects of allogeneic versus autologous MSCs (Hare et al. 2012). The MSC-HF trial involving transendocardial injection of autologous BM-MSCs gave an insight into the sustainability of these cells' regenerative potential as it reported elevated LEVF in the treatment group compared to the placebo recipients up till 1-year after follow-up. Other remarkable finding that could be gleaned from this trial was the congruence between the cell dose injected and the improvement observed in cardiac physiology (Mathiasen et al. 2020).

Dilated Cardiomyopathy/Heart Failure

For dilated cardiomyopathy (not of ischemic etiology), BM stem cells' therapeutic potential has been demonstrated. The TOPCARE-DCM (transplantation of progenitor cells and recovery of LV function in patients with non-ischemic dilated cardiomyopathy) trial showed improved LEVF and wall kinetics at 3 months and reduced NT-proBNP levels during 1 year follow-up (Fischer-Rasokat et al. 2009). In the same manner, another trial dubbed the ABCD (autologous bone marrow cells in dilated cardiomyopathy) also had remarkable findings such that treated patients reported improved QoL (Seth et al. 2010). The POSEIDON-DCM trial (percutaneous stem cells injection delivery effects on neomyogenesis in dilated cardiomyopathy) only noted an improvement in LVEF and 6-MWD in those receiving allogenic stem cells compared to the autologous treatment category. However, both treatment arms showed a decrease in serum tumor necrosis factor (TNF)-α levels (Hare et al. 2017).

The CELLWAVE study conducted by Assmus et al. involved a combination of pre-treatment with shock wave technology and administration of BM-MSCs. The goal was to improve cell retention by expressing chemo-attractants, like VEGF and stromal-derived growth factor-1α (SDF-1α). BM-MSCs + shock wave increased LEVF by 3% compared to the control group (BM-MSCs + placebo), which increased LEVF by only 1%.

Mesenchymal Stem Cells

According to the International Society for Cellular Therapy (ISCT), MSCs are plastic-adherent and capable of differentiating into adipocytes, chondroblast, and osteoblast lineages when maintained in standard culture (Dominici et al. 2006). They express the cluster differentiating factors CD73, CD90, and CD105 and lack hematopoietic lineage markers like CD14, CD11b, CD19, CD34, CD45, CD79**α,** and HLA-DR surface molecules (Dominici et al. 2006). Although only a few studies on MSCs in cardiovascular disease (CVD) have strictly followed these criteria, those who did, noted significant cardiac function improvement (Amado et al. 2005; Xu et al. 2011). However, no single marker has been reported to define MSCs in vivo (Watt et al. 2013). Adult MSCs can be derived from stem cells in various sources, including BM (Zuk et al. 2001), adipocytes (Miyahara et al. 2006; Bayes-Genis et al. 2010; Gaebel et al. 2011), umbilical cord endothelium (Gaebel et al. 2011; Zhao et al. 2011a, b), amniotic membrane (Tsuji et al. 2010), lungs (Sabatini et al. 2005), synovial membrane (Acquistapace et al. 2011), and peripheral blood (Zvaifler et al. 2000). They have low immunogenicity and high plasticity (Rangappa et al. 2003), thus rendering them an appealing source for cardiac repair and regeneration. They can migrate to the site of injury post-ischemic insult (Rangappa et al. 2003; Oswald et al. 2004). Studies have shown that injecting MSCs to areas of cardiac injury significantly improved cardiac function (Miyahara et al. 2006; Williams et al. 2011). However, the significance of this improvement in cardiac function largely depends on the donor MSCs' tissue of origin (Gaebel et al. 2011).

There are four primary mechanisms proposed to explain the cardiac function improvement observed in different studies. In the first mechanism, MSCs could reprogram the host CPCs into functionally competent CMs, thus promoting repopulation of the infarcted myocardium (Hatzistergos et al. 2010; Acquistapace et al. 2011). A second mechanism is that the donor MSCs transdifferentiate into morphofunctional competent CMs, that contribute to improved cardiac function (Bayes-Genis et al. 2010; Numasawa et al. 2011).

The third mechanism involves the release of paracrine factors from the transplanted MSCs that enhance the functionality of CMs or activate previously latent resident CPCs, thereby limiting cardiac remodeling after injury (Boni et al. 2008; Sassoli et al. 2011). Last, there is the possibility of direct electrophysiological coupling of MSCs with CMs through gap junctions (Chang et al. 2006). In the following sections, we will discuss the different cell sources and their evolution in CVD therapy.

7.2.2 Adipose-Tissue Derived Stem Cells

Adipose tissue-derived MSCs are isolated from the stromal compartment of adipose tissue. They can differentiate into several cell lineages of mesenchymal origin (Wu et al. 2006). Adipose-derived stem cells (ADSCs) have unique characteristics that also include their easy availability in a large number (Wu et al. 2006a, b, c). ADSCs have more proliferative capacity than BMSCs; they secrete a plethora of bioactive molecules such as basic fibroblast growth factor (bFGF), Interferon-gamma (IFN- γ), and Insulin-like growth factor-1 (IGF-1), and many more with immunomodulatory effects (Li et al. 2015). However, BMSCs are more advantageous in chondrogenic and osteogenic differentiation. This is an important consideration when deciding on stem cell sources for conditions like acute myocardial, lung, renal, lung, neural, or hepatic injury (Li et al. 2015).

Planat-Benard et al. successfully isolated from adipose stroma a cell population that was phenotypically similar to cardiac cells. On further investigation, they observed that the cells expressed cardiac-specific markers. Ultrastructural analysis and immunocytochemical staining also revealed the presence of atrial- and ventricular-like cells (Planat-Benard et al. 2004). Furthermore, cells from the adipose-lineage were capable of proliferation and differentiated into endothelial cells to participate in neovascularization in the ischaemic tissues (Planat-Benard et al. 2004). A few years later, Mazo et al. compared the effect of ADSCs and their derivative cardiomyogenic cells (ADCMG) with BM-MNC in a chronic model of experimental MI in rats. These cells were introduced through direct intramyocardial (IM) injection and followed-up at 1 month. ADSCs were found to improve cardiac function and tissue metabolism, significantly promoted angiogenesis, and reduced fibrosis compared to ADCMG BM-MNC (Mazo et al. 2008). Bayes-Genis et al. transplanted human cardiac adipose tissue-derived progenitor cells (CATDPCs) into infarcted mouse and rat hearts. These cells expressed endothelial and cardiac-specific markers and reduced infarct size and increased vascularization compared to the control group of animals. Furthermore, a significant difference was noted in LVEF and LVFS, thus making CATDPCs a valid candidate for future use in myocardial cell therapy (Bayes-Genis et al. 2010). The first clinical trial involving ADSCs, the APOLLO trial, was carried out in relatively few patients. The trial was a randomized double-blinded control study involving 14 patients with anterior wall MI (Fig. 7.2).

The study results showed that intracoronary infusion of ADSCs to patients with acute myocardial injury was feasible, safe, and did not result in interruption of coronary flow. There was also a trend toward increased LVEF, although this was statistically insignificant, possibly due to the small sample size (Houtgraaf et al. 2012).

7.3 Second Generation

7.3.1 Cardiac Stem Cells in Cardiovascular Regeneration

CMs have long been regarded as post-mitotic cells, incapable of repair, and regeneration. However, over the last two decades, increasing evidence has proven otherwise. The first proof resident CSCs came from Quaini et al. following analysis of sex-mismatched heart transplantation (Quaini et al. 2002). Male recipients who got donor hearts from females showed Y-positive CPCs translocated into the graft. The Y-chromosome was used to differentiate between primitive cells derived from the recipient and those from the donor. Y-chromosome positive CMs from these primitive recipient cells made up 7–10% of those in the donor's hearts and were highly proliferative. Since the publication of these data, multiple studies have confirmed the existence of resident CPCs in the heart (Dawn et al. 2005; van Laake et al. 2006).

Resident precursor cells, that is, CPCs and CSCs, can reenter the cell cycle, besides having the ability to differentiate into adult CMS, endothelial cells, and smooth muscle cells. These stem cells are responsible for physiologic turnover in the uninjured heart, although at a low rate of about 1% in a year (Dawn et al. 2005; van Laake et al. 2006). Furthermore, increased allograft chimerism has been observed in hearts that have suffered acute injury compared to the uninjured hearts. A marked difference has also been observed in the recruitment of CPCs in the hearts of patients who have died from AMI compared to those who died from more chronic diseases thus suggesting that increased CPCs' recruitment is injury-dependent (Hochtzeisberg 2004; Urbanek et al. 2005).

7.3.1.1 Brief History of Cardiac Stem Cells

Following the discovery of CSCs, a lineage-negative, c-kit-positive (Lin^{-} $c\text{-}kit^{+}$) CPCs population was successfully isolated from the adult rat hearts (Beltrami et al. 2003). These cells were multipotent and capable of differentiating into CMs, smooth muscle cells, and endothelial cells (Beltrami et al. 2003; Tillmanns et al. 2008). During the same year, Oh

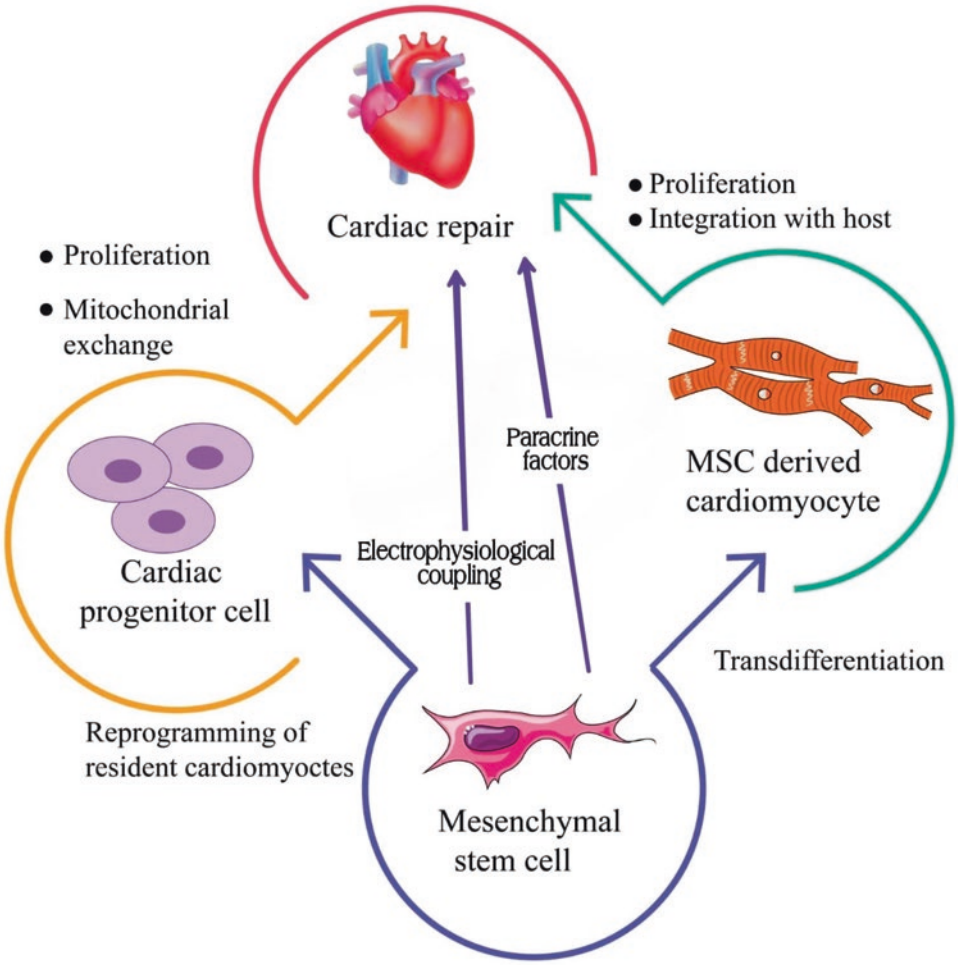

Fig. 7.2 Mechanism of cardiac repair with MSCs. MSCs secrete proangiogenic paracrine factors like vascular endothelial growth factor and platelet-derived growth factor, which promotes angiogenesis. MSCs also have a large differentiation potential into different cells such as osteoblasts, adipocytes, and chondrocytes and especially cardiomyocytes when the target is cardiac repair. They also reprogram cardiomyocytes to express more adhesion molecules, which leads to increased cellular adhesion and cardiac function following MI

et al. identified Sca-1+ CSCs from the adult mouse, capable of differentiating into CMs (Oh et al. 2003). Martin et al. defined a subpopulation of cells that expressed ATP-binding cassette transporter (ABCG2) in addition to Sca-1+ cells. However, these cells needed to be cultured with a bulk of cardiac cells to differentiate into cardiomyocytes (Martin et al. 2004). Concurrently, a mixed group of cells that expressed c-kit, Sca-1, and Flk-1 were reported with the potential to undergo cardiogenic differentiation (Messina et al. 2004).

7.3.1.2 Preclinical Evolution of CSCs in the Repair of Cardiovascular Diseases

As a result of such promising discoveries, stem cell research has explored the therapeutic use of resident CPCs in CVD during the last decade. Pivotal to this research uses autologous resident CSCs harvested from the interventricular septum or explanted from the right atrial appendages after in vitro expansion in cell culture. These cells expressed c-kit, CD31, CD34, and CD90 and formed self-adherent clusters, called cardiospheres (Chamuleau et al. 2009). In 2007, Smith et al. showed that these CSCs/ CPCs isolated from cardiospheres could proliferate in vitro and 20 mg of human heart samples could yield approximately 1.5 million CDCs in 45 days (Smith et al. 2007). Furthermore, in 2009, Johnston et al. isolated endomyocardial biopsies from pigs to isolate CDCs. Subsequently, these cells were delivered via intracoronary infusion to pigs 4 weeks post-MI. Results showed an improvement in hemodynamic parameters, attenuation of cardiac remodeling, reduced infarct size, and formation of new cardiac tissue (Johnston et al. 2009). Lee et al. used IM injection to assess CDCs' safety and efficacy, and their 3D precursors, cardiospheres in mini-pigs with heart failure. LVEF was higher in both cell-treated groups than in the placebo-treated group after 8 weeks (Table 7.2); however, cardiospheres showed superiority in reducing LV remodeling, improving hemodynamic parameters, and regional LV function. The most remarkable finding in this study was that the successful donor cell engraftment was inversely proportional to the number of cells implanted (Lee et al. 2011). In other words, "less was more" in this case; however, it was proposed that the treatment benefits may be attributed to the paracrine activity of the transplanted cells via mobilization and activation of endogenous stem/progenitor cells, cytopro-

Table 7.2 Pre-clinical Studies involving CSCs in cardiovascular disease repair

References	Description	Outcome
Zimmerman et al. (2006)	Cardiac tissue engineering involving rat cardiomyocytes transplanted on collagen I and matrigel	↑ LV function including neovascularization
Bearzi et al. (2007)	A trial of human C-kit + CSCs transplanted into injured immunocompromised rat myocardium	↑ LV
Smith et al. (2007)	Human CDCs transplanted on infarcted rat myocardium	↑LVEF (42.8 ± 3.3%)
Tang et al. (2010)	Intracoronary injection of CPCs to infarcted rat hearts	↑LV function; ↓fibrosis; ↑expression and proliferation of endogenous CPCs
Li et al. (2011)	Intracoronary delivery of CSCs to infarcted murine myocardium	↑LV systolic and diastolic function
Bolli et al. (2013)	Autologous intracoronary injection of porcine CSCs	↑LVEF (51.7 ± 2.0% vs 42.9 ± 2.3%,P < 0.01), ↓LV end-diastolic pressure, increased thickening of infarcted LV wall
Yee et al. (2014)	Allogenic cardiospheres delivered through transendocardial injection	↑LV viable wall thickening; ↓scar size
Kulandavelu et al. (2016)	Human CSCs or hckit+ CSCs overexpressing Pim1, delivered via IM injection to immunosuppressed Yorkshire swine	Pim1+ group showed three fold ↓ in scar mass. Both groups reduced afterload and significantly ↑EF
Murphy et al. (2019)	Human engineered cardiac tissue supplemented with human CSCs in vitro	↑contractile function

tection of existing cardiac cells, and promoting survival of cardiomyocytes by creating a favorable extracellular environment (Laflamme et al. 2007). Landmark pre-clinical studies are summarized in Table 7.2.

7.3.1.3 Clinical Studies Involving CSCs in Cardiovascular Disease

A summary of key clinical trials involving the use of CSCs in cardiovascular disease is summarized in Table 7.3.

By 2012, the results of the first clinical study, CADDAEUS, were due. Although Makkar et al. reported remarkable improvement in myocardial function after an AMI after intracoronary delivery of CPCs (Makkar et al. 2012), the researchers argued that the observed functional improvement was clinically insignificant. The clinical insignificance of the reported data was ascribed to the cell attrition post-transplantation which led to significant loss of the transplanted cells. The contributory factors to the massive loss of the donor cells was the hostile post-infarct cardiac environment which was nutrient deficient, hypoxic, and acidotic due to the accumulation of toxic waste (Balsam et al. 2004; Stamm et al. 2009). According to the published data, only 40% of the cells were retained at 5 h of cell transplantation which decreased to only half by 24 h (Qiao et al. 2009). Conservation of donor stem cell viability especially during the acute phase after MI episode has always remained a problem that necessitates the development of strategies to counter this issue.

Following these challenges, there has been an evolution in tissue engineering in cardiovascular-related regenerative medicine. To enhance the number of stem/progenitor cells available at the injury site for angiomyogenesis and to ensure their functional integration with the host CMs, much research has been focused on viable scaffolds (Codina et al. 2010). Tissue engineering in cardiac regenerative medicine involves the use of biodegradable three-dimensional (3D) scaffolds seeded with CSCs/CPCs. These cell-seeded scaffolds serve as an alternative to the extracellular matrix (ECM), providing structural and functional support to the donor cells during early phase after delivery, encouraging retention, proliferation, and overall survival of the seeded cells (Langer and Vacanti 1993). On the other hand, there is the possibility of generating whole organs from scaffold seeded tissues. However, cardiac-specific scaffolds need to conform to the heart muscle's intricate structure and complex organization to successfully promote neovascularization and donor cell coupling with the host CMs (Forte et al. 2013).

As an alternative to cell transplantation therapy, tissue engineering can potentially be used in acquired and congenital heart defects, including complete regeneration of a failing heart (Wu et al. 2006a, b, c). Zimmerman et al. successfully produced 3D heart tissue containing neonatal rat CMs cultured on collagen type I, and a basement membrane protein mix. The 3D heart tissue exhibited key features of the natural myocardial tissue, such as spontaneous and synchronous beating. As a result of this discovery, the engineered tissue was transplanted into an experimentally injured rat heart, which showed successful electrical coupling with the injured myocytes and that too without any arrhythmias. Improvement in LVFS and LV systolic wall thickness was also observed (Zimmermann et al. 2006). On the same note, Ott et al. successfully repopulated a de-cellularized rat heart (only extracellular material) with seeded cardiac-derived cells. The re-cellularized construct was contracting and generating sufficient pump function on eighth day of culture (Ott et al. 2008).

Table 7.3 Clinical trials: Cardiac stem cells in cardiovascular disease

Trial	References	Study design/year	Cell origin	Patients	Follow up period	Route of delivery	Outcome	Adverse Effects
CADUCEUS	Makkar et al. 2012	Phase I open-label, randomized controlled/2009–2011	Interventricular septum – Cardioshpere derived cells	Treated = 17; Controls = 8	6 and 12 month	Intracoronary	No changes in LVEF; ↑viable myocardium(+22.6 ± 9.4 g); ↓scar size (−11.1% ± 4.6)	Safe; increased viable myocardium; EDV, ESV, LVEF unchanged
ALCADIA	Duckers, et al. 2011	Phase I, open-label, single group assignment (non-randomized)	Cardiosphere derived with controlled release bFGF	Treated = 6, no controls	6 and 12 months	IM injection	↑LVEF (9–12%); ↓scar size after 6 months (−3.3%)	One congestive heart failure
CONCERT-HF	Bolli et al. 2018	Phase II, randomized, placebo-controlled	c-KIT+ CSC and/or autologous BMSC	144 estimated enrollees	6, 12, and 24 months	Transendocardial	In progress	In progress?
ALLSTAR	Makkar et al. 2020	Phase I/II, randomized, double blind, placebo-controlled	Allogenic cardiosphere derived cells	134 estimated enrollees	6 and 12 months	Intracoronary	Confirmed safety. ↓LVEDV↓LVESV ↓NT-proBNP	None
DYNAMIC	Chakravarty et al. 2020	Phase I, randomized, double blind, placebo-controlled	Cardiosphere-derived cells	42 patients	6 and 12 months	Intracoronary	Short-term increase in LVEF (+17.5%) – Still in progress	No preliminary major adverse events
CAREMI	Sanz-Ruiz et al. 2017	Phase I/II, randomized double-blind, placebo-controlled.	Allogenic human CSC	55 patients	6 and 12 months	Intracoronary	Safe	No major adverse cardiac events

Put together, future research into stem cells in cardiac tissue engineering needs to meet the following requirements.

- Scaffold biocompatibility with the host tissue to prevent immune or inflammatory response.
- Be sourced from cells morphologically similar to the native myocardium. This would boost cardiomyocytes differentiation and alignment, promoting good contractibility of the graft.
- Maintain cell viability before and after implantation into the host tissue. Bioreactors are used to achieve this in vitro, but once delivered, viability is fostered through adequate blood perfusion; hence, assimilation by host heart tissues.
- Allow adequate oxygen diffusion. As a result of this, viable engineered tissue need be limited to 200 mm.
- The ECM graft needs to be biodegradable at an optimal rate that promotes the assimilation of donor cells and sync with the host tissue repair rate.
- Facilitate cell–cell adhesion through the formation of membrane channels and gap junctions.
- Possess contractility characteristics similar to the native tissue and capable of conducting electrical signals without provoking arrhythmias.
- Confer sufficient support and integrity due to its mechanical strength to allow in vitro manipulation. Moreover, it will prevent fibrous scar expansion and promote repair/regeneration in host tissue (Radisic et al. 2006; Jawad et al. 2008; Iyer et al. 2011; Georgiadis et al. 2014).

7.3.1.4 Pros and Cons of CSCs

A significant advantage of the use of CSCs over other sources is safety. It is an attractive choice without the risk of tumorigenicity, arrhythmogenicity, or immunogenicity in addition to low post-treatment mortality (Hsiao et al. 2013). A significant disadvantage is the low regenerative potential of CSCs that remains a challenge for tissue repair. However, more clinical research needs to be carried out, not only focused on the outcome but also standardization of the cell preparation process, rate of donor cell retention, and the optimal time of cell delivery post-injury.

7.3.2 Embryonic Stem Cells

Embryonic stem cells (ESCs) are groups of cells acquired from the inner cell mass of the blastocyst phase of embryo development and have the unique configuration of indefinite growth when undifferentiated and concomitant differentiability into all cell types in the adult body (Fig. 7.3) (Chao et al. 2014).

7.3.2.1 Embryonic Stem Cells by the Years

A historical timeline of ESCs in research shows their first isolation from a mouse embryo in the early 1980s (Yu et al. 2013). The human equivalent was successfully isolated by Thomson et al. (Thomson et al. 1998). Spontaneous cardiac differentiation of mouse ESCs (mESCs) was first elicited by Kehat et al. who reported small islands of 3–20 cells per island, when provided with the optimum milieu, and developed contractile elements in 8% of the sample on day 20 after a transient embryoid body stage (Kehat et al. 2001). Similar data were reported by Xu et al. with a whopping 70% cardiac differentiation during the same time frame (Xu et al. 2002). Human ESCs (hESCs) have been touted as undergoing cardiac differentiation at 1–25% conversion rate after several weeks. This limitation prompted Mummery et al. and Passier et al. to innovatively apply co-culture of hESCs with visceral endodermal cells (END-2) or use END-2 infused medium for hESC-derived embryoid bodies cultures, as the visceral endoderm is key to CPCs differentiation during embryogenesis (Mummery et al. 2003; Passier and Mummery 2005).

7.3.2.2 ESCs Transplantation and Integration into Host Tissue

Effective successful electrical coupling of intramuscularly transplanted ESC-derived CMs into the recipient heart was demonstrated by Kehat et al. (Kehat et al. 2004) while Shiba et al. showed that hESC-derived CMs were protective against arrhythmias and permitted synchronous contraction with the improvement of cardiac function (Shiba et al. 2012). Chong et al. led the revitalization of the infarcted heart muscle with the persistence of the risk of arrhythmogenicity (Chong et al. 2014). Most of the studies on the integration were plagued by limited cell retention rates. Laflamme et al., however, developed a blend of pro-survival factors to combat this issue, thereby enhancing donor cell survival, while Zhang et al. achieved increased donor cell retention by activating the Akt pathway or via heat-shock pre-conditioning before transplantation (Zhang et al. 2001; Laflamme et al. 2007).

7.3.2.3 Preclinical Studies on Cardiovascular Diseases with ESCs

Using experimental animal models, many studies have demonstrated the therapeutic potential of ESCs for the treatment of CVD as well as peripheral vascular disease. A summary of some of the landmark studies is given in Table 7.4. Caspi et al. demonstrated an increase in LVFS and a decrease in LV end-diastolic diameter (LVEDD) (Table 7.4), thereby signaling an improvement in cardiac function (Caspi et al. 2007). Similar results were obtained by other research groups reporting an increase in LVEF and LV systolic wall thickness (SWT) as additional hemodynamic parameters (Ebelt et al.

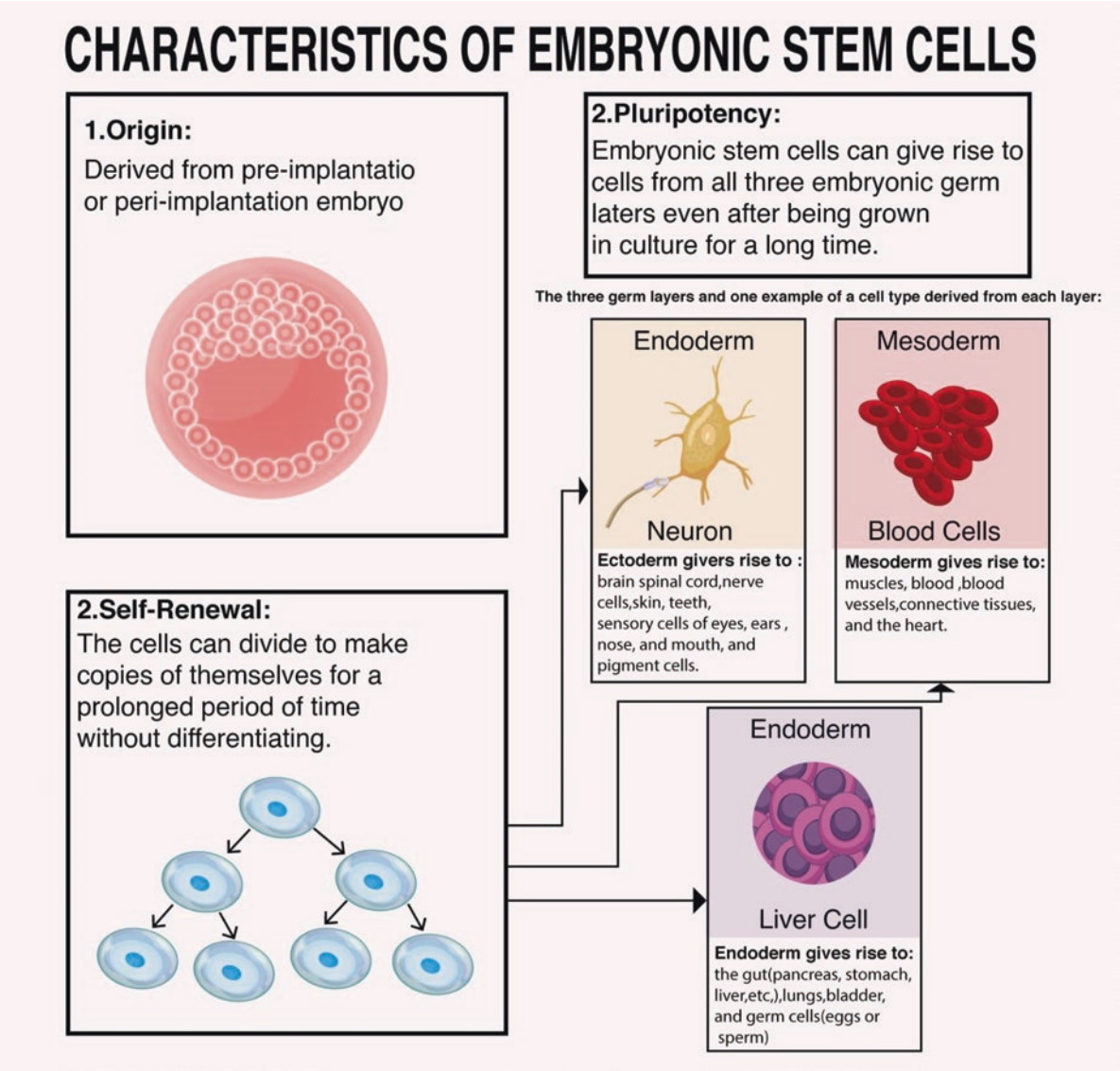

Fig. 7.3 Diagram showing some characteristics of ESCs. These cells are harvested at the blastocyst stage of the embryo which adds to the ethical dilemma that comes with their potential use. Also, pluripotency of ESCs means they are prone to tumor formation especially tumors composed of derivatives from multiple cell lines-teratomas

2007; Laflamme et al. 2007; Xiong et al. 2011, 2012). Leor et al. noticed a significant elevation in LVEF after transplantation of hESC-derived cardiomyocytes in a rodent model post-MI, as did van Laake et al. and Li et al. noticed an increase in LVFS and a decrease in LVEDV (Leor et al. 2007; van Laake et al. 2007; Li et al. 2009). Other studies focused on limb revascularization using an experimental model of hind limb ischemia provided insights into ESCs' angiogenic potential via paracrine mechanisms. Cho et al. and Yamahara et al. both reported increased limb neovascularization following pre-clinical studies involving hESCs-derived endothelial cells delivery to treat experimental murine model of limb ischemia (see Table 7.4) (Cho et al. 2007; Yamahara and Itch 2009).

7.3.2.4 Clinical Studies Involving hESCs in Heart Disease

Only one clinical trial has been conducted using hESC for myocardial repair. This clinical trial began in 2014 in France, involving six patients with severe LV dysfunction (LEVF <35%) due to a prior episode of MI (Menasché et al. 2018). A fibrin patch seeded with hESCs-derived CD-15+ ISL-1+ (transcription factor Isl-1 expression) progenitor cells was transplanted into the infarcted epicardium and circumscribed by a nutrient-rich pericardial flap to enhance the survival of the transplanted cells. The trial was successful in terms of primary and secondary endpoints of safety and efficacy, respectively, albeit a small sample size of six patients, diminishing the generalizability of results. There were no terato-

Table 7.4 Preclinical studies on the therapeutic potential of stem cells in cardiovascular disease

Tissue defect	References	Brief description	Outcomes
Myocardial infarction	Caspi et al. (2007)	Injection of undifferentiated hESCs, hESC-CMs, non-CM derivatives, or saline into immunosuppressed and infarcted hearts with histological and echocardiography analysis as study endpoints after 30–60 days	↑LVFS; ↓EDD
	Ebelt et al. (2007)	A comparison of SKMs and ESC-derived CMs after transplantation into mice hearts with MI through histological and echocardiographic after 4 weeks	↑LVFS; ↓EDD
	van Laake et al. (2007)	A magnetic resonance imaging analysis after 4 and 12 weeks of transplanted hESC-derived CMs after IM injection into healthy, immunocompromised, and infarcted mice hearts versus controls	4 weeks: ↑LVEF;
	Laflamme et al. (2007)	Integration of hESC-derived CMs into an infarcted rat myocardium while using activin A and BMP4 in the differentiation process to achieve CMs of high purity	↑LVFS; ↓EDD; ↑LVEF; ↑SWT
	Leor et al. (2007)	An investigation into the influence of hESC-derived CMs on the functioning of a rat model of MI	↑LVEF
	Xiong et al. (2011, 2012)	An investigation into the effects of transplanted hESC-derived endothelial and smooth muscle cells on post-MI LV remodeling in a pig. These cells were embedded in a fibrin 3D porous scaffold biomatrix	2011: ↑FS; ↑LVEF 2012: LVEF
	Yu et al. (2019)	An investigation into the effectiveness of hESC-derived CMs on permanent ischemia (PI) and myocardial ischemia-reperfusion (IR) in mice	Preservation of cardiac function in PI mice
	Liu et al. (2018)	To assess the re-muscularization of infarcted myocardium of macaque monkeys following transplantation of hESC-derived CMs	↑LVEF
	Yamahara and Itoh, (2009)	An investigation into the neovascularization attributes of human vascular progenitor cells derived from hESCs when transplanted into nude mice with ischemic hind limbs	↑vascular regeneration ↑ neovascularization
Limb ischemia	Cho et al. (2007)	Evaluation after 4 weeks of intramuscularly injected EC derived from hESCs into induced ischemic hind limbs in athymic mice	↑perfusion and limb salvage ↑ neovascularization
	Huang et al. (2010)	To study the neovascularization potential of ESC-ECs delivered intramuscularly and via the intra-femoral artery and veins. Functional assessment of perfusion was conducted using laser Doppler perfusion	↑engraftment into limb vasculature ↑limb perfusion

mas, arrhythmias, and alloimmunization among the study subjects receiving cell therapy. A 1-year follow-up revealed symptomatic improvement as well as an improved LEVF from 26 to 38.5% (Menasché et al. 2018).

Cardiomyocytes = CMs; EDV = End Diastolic Volume; EDD = End Diastolic Diameter; LV = Left ventricle; LVFS = Left ventricular frictional shortening; LVEF = Left ventricular ejection fraction; SkMs = Skeletal myoblasts; SV = Systolic volume; SWT = Systolic wall thickness; MI = Myocardial infarction.

7.3.2.5 Problems with ESCs

The ethical issues arising from the use of ESCs' derivative cells thwart their potential for therapeutic applications and remain contentious in stem cell research (Fig. 7.3). Another problem encountered in ESCs research is generating a large number of differentiated cells from a distinct lineage with GMP and clinical standards of purity. Chong et al. optimized protocols to successfully generate large quantities of ESC-derivative cells that were later used in cell transplantation studies in experimental animal models of MI (Chong et al. 2014). Thus, the data generated showed great promise for the ESCs and their derivative cells for cell-based therapy to treat CVD. The data from small experimental animal studies obviously cannot be extrapolated to humans due to the wide gap in species' physiological comparison (Leor et al. 2007). For example, a murine cardiac rhythm at a frequency of ~500 beats per minute erodes any arrhythmogenicity evoked by an ectopic pacemaker, making a case for their unreliability. Indeed, the arrhythmogenicity of transplanted ESCs remains a significant concern in their application and use in humans for CVD. Retention and survival of transplanted tissue also appeared to be minimal until Kofidis et al. delivered a composite mixture of murine cells seeded on a matrix base, and Laflamme added survival factors that inherently prevented apoptosis by initiating anti-apoptotic signaling as alternative delivery methods (Kofidis et al. 2005; Laflamme et al. 2007).

Although hESCs lack the expression of MHC Class II molecules and cannot directly activate T lymphocytes, they still can cause an immune response in various ways. First is the activation of allogenic killer NK cells (Draper et al. 2002; Li 2004a, b; Drukker et al. 2006; English and Wood 2011). Secondly, they can undergo uncontrolled differentiation into cell derivatives that express both MHC Class I and II mole-

cules, thereby unleashing an immunogenic storm (Dressel et al. 2010; Perez-Cunningham et al. 2014). Thirdly, it can be due to the interaction between the MHC Class I molecule and Oct4 on the surface of hESCs, which may lead to indirect T-cell activation via antigen presentation (Zhao et al. 2011a, b; 2015).

And lastly, their malignant transformation, most commonly teratoma formation, enables them to differentiate into different cell lines, thus becoming the Achilles heel of researchers to control the outcome (Fig. 7.3) (Li 2004; Drukker et al. 2006). Teratomas characteristically contain cells from all three germ layers. The probability of their formation ranges from 33 to 100%, depending upon various factors, including the site of transplantation, purity of the cell preparation, cell maturation, and implantation technique (Nussbaum et al. 2007; Prokhorova et al. 2009). The strategies to prevent teratoma formation include developing a transplantation protocol that ensures complete differentiation into the target organ's mature cell type and rigorous checks to eliminate undifferentiated cells. This strategy alleviated teratogenicity in more than 200 animals receiving hESC-derived CMs transplantation (Laflamme et al. 2007). This strategy's limitation is that unwanted differentiation of the cells may persist as primitive forms resulting from the differentiation of committed precursors (Roy et al. 2006).

Make no mistake; the potential of ESCs in CVD treatment is enormous. Considering all that has been discussed, the value of ESCs as donor choice cells probably lies in their contribution to the body of knowledge on cardiomyocyte differentiation, which typically involves signal transduction factors like Wnt, activin/Nodal/transforming growth factor-ß (TGFß), bone morphogenetic protein (BMP), and fibroblast growth factor (FGF).

7.4 Third Generation

7.4.1 Induced Pluripotent Stem Cells

iPSCs are derived from adult somatic cells through genetic reprogramming (Ibrahim et al. 2016). Like ESCs, iPSCs have the ability to self-renewal and differentiation into other cell types, including CMs (Rikhtegar et al. 2019). Numerous embryos were destroyed to generate ESCs lines, raising moral and ethical concerns about their generation and subsequent experimental use. Hence, it gave room for the development of IPSCs by reprogramming adult cells. The primary purpose for reprogramming somatic cells and generation of iPSCs was to eliminate the cellular identity of somatic cells and reverse them to the embryonic inner mass status of pluripotency, thus serving as surrogate ESCs (Samak and Hinkel 2019).

Given the simple and ethically less offensive protocols for induction of iPSCs than ESCs, they are regarded as the most suitable cardiac engineering source as well as for in vitro cardiac disease modeling (Brenner and Franz 2011; Cagavi et al. 2018). Also, iPSCs can be expanded and differentiated in vitro to produce a large amount of any cell type required for myocardial repair and regeneration (Lalit et al. 2014).

Over the years, meaningful progress has been made in developing strategies to direct iPSCs to cardiovascular cells. The first protocol of somatic cell reprogramming was reported by Takahashi and Yamanaka (Takahashi et al. 2006). Their experiment demonstrated that iPSCs could be produced from somatic cells like skin fibroblasts by overexpressing a quartet of transcription factors essential for cellular reprogramming to a pluripotent status akin to ESCs. These important transcription factors included octane binding transcription factor 4 (Oct4), sex-determining region Y box 2 (Sox2), myelocytomatosis viral oncogene homologous (c-Myc), and Kruppel-like factor 4 (Klf4) (Takahashi et al. 2006). One year later, Yu et al. were the first to successfully reprogram human somatic cells to iPSCs using Sox2, Oct4, Nanog, and Lin28 (Yu et al. 2007). These IPSs have shown the capacity to differentiate into functional CMs.

Further research advancements revealed that instead of dermal fibroblast, other cell types, including skeletal myoblasts (SkMs), BM-derived MSCs, T-cells, fat tissue, cord blood, amniotic fluid cells, and renal tubular cells, could also be utilized to generate iPSC (Ahmed et al. 2011a, b; Buccini et al. 2012). It was found that juvenile human keratinocytes were much more efficient and faster at generating iPSCs than dermal fibroblasts (Aasen et al. 2008).

The protocol of reprogramming of somatic cells has evolved with time. Retroviral and lentiviral-based protocols for somatic cell reprogramming were first developed and employed successfully but had the limitation of mutagenesis (Lalit et al. 2014). Their use in reprogramming protocols was substituted with Sendai viruses, which, unlike retroviruses, did not integrate into the host genome, thus carrying a low risk of mutagenesis (Ban et al. 2011). To make it safe for human use, protocols based on non-viral and non-integrating approaches, that is, simple plasmids, episomal vectors, and minicircle-based vectors were developed; however, they were less efficient than virus-based vectors (Lalit et al. 2014). More recent advancement in cellular uses modified mRNA-based delivery but again this is not as efficient as viral-based methods (Warren et al. 2010). Epigenetic modifiers, such as HDAC inhibitors and DNA demethylation, have been used to improve reprogramming efficiency and can be combined with other methods (Lalit et al. 2014). The applications of hiPSCs range from CVD modeling to precision medicine (Cagavi et al. 2018). Consensus molecular subtypes (CMSs) produced from hiPSCs can help identify various CVDs, that is, hypertrophic cardiomyopathy, dilated

cardiomyopathy, and arrhythmogenic cardiomyopathy (Rikhtegar et al. 2019).

Previously, there were reports of differences between hESCs and iPSCs that may have been acquired in the process of reprogramming. However, a more recent study has shown that despite minor transcriptional differences between the two, they have molecular similarities. It has also been demonstrated that iPSCs carry a higher risk of mutagenesis than ESCs, and they can keep epigenetic memory of their mother cells from which they were derived (Brenner and Franz 2011).

7.4.1.1 Pre-Clinical Studies on Cardiovascular Diseases with iPSCs

Several animal studies have tested the use of both differentiated and undifferentiated iPSCs post-MI repair. Earlier studies have made use of undifferentiated cells in animal models (Brenner and Franz 2011). Nelson et al. were the first to demonstrate the use of iPSCs for post-MI repair. Using human transcription factors Oct4, Sox2, Klfa4, c-Myc, they successfully reprogrammed murine embryonic fibroblasts to murine IPSCs (miPSCs) (Nelson et al. 2009). Undifferentiated miPSCs were injected intramyocardially into either immunocompetent or immunodeficient mice with LAD ligation. They reported significant LV function improvement and reduced cardiac remodeling 4 weeks after iPSCs transplantation without tumor formation in immunocompetent mice. On the contrary, most of the immunodeficient mice developed tumors 2 weeks after transplantation (Lalit et al. 2014; Buccini et al. 2012). This finding was similar to the previously published results of a study which used undifferentiated miPSCs derived from H9c2 cardiomyoblasts isolated from embryonic ventricular tissue. The cells were transplanted into the infracted region of an immunocompetent mouse that showed improved LV function and reduced CM apoptosis 2 weeks after transplant without tumor formation (Singla et al. 2011). These findings supported the hypothesis that the transplantation of low numbers of pluripotent cells into immunocompetent post-MI mouse hearts resulted in cardiac-specific differentiation of iPSCs and cardiac repair with a low risk of tumor formation.

On the contrary, Ahmed et al. reported tumor formation in post-MI hearts injected with SkMs-derived iPSCs that were independent of cell dose, transplant duration, and the presence or absence of MI, thus challenging the initial claim that cardiac environment was enough to direct pluripotent cells to cardiac lineages (Ahmed et al. 2011a, b). It was generally concluded that undifferentiated pluripotent cells should be used with caution due to their tumorigenic potential. To address the tumorigenicity of iPSCs, Pasha et al. transplanted pre-differentiated iPSCs-derived CMs into immunocompetent mice following LAD ligation and reported improved ventricular contractility and reduced cardiac remodeling 4 weeks post-transplantation without tumor formation (Pasha et al. 2011).

To transition to clinical studies, pre-clinical testing using human iPSCs and larger animal models was necessary. Templin et al. (see Table 7.5) reported using engineered transgenic human iPSCs in a pig model. They intramyocardially injected hiPSCs/hiPSCs + hMSCs 10 days after inducing MI in pigs. Results showed that hMSCs co-injection was essential for long-term survival and engraftment of hiPSCs (Templin et al. 2012).

Results from a study carried out by Kawamura et al. revealed improved LV function, myocardial metabolism, decreased infarct size, reduced ventricular wall stress 8 weeks following transplantation of cell sheets containing cardiac myocytes derived from hiPSCs in addition to SMC and endothelial cells with a 3D fibrin patch comprising IGF-1 in a porcine model of MI without tumor formation and arrhythmias (Kawamura et al. 2013). Most of the benefits attributed to these studies were attributed to paracrine signaling (Lalit et al. 2014). These studies demonstrated the first use of hiPSCs in large animal pre-clinical studies. Hopefully, more studies will be carried out to determine optimum dose, delivery methods, and safety, which will give way to clinical trials.

7.4.1.2 Limitations/Shortcomings with the Use of iPSCs

One major limitation with iPSCs is their potential tumorigenic nature, especially when they are used in undifferentiated state, which is of concern for their use in clinical settings. The risk of tumorgenicity usually arises further due to the prolonged culture of iPSCs as it may incur genetic abnormalities in the cells. Also, certain factors used in the reprogramming process, like c-Myc, significantly contribute to their tumorigenic potential. Genetic abnormalities in human iPSCs lines can also limit the safe therapeutic applications. These abnormalities usually arise from somatic cells used in the reprogramming and their culture adaptation. A study of 22 human iPSCs showed that half of the genetic mutations arose from fibroblasts used for reprogramming, while the other half arose from the reprogramming process itself (Gore et al. 2011). To overcome this challenge, karyotyping is used to identify and exclude the cell lines with genetic abnormalities. High-resolution evaluation of chromosomal integrity can also be done using comparative genomic hybridization.

The cells derived autologously are generally considered safe, immunotolerant, showing a higher rate of survival and acceptability after transplantation. However, immune-rejection and teratogenicity of autologous transplanted iPSCs have also been reported (Zhao et al. 2009). Further studies did not report immune rejection of iPSCs-derived differentiated cells transplanted in syngeneic recipients (Lalit

Table 7.5 Pre-clinical studies on the use of iPSCs in cardiovascular diseases

Tissue defect	References	Study description	Key findings
Myocardial Infarction	Nelson et al. (2009)	iPSCs reprogrammed from MEFs using lentiviral-reprogramming method and IM injection in a mouse model and followed-up for 4 weeks	↓LV function, ↓pathological remodeling differentiation into CMs, SkMs, ECs. No teratomas in immunocompetent mice but teratomas in immunodeficient mice
	Singla et al. (2011)	IM injection of iPSCs derived from reprogrammed mouse H9c2 cardiomyoblasts and transplanted into a mouse model of MI for 2 weeks	↑ventricular function, ↓apoptosis. No teratomas in immunocompetent mice
	Zhao et al. (2011a, b)	iPSCs reprogrammed from rat BM using the human lentiviral -based protocol f reprogramming and IM injection into rat model for 3–6 weeks	Tumorigenic independent of MI presence or absence, cell dose or duration
	Ahmed et al. (2011a, b)	iPSCs reprogrammed from mouse SkMs using retrovirus–based reprogramming method and IM injection in a mouse model and followed up for 4 weeks	Tumorigenic in 40% of immunocompetent mice.
Myocardial infarction	Mauritz et al. (2011)	iPSCs from MEFs using retrovirus reprogramming method and IM injection into mouse model of MI and followed up for 2 weeks	↑graft size ↑LV wall thickening
	Templin et al. (2012)	iPSCs from human cord blood using lentiviral–reprogramming method. IM injection into immunosuppressed pig model of MI and followed up for 12–15 weeks	iPSCs co-injected with hMSCs survived and differentiated into endothelial cells while iPSCs injected alone did not survive
	Kawamura et al. (2012)	CMs and human dermal fibroblasts derived from reprogrammed human dermal fibroblasts and delivered using scaffold-free cell patch in pig models with MI and followed up for 8 weeks	↑cardiac performance ↓ventricular remodeling Few hiPSCs-CMs were retained at the infarct site.
Myocardial Infarction	Xiong et al. (2013)	Immunosuppressed Yorkshire pigs separated into MI group, cell-treatment group and normal group.	↑LV function scar size Mobilization of endogenous progenitors.

et al. 2014). Immunogenicity is considered related to the origin of somatic cells. For example, iPSCs derived from the umbilical cord were less immunogenic than those differentiated from skin fibroblasts (Liu et al. 2013). To overcome this challenge, several research groups have attempted to use purified pre-differentiated cells for transplantation studies. An alternative approach is to identify cardiac genes that could directly reprogram fibroblasts to CMs without undergoing de-differentiation to a progenitor stage (Hanson and Lendahl 2013).

7.4.2 Skeletal Myoblasts

SkMs are the skeletal muscle-derived progenitor cells (satellite cells). They have a high proliferative capacity when cultured under optimal conditions and share similar functional and histological features with cardiac muscle (Rikhtegar et al., 2019). They are resistant to ischemia with a low risk of tumorigenicity. All this accounts for their promise in using stem cell transplantation to treat CVD. The first attempt to successfully transplant SkMs into a damaged myocardium was carried out in 1994 (Zibaitis et al. 1994). The first SkMs transplantation in patient was carried out as an adjunct to CABG by Menache et al. using autologous SkMs in a 72-year-old male patient. Results showed an improvement from NYHA class 3 to NYHA class 2 and improved LVEF after 5 months of follow-up (Wu et al. 2006a, b, c). Several pre-clinical studies in experimental animal models using SkMs to treat CVD yielded encouraging results. A study carried out in rabbits using SkMs to replace damaged myocardium results revealed improved cardiac function (Suzuki et al. 2001).

Another study in rats demonstrated stable and long-term therapeutic benefits of SkMs until 1 year after cell therapy. The study results showed markedly improved contractile function in the cell therapy group than in the control group (Alattar et al. 2003). These data also showed

that autologous SkMs transplantation improved LV function and reduced cardiac remodeling in 19 dogs with experimentally induced heart failure (He et al. 2005). The SkMs transplantation benefits are not limited to the infarcted heart; they have also shown therapeutic benefits in the non-ischemic myocardium (Pouly et al. 2004). Encouraging results from these pre-clinical studies have paved the way for their use in humans. The first pilot study was carried out on five patients to demonstrate the possibility of using the percutaneous cell delivery approach as the sole therapy for patients with post-MI heart failure. The results of the study showed improved LVEF during a 3-months follow-up as compared to their respective baseline LVEF (Smits et al. 2003). Similar encouraging results were reported in several subsequent pilot studies, which led to randomized clinical trials. The Ponzan trial by Siminiak et al. revealed that treatment of post-MI heart failure patients with transvenous transplantation of autologous SkMs improved both the NYHA class and LVEF during a 6-months follow-up (Siminiak et al. 2005). This was similar to the results reported with the SEISMIC trial (Duckers et al. 2011). On the contrary, the MAGIC trial led by Menache et al. failed to record any clinical efficacy and was terminated due to high incidence of arrhythmias in the patients receiving SkMs therapy (Menasché et al. 2008).

7.4.2.1 Limitations

Despite the promising results from pre-clinical experimental studies and clinical trials with SkMs in the treatment of heart failure, several limitations have been ascribed to the use of SkMs. One such limitation was the increased occurrence of arrhythmias associated with SkMs treatment. SkMs are unable to express adhesion molecules and failed to develop gap junctions much needed to electromechanically couple each other and with the host CMs. Failure to establish gap junctions thus hindered them from beating synchronously with the host CMs, thus pre-disposing the development of arrhythmias (Abraham et al. 2005). However, the Ponzan trial showed that treatment of post-MI heart failure patients with transvenous transplantation of autologous SkMs did not result in arrhythmias in patients pre-treated with amiodarone (Siminiak et al. 2005). Another limitation of autologous SkMs was hinged on the observation that the grafted SkMs differentiated into skeletal muscle rather than CMs (Siminiak et al. 2005) (Table 7.6 and 7.7).

7.4.3 Endometrial/Menstrual Blood-Derived Stem Cells

Menstrual blood-derived stem cells (MenSCs) demonstrated their benefits following research into their potential use to ameliorate disease progression in Duchenne's muscular dystrophy (DMD) murine models. These cells evoke sarcolemmal dystrophin expression in host myocytes through intercellular transfer (Cui et al. 2007). Also, in an experiment on comparative efficiency compared to their BM counterparts, improved cardiac function with increased LVFS and a decreased infarct size was observed with MenSCs (Hida et al. 2008). The exact mechanism of improved cardiac function elicited by MenSCs is primarily through paracrine mechanisms (Fig. 7.4). Jiang et al. reported that IM injection of MenSCs after LAD ligation improved cardiac function by activating survival signaling pathways and endogenous c-kit+ cell recruitment (Jiang et al. 2013). Another mechanism advocated by Lan et al. 2017 and Xu et al. 2017 is that MenSCs improve cardiac function by immunomodulation mainly through suppression of humoral immune response,

Table 7.6 Pre-clinical studies on the therapeutic potential of SkMs in cardiovascular disease

Tissue defect	References	Description	Outcomes
Heart failure	Suzuki et al. (2001)	Intracoronary injection of SkMs following doxorubicin-induced heart failure in rats	↓mortality. Improved hemodynamic parameters
Dilated cardiomyopathy	Pouly et al. (2004)	IM infusion of SkMs in Syrian hamsters with induced dilated cardiomyopathy	↑FAC; ↓fibrosis
Chronic myocardial infarction.	Gavira et al. (2009)	Gottingen mini pigs with chronic infarction divided into four groups that received either the control or one, two or three doses of SkMs and followed up for 7 months	↑LVEF, ↑tissue vasculogenesis and ↓fibrosis in pigs that received three doses vs those that received one dose
Post-infarction chronic heart failure	Fukushima et al. (2008)	IM or intracoronary transplantation of SkMs in rats after 3 weeks of coronary artery ligation	↑cardiac function; ↑physical activity ↓cardiomyocyte differentiation and fibrosis

Table 7.7 Clinical trials on the therapeutic potential of SkMs in cardiovascular disease

Tissue defect	References	Study description	Delivery methods	Key findings
Post-infarction LV dysfunction	Menasché et al. (2003)	Nonrandomized uncontrolled study with ten patients in the treatment group	IM injection	↑LVEF ↑regional wall ↓NYHA class
Non-acute myocardial infarction	Herreros et al. (2003)	Nonrandomized uncontrolled study with twelve patients in the treatment group	Transepicardial	↑EF
Post-infarction with severe LV dysfunction	Ince et al. (2004)	Matched controlled with six patients in both the control and treatment group	Transendocardial	↑LVEF Ventricular tachycardia
Post-infarction myocardial impairment	Siminiak et al. (2005)	Nonrandomized uncontrolled study with ten patients in the treatment group. (POZNAN TRIAL)	Percutaneous trans-coronary venous	↑LVEF ↓NYHA class
Ischemic heart failure	Hagege et al. (2006)	Long- term follow up of the first phase I of a cohort of nine patients	IM injection during CABG.	↑LVEF ↓NYHA class Ventricular arrhythmias in 5 patients
Ischemic cardiomyopathy	Menasché et al. (2008)	First randomized placebo-controlled study in 97 patients (treatment group & 30 patients (control group). Received high dose cells (800×10^6) and low dose cells ($400 \times 10^{6)}$ (MAGIC TRIAL)	IM injection during CABG.	↓LVESV ↓LVEDV in group that received high dose. Ventricular arrhythmias in 5 patients and 4 deaths in high dose group
Congestive heart failure	Duckers et al. (2011)	Final results of a phase IIa randomized open-label trial consisting of 26 patients in the treatment group and 14 patients in control. (SEIMIC TRIAL)	IM injection Transendocardial	↓NYHA Arrhythmias in 12 patients and 1 death.

making them an exciting prospect in cardio-regenerative therapy because of the potential lack of allograft rejection (Lan et al. 2017; Xu et al. 2017).

The therapeutic benefits of MenSCs in humans have been studied in a recent clinical study, which showed increased muscular strength and normalization of dystrophin levels in a 23-year-old male with DMD (Ichim et al. 2010a, b). From a cardiovascular therapy standpoint, one study was reported to have demonstrated an improved LVEF in a 74-year-old patient with heart failure besides a decrease in Pro-BNP (Ichim et al. 2010a, b). A double-blinded placebo-controlled trial, RECOVER-ERC, launched by Medistem in 2012 showed the safety and efficacy of the transplanted MenSCs in patients with heart failure (HF) (Bockeria et al. 2013). Another pilot study by Murphy et al. in a pre-clinical setting proposed using MenSCs in limb ischemia after their research revealed that intramuscular injections of these cells in rats' hind limbs successfully stopped necrosis via restoration of blood circulation (Murphy et al. 2008).

7.4.3.1 The Case for Endometrial-Derived Stem Cells

MenSCs have the attributes of tremendous proliferative capacity (>30 population doublings) compared to other stromal cells derived from BM and dental pulp, and their immature phenotypic constitution, which allows them to differentiate into the cells from all three germ layers (Meng et al. 2007; Patel et al. 2008). Also, endometrium-derived cells have strong angiogenic properties noticeable from the high-level expression of VEGF receptors, which may be responsible for the growth of the decidua and enable the implantation of the embryo (Fan et al. 2008). It is noteworthy that these cells have a protracted telomerase activity compared to BM stromal cells, which implies delayed aging (Nesselmann et al. 2009; Miranović 2016).

A pre-clinical study conducted by Hida et al. provided the best evidence yet make a case for MenSCs compared to BM cells. They reported remarkable improvement in LVFS and reduced infarct size 2 weeks after MenSCs transplantation in

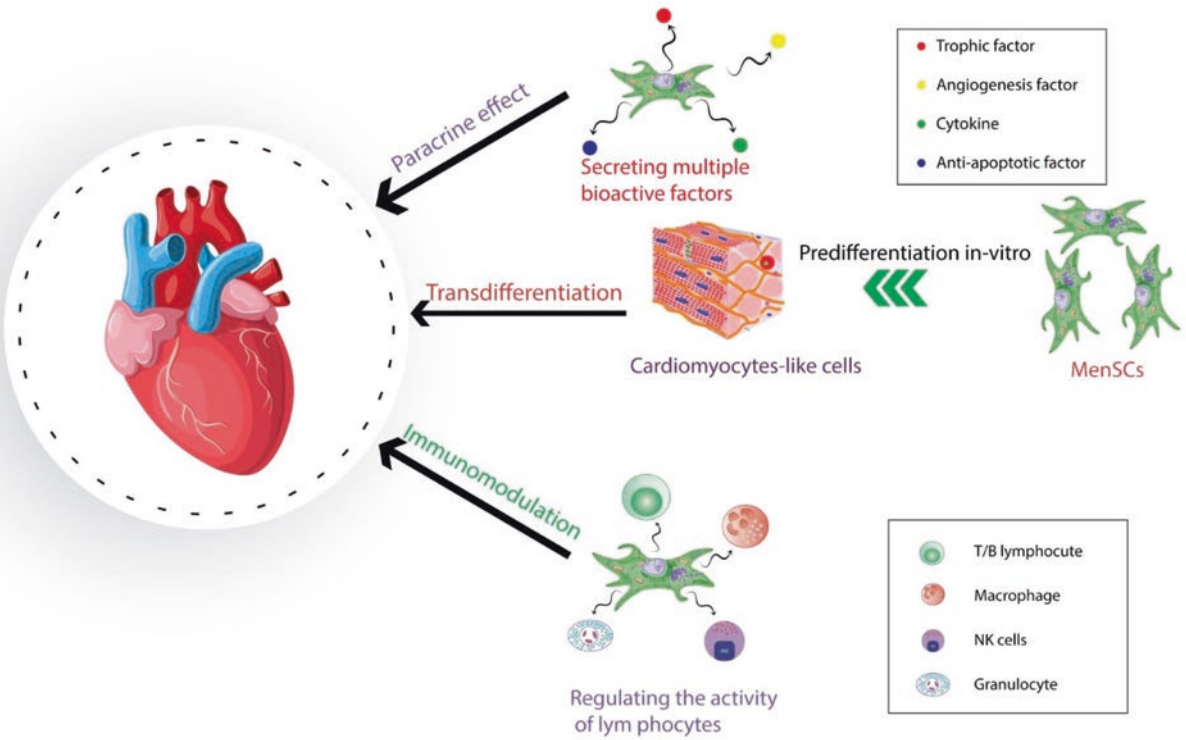

Fig. 7.4 Possible mechanisms of cardio-regeneration by menstrual blood-derived stem cells**.** The plasticity of menstrual blood-derived stem cells means transdifferentiation to cardiac cells. Immune regulation potential of these cells involves the inhibition of B- and T- lymphocytes and natural killer (NK) cells thereby increasing homing of the transplanted stem cells

rat MI model compared to those that received BM-derived stromal cells. MenSCs' higher cardiomyogenic differentiation potential was implied as a reason for the reported data (Hida et al. 2008).

7.5 Exosomes

An exciting new field that has emerged in cardio-regenerative therapy is the use of exosomes as part of the fast-emerging cell-free therapy approach (Haider and Aslam 2018) (Fig. 7.1). Exosomes are extracellular vesicles that carry nucleic acids and proteins as their payload for intercellular exchange and communication (Gezer et al. 2014; Ahadi et al. 2016). They have been implicated as the primary paracrine mechanism for stem cells' cardio-regenerative ability (Gartz and Strande 2018). Various studies have used exosomes derived from different stem cell types in cardiovascular therapy (Haider and Aramini 2020). Key studies are discussed below.

7.5.1 Pluripotent Stem Cell-Derived Exosomes

A study by Adamiak et al. showed the iPSC-derived exosomes are cytoprotective and cause in vivo LV, vascularization, and reversal of hypertrophy in murine models (Adamiak et al. 2018). Meanwhile, Khan et al. had demonstrated that mouse ESCs-derived exosomes promoted repair of cardiomyocytes following MI induced by ligation of LAD (Khan et al. 2015). The ability of exosomes derived from pluripotent stem cells to stimulate cardiac repair is attributed to numerous micro-ribonucleic acids (miRNAs) and proteins payload which they carry such as bone morphogenetic protein 4 (BMP4), platelet-derived growth factor alpha (PDGFA), teratocarcinoma-derived growth factor 1 (TDGF1), thrombospondin 1 (THBS1), and vascular endothelial growth factor C (VEGFC) (Wang et al. 2017).

7.5.2 Multipotent MSC-Derived Exosomes

Evidence from the study conducted by Wang and his colleagues (2017) showed that exosomes derived from endometrial-derived MSCs were cardioprotective in the domains of cell survival and angiogenesis (Wang et al. 2017). The key cytoprotective agents identified in these vesicles were a list of miRNAs, in particular miR-21. However, Ma et al. reported the cardiac regenerative potential of exosomes extracted from Akt-overexpressing human umbilical cord MSCs (Ma et al. 2017). The main mechanism theorized for their cardio-regenerative and

angiogenic ability was through the activation of platelet-derived growth factor-D (PDGF-D). On the same note, exosomes derived from BM-MSCs enhanced cardiac repair (Zhu et al. 2018). The identified factors in promoting cardiac repair in these cells were miR-210 and neutral sphingomyelinase 2 (nSMase2).

7.5.3 Multipotent Cardiac Stem and Progenitor Cell-Derived Exosomes

Encouraged by the success demonstrated by exosomes derived from MSCs, other scientists also experimented with exosomes derived from other adult stem cells. Exosomes from CPCs have emerged as a major focus due to their ability to repair and regenerate ischemic tissue. Tseliou et al. demonstrated that CDCs-derived exosomes converted dermal fibroblasts into active cells with scar-reducing capabilities (Tseliou et al. 2015). The same exosomes, when injected into the infarcted myocardium of pigs following reperfusion, resulted in infarct size reduction, and the improved global cardiac function after a month of treatment (Gallet et al. 2017).

7.5.4 Advantages of Exosomes Over Traditional Stem Cell Therapies

Being smaller and less complex than stem cells, exosomes offer the unique advantage of easy storage, reconstitution, and production in large quantities (Dougherty et al. 2017). Moreover, their use is safer as they lack tumorigenic potential and immunogenicity (Dougherty et al. 2017). The potential for targeted therapy using exosomes is limitless since they can be selectively harvested and their uptake is swift at the cellular level (Mathiyalagan et al. 2017). Indeed, cellular targeting using exosomes was demonstrated in a rat heart model of MI with human platelet-derived microparticles. The data showed significantly increased angiogenesis in the infarct region (Brill et al. 2005). Vicencio et al. demonstrated attenuation of infarct size expansion in the post-MI rat heart model after tail vein injection of plasma exosomes (Vicencio et al. 2015). When specificity is the goal, the harvested exosomes were targeted to the specific cell type (Alvarez-Erviti et al. 2011). For example, dendritic cells were engineered to express Lamp2b, an exosomal protein with specificity for acetylcholine receptors. However, despite having these advantages, the main disadvantage of exosomes is the labor-intensive large-scale generation processes and their inability to regenerate (Martin-Rendon et al. 2008; Vakhshiteh et al. 2019).

7.6 Stem Cells as the Future of Cardiovascular Disease Therapy

With all the trials documented so far, it is clear that stem cells are the future for the treatment of CVD. More than two decades of research has led to this point with potential ramifications for therapeutic use, yet adoption is critically low. What does this say about the future of stem cells in cardiovascular regeneration? For starters, ethical consideration will always set the pace especially for first- and second-generation stem cells. There is still that gray area for the translation of pre-clinical studies to human trials. Next-generation products do not carry these many ethical concerns and hence maybe the path forward if cardiac regenerative therapy with stem cells becomes part of the standard practice. The contributions of other generation products should not in any way be dismissed. Through research hypotheses and theories by scientists, they have led to longitudinal advances that have helped us to understand the physiologic functions of the cardiovascular system and disease pathophysiology involved therein.

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Stem-Cell-Based Cardiac Regeneration: Is There a Place For Optimism in the Future?

8

Alexander E. Berezin and Alexander A. Berezin

Abbreviations

AE Adverse effect
AMI Acute myocardial infarction
BMMNCs Bone marrow mononuclear cells
CABG Coronary artery bypass grafting
CDCs Cardiosphere-derived cells
CMGs Cardiomyogenic cells
CSCs Cardiac stem cells
CV Cardiovascular
EF Ejection fraction
EPCs Endothelial progenitor cells
ESCs Embryonic stem cells
EVs Extracellular vesicles
HF Heart failure
HFrEF Heart failure with reduced ejection fraction
HRV Heart rate variability
HSCs Hematopoietic stem cells
ISCT International society for cellular therapy
iPSCs Induced pluripotent stem cells
LV Left ventricle
LVEDV Left ventricular end-diastolic volume
LVESV Left ventricular end-systolic volume
MACE Major adverse cardiovascular event
MI Myocardial infarction
MPCs Mononuclear progenitor cells
MSCs Mesenchymal stromal cells
NYHA New York Heart Association
PET Positron emission tomography
PI3K Phosphoinositide 3-kinases
RCT Randomized clinical trial
SAE Serious adverse effect
SDNN Standard deviation of NN intervals
SPECT Single-photon emission computerized tomography

A. E. Berezin (✉)
Internal Medicine Department, Zaporozhye Medical University, Zaporozhye, Ukraine

A. A. Berezin
Internal Medicine Department, Medical Academy of Postgraduating Education, Zaporozhye, Ukraine

8.1 Introduction

Previously, it was postulated that human bone-marrow (BM)-derived mesenchymal stem cells (MSCs)/human-induced pluripotent stem cells (iPSCs) or their secretome could be sources for new/rejuvenated cardiomyocytes. Unfortunately, experimental studies during the last decade have not shown clinical benefits resulting from stem-cell therapy for heart failure (HF) with reduced left ventricular (LV) ejection fraction (LVEF) and reported inconsistent improvement after treatment with stem cells in patients after myocardial infarction (MI). It is argued that MSCs injected into the myocardium alone may not be the answer to HF progression in patients with MI and dilated cardiomyopathy. Developing a multipronged approach based on simultaneous intramyocardial delivery of both major types of stem cells would be able to induce angiogenesis, vascular reparation, and regeneration, as well as improve the endothelial cell (EPC) survival and cardiomyocytes rejuvenation, thus leading to attenuated infarct size. These effects are directly related to modifications in the cardiac-specific genes' expression, which contributes to the synthesis of structural as well as ion channels' proteins, declines mitochondrial distress, supports cellular metabolism, and enhances the microenvironmental factors including fibroblasts, progenitor EPCs, and cardiomyocytes. Probably, this approach would reduce the potential risk of fatal arrhythmias and prevent severe contractile dysfunction. Although there is a large body of evidence regarding the low efficacy of cell therapy in HF patients, novel stem-cell-based methodologies are discussed in this chapter.

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8.2 Stem-Cell-Based Therapy of Failing Heart

Stem-cell-based therapy of the failing heart has been considered as an important alternative treatment strategy to durably restore LV-function and improve clinical outcome in patients with known HF due to several reasons, either ischemic or nonischemic (Menasché and Vanneaux 2016). Although numerous completed clinical trials have highlighted several controversial issues toward survival and quality of life in the patients with acute myocardial infarction (AMI) and severe refractory HF with reduced LV ejection fraction (HFrEF), the best clinical outcome that seems to be achieved by stem cells is the immune phenotype, which strongly matches with the target tissue (Nguyen et al. 2016). Indeed, stem cells, derived from the BM or umbilical cord blood, have been infused into the coronary arteries or injected directly into the myocardium and had a positive impact on adverse cardiac remodeling and global myocardial function; the long-term prognosis lacked any significant improvement (Menasché 2017). Moreover, there were several difficulties to translate various methodologies of stem-cell harvesting, isolation, and purification in the routine clinical practice.

There are at least three generations of stem cells that either were used or are currently being used in clinical practice. The first-generation stem-cell therapy was based on the utility of heterogeneous populations of cells, such as bone-marrow mononuclear cells (BMMNCs), MSCs, and EPCs predominantly isolated from the BM or rarely from peripheral blood. The second-generation stem cells utilized purified cardiac cell populations (c-kit+ cardiac stem cells (CSCs) and cardiosphere-derived cells, embryonic stem cell (ESCs)-derived cells, allogeneic cells, cardiopoietic cells, and their combinations). The newly proposed third-generation cell therapy is using several stem and progenitor cells derived from the placenta and umbilical cord cells, iPSCs, stem cell-derived exosomes, and cell patches (Turner et al. 2020).

However, the use of cardiac-committed cells, including the iPSC-derived cardiac progeny, has been particularly attractive for clinical application. Although adult cardiac-committed stem cells were once believed to have the potency to directly create new cardiac tissues or replace injured and nonviable myocardium (Wollert and Drexler 2010), preclinical and clinical studies had yielded that these stem cells release cardioprotective paracrine factors, which act as activators of endogenous repair mechanisms leading to myocardial reparation and preserved myocardial function (Goradel et al. 2018). Effective translation of stem-cell-based therapy in routine clinical practice remains challenging due to inconclusive clinical trial results and the ability of stem cells to improve cardiac function and reduce mortality.

8.3 Primary Mechanisms of Action of Cellular Cell Therapy

There are at least two paradigms that explain the effect of implanted pluripotent stem cells on structure and function of a failing heart. Figure 8.1 consists of pathophysiological mechanisms that explain the positive effects of various stem cell populations. The first one is based on the idea that implanted adult stem cells could be structurally integrated into the recipient myocardium and directly repairs the cardiac tissue (Orlic et al. 2001). Thus, according to this hypothesis, the implanted adult stem cells can directly differentiate into cardiomyocytes, EPCs, smooth muscle cells, fibroblasts, and engraft into the myocardium, vasculature, and interstitial tissue (Balsam et al. 2004).

This suggestion has been supported by the evidence of lack of myocardial regeneration from blood-borne partner-derived cells and long-term reconstitution of hematopoietic stem cells (HSCs) has been adopted by only traditional hematopoietic fates (Balsam et al. 2004; Murry et al. 2004). On the other hand, there is an evidence of the fact that non-hematopoietic MSCs, which were transplanted directly into BM prior to experimental MI in mice, were observed mobilized and homed-in to the ischemic myocardium in response to granulocyte colony-stimulating factor (G-CSF) treatment (Kawada et al. 2004). These data supported the assumption that cardiomyogenic cells mobilized from the BM could differentiate into cardiomyocytes. Although MSCs have yielded a decrease in the fibrotic heart area, increase in microvessel density, and a significant reduction in the apoptotic positive index in the ischemic myocardium, they have demonstrated poor viability postengraftment (Song et al. 2007, 2009). Surprisingly, genetically engineered MSCs overexpressing tissue transglutaminase were able to enhance adhesion and ultimately better cell survival after implantation via increased phosphorylation of focal adhesion-related kinases FAK, proto-oncogene tyrosine-protein kinase Src, and phosphoinositide 3-kinases (PI3K)/Akt/mTOR, thereby leading to preserved cardiac function (Song et al. 2007; Guertin et al. 2006; Hemmings and Restuccia 2012). Thus, the enhancement of engrafted MSC adhesion improves the survival of MSCs and their ability to transdifferentiate into cardiomyocytes (Song et al. 2010).

The second paradigm was proposed recently and suggested that secretome of implanted stem cells is the cargo of biomolecules that ensure stimulation of endogenous repair processes (Fadini et al. 2012; Berezin 2019). The primary cardioprotective mechanism of stem cells, either infused into circulation or transplanted directly into the myocardium, is the transduction of paracrine signals that mediate differentiation, growth, and proliferation of a wide spectrum of progenitor cells and their precursors (Moccia

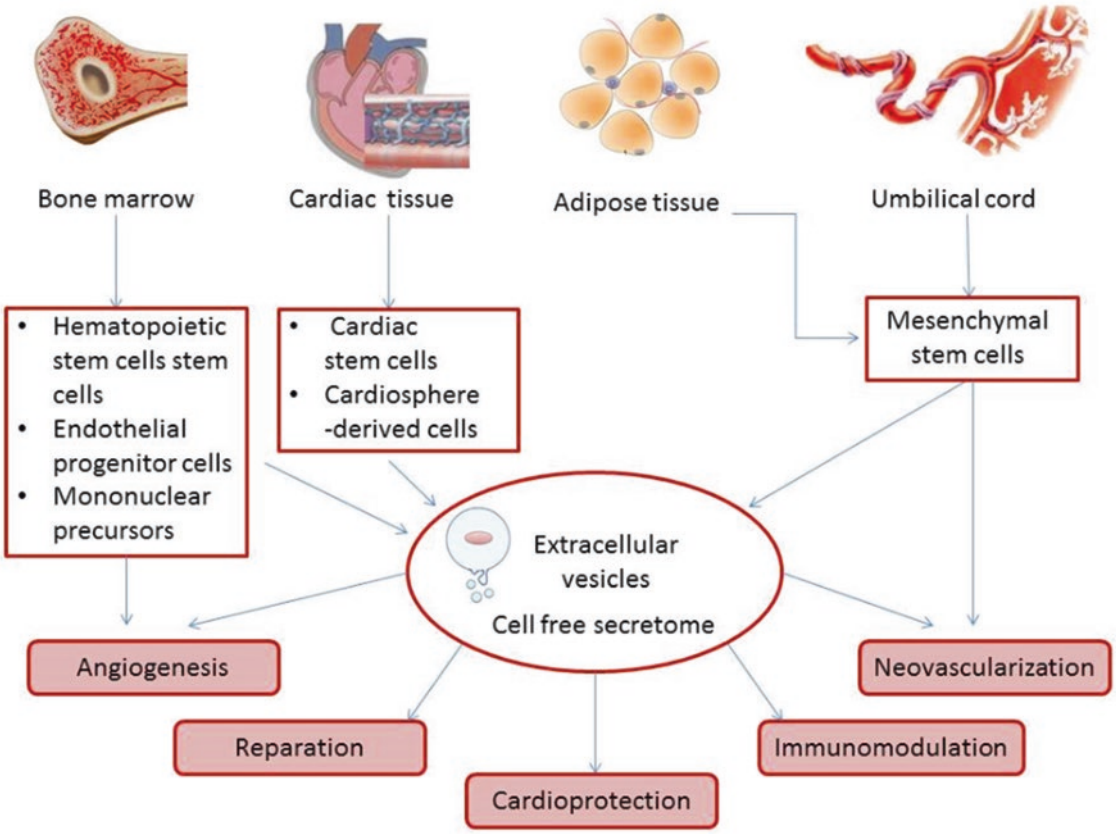

Fig. 8.1 The direct and indirect mechanisms of actions of various stem cell populations on reparation and cardioprotection

et al. 2013; Berezin and Berezin 2020). The paracrine hypothesis seems to be more flexible for allogeneic cell transplantation because risk of early rejection, infection, immunomodulation, and monoclonal activation are low while the transient engraftment ensures cardiac protection. Therefore, allogeneic stem cells can translate their effects through secretion of a wide range of extracellular vesicle-embarked growth factors (transforming growth factor-β, vascular endothelial growth factor), regulatory peptides and enzymes (integrin-linked kinase), active molecules (α2-integrins), noncoding RNAs (microRNAs, small interfering RNAs), lipids, fragments of chromatin, which are powerful cues for (trans)differentiation, proliferation, and migration of progenitor cells including pro-cardiomyocytes and endothelial precursors (Gnecchi et al. 2005; Takahashi et al. 2006). Although numerous experimental animal studies have shown favorable effects of the stem cells and their derivative extracellular vesicles (EVs) (Huang and Lai 2019), large clinical studies are required to confirm whether EVs can substitute for adult stem cells and provide more profound and clinically relevant effects on cardiac tissues. The delivery of EVs seems to be attractive in point-of-care therapy, because it alleviates the ethical issues besides minimizing the risk of autoimmune response (Carotenuto et al. 2020).

8.4 Stem Cells in Adverse Cardiac Remodeling

Previous clinical studies have evaluated the efficacy of several populations of adult stem cells including BMMNCs, MSCs, and CSCs (Kubal et al. 2006; Lai et al. 2009; Ripa 2012; Der Sarkissian et al. 2017). Therapeutic remodeling of failing heart is enhanced by several pathophysiological processes that can be induced and supported by the engrafted stem cells. The potential mechanisms by which implanted stem cells reduce adverse cardiac remodeling and improve survival have been summarized in Fig. 8.2.

8.4.1 Bone-Marrow-Derived Mononuclear Cells

The human BM stromal cells being progenitors of numerous tissue components contain a small fraction of various populations of adult stem cells having multipotent capacity, which include HSCs, EPCs, MSCs, and tissue-committed stem cells, as well as other cells, such as MSCs, very small embryonic-like stem cells, and

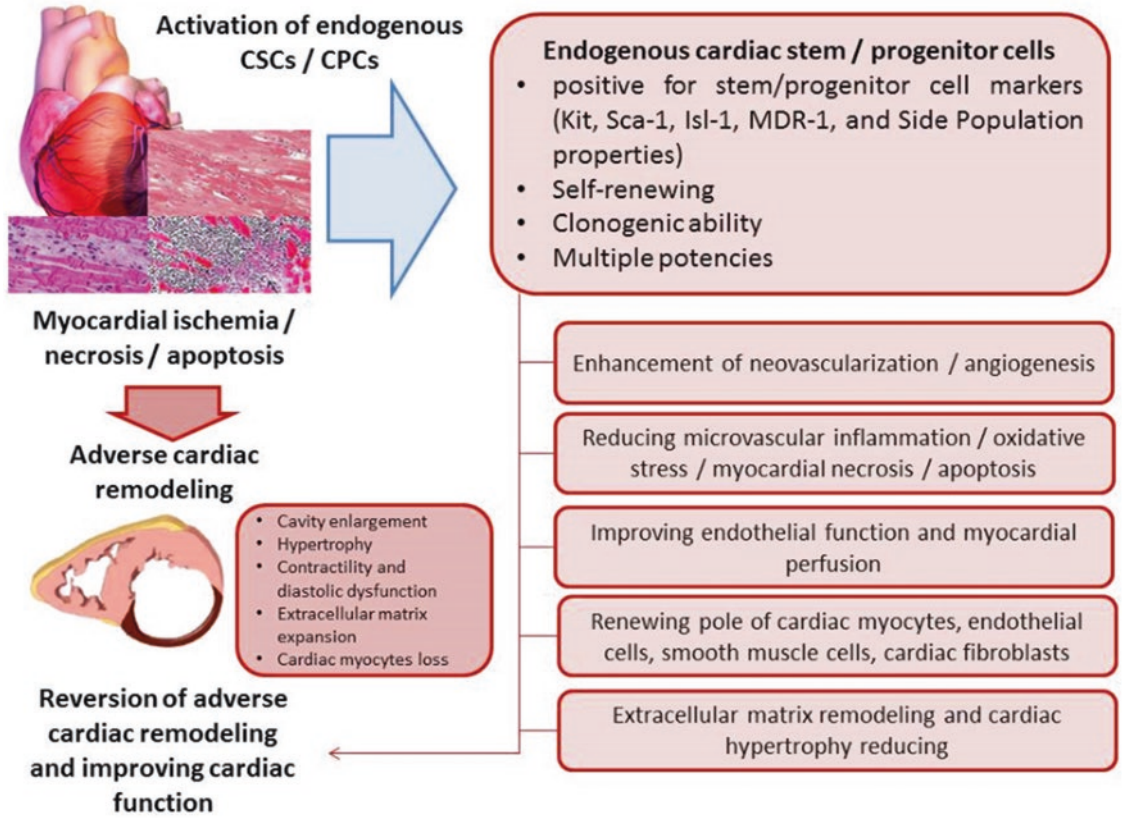

Fig. 8.2 Potential pathophysiological mechanisms by which implanted stem cells reduce adverse cardiac remodeling and improve survival. Abbreviations: *CSCs* cardiac stem cells, *CPCs* cardiac progenitor cells, *Sca-1* stem cell antigen 1, *MDR-1* multidrug resistance protein 1, *Isl-1* insulin gene enhancer protein

hemangioblasts (Bianco et al. 2001; Krause et al. 2010). These fractions can be purified using a density gradient centrifugation, magnetic separation, and concentration to obtain a cell mixture with similar density and size, but different from the myeloid cells and red blood cell progenitors, commonly known as BMMNCs (Cuende et al. 2012). BMMNCs contain various hematopoietic progenitor cells at different stages of maturation as well as lymphoid cells (T- and B lymphocytes, plasma cells), monocytes, mononuclear fractions, and macrophages (Miyamoto et al. 2007). Some fractions of the BMMNCs, which express CD34 or CD133 markers, have yielded reparative potency due to the expression of higher levels of microRNAs for angiogenic cytokines (including vascular endothelial growth factor A, fibroblast growth factor 2, and hepatocyte growth factor) (Wang et al. 2014).

There is mounting evidence that the BMMNCs are mobilized from remote tissues in response to ischemia-induced cytokines through FOXO3a/NF-κB/CXCR7-dependent mechanism and the circulating levels of the BMMNCs dramatically increase in the peripheral blood circulation (Fan et al. 2020; Spinetti et al. 2013; Gremmels et al. 2019), while some patients with severe diseases, such as sepsis, autoimmune, and connective tissue diseases, diabetes mellitus, had lower levels of the BMMNCs in circulation (Kollet et al. 2003; Schmidt-Lucke et al. 2005; Berezin 2016). Given that these cells contribute to tissue regeneration and cell renovation under physiological circumstances as part of homeostasis, altered function and lower levels of ones are considered as a weakness of endogenous repair system (Berezin 2017; Wang et al. 2020). These cells incorporate into foci of neovascularization, neoangiogenesis, which is consistent with postnatal vasculogenesis, and regeneration of the damaged tissue (Asahara et al. 1999; Bauer et al. 2006; Murohara 2003). Moreover, lower levels of circulating BMMNCs besides EPCs and mononuclear progenitor cells strongly predict new cardiovascular events in the future (Hill et al. 2003; Kaihan et al. 2019; Schreier and Triampo 2020). In this context, predominant depletion of the progenitor cell-reserve in the circulation, rather than decreased mobilization, underlies the association between BMMNCs numbers and CV risk (Gremmels et al. 2019; Vasa et al. 2001).

8.4.2 Mesenchymal Stromal Cells

According to the International Society for Cellular Therapy, MSCs are defined as spindle-shaped plastic-adherent mesoderm-derived stem cells isolated from numerous tissues including the BM, adipose, and connective tissues, umbilical cord blood, skeletal muscles, and cardiac tissue (Horwitz et al. 2005). Like BMMNCs, isolated MSCs are not a homogenous population of stem cells and include numerous fractions of the cells with different origins, immune phenotypes, size and sedimentation, ability of plastic adherence, and tri-lineage differentiation (Muguruma et al. 2006). However, various populations of MSCs express CD105, CD73, and CD90 and lack the expression of CD45, CD34, CD14 or CD11b, CD79-alpha/CD19, and HLA-DR surface antigens (Dominici et al. 2006; McNiece 2007). Additionally, MSCs cultured in vitro should undergo tri-lineage differentiation to adopt osteogenic, adipogenic, and chondrogenic phenotypes. Intravenously infused MSCs may home in and incorporate in the desired tissue to participate in the repair process. MSCs also have immunosuppressive potential and enjoy immunoprivileged status. There is no evidence of how biologically similar MSCs from various tissues including adipose tissue-derived MSCs and cardiac tissue-derived MSCs exhibit diverging cardio-protective and angiopoietic abilities (Le Blanc and Ringdén 2005). It is interesting to note that successful engraftment of MSCs was qualified as tissue and disease-specific regardless of either delivery methods or origin of MSCs (Lazarus et al. 2005). Isolation, purification, and in-vitro expansion MSCs are tedious and time-intensive processes that have some restrictions for the large-scale production of MSCs for routine clinical utility.

8.4.3 Cardiac-Derived Stem Cells

Several populationsof resident Lin-negative c-kit-positive cardiac progenitor cells (CPCs) exist in post-natal human cardiac tissue. CSCs with self-renewing, clonogenic, and multipotentiality have demonstrated the ability to differentiate into cardiomyocytes (Bearzi et al. 2007; Beltrami et al. 2003). Besides CSCs, there is a population of cardiosphere-forming cells (known as cardiosphere-derived cells—CDCs) with stem-cell-like characteristics (Smith et al. 2007). Experimental animal studies have shown that after direct injection into the border zone or center of the ischemic myocardium, these cells and their clonal progeny undergo differentiation into morphofunctionally competent cardiomyocytes and blood vessel-forming myocytes to reconstitute the injured myocardium (Yoon et al. 2005; Smith et al. 2007). However, isolation of cardiac tissue-derived stem cells requires sophisticated harvesting of the donor cardiac tissue through percutaneous endomyocardial biopsies or other invasive surgical procedures (Barile et al. 2007). Consequently, further processes associated with digestion, expansion, and purification of cardiac-derived stem cells are time- and labor-intensive to generate an appropriate number of cells to engraft (Carvalho et al. 2013).

8.4.4 Pluripotent Stem Cells

Pluripotent stem cells have been extensively evaluated for cardiac regeneration and rejuvenation, but their use is associated with the problems of tumorigenesis, arrhythmias, and rejection by the recipient which has seriously hampered their progress to the clinics for routine applications. For instance, ESCs have been investigated in preclinical experimental animal studies with positive effect on ischemia-damaged myocardium (Levenberg et al. 2010). Similarly, iPSCs are considered as promising alternatives to ESCs because they have autologous availability and incredible differentiation potential and can be derived from various somatic cells (Duelen and Sampaolesi 2017). The major concerns regarding their clinical usage are tumorigenesis and arrhythmias in the recipients after their implantation (Kempf et al. 2014).

Put together, early experimental studies have yielded clear evidence about the benefits of the transplanted stem and progenitor cells including BMMNCs, CSCs, and EPCs in terms of overcoming the post-MI adverse cardiac remodeling (Rosenzweig 2006). The safety and feasibility of autologous stem and progenitor cell transplantation in patients with ischemia/MI-induced cardiac remodeling had been identified by numerous investigators as acceptable (Ng 2004).

8.5 Stem Cells in Clinical Trials for Patients with Ischemia-Induced Adverse Cardiac Remodeling

There are a large number of clinical trials wherein intracoronary infusion of the autologous stem and progenitor cells was performed to evaluate their beneficial effects to prevent postinfarction adverse cardiac remodeling and preserve LV function in short-term and long-term perspectives (Table 8.1). TOPCARE-AMI (Transplantation of Progenitor Cells and Regeneration Enhancement in AMI) randomized clinical trial (RCT) prospectively enrolled 20 AMI patients who were stabilized by routine reperfusion intervention and subsequently received intracoronary infusion of either BM-derived or circulating blood-derived progenitor cells into the culprit coronary artery (Assmus et al. 2002). Authors have reported that intracoronary infusion of progenitor cells

Table 8.1 Summary of the clinical trials using autologous stem and progenitor cells to evaluate their beneficial effects to prevent postinfarction adverse cardiac remodeling and preserve LV function in short- and long-term perspectives

RTC name	n	Delivery	Duration	Outcomes	References
Engrafted cell types: autologous BMMNCS					
TOPCARE-AMI	31	i/c	4 months	↑ LVEF, perfusion and viability	Assmus et al. (2002)
BOOST	60	i/c	18 months	Lack of improvement of cardiac function and structure	Drexler et al. (2006)
LEUVEN-AMI	67	i/c	4 months	↑ LVEF, ↓ infarct size	Janssens et al. (2006)
REPAIR-AMI	204	i/c	12 months	↑ LVEF, ↑ regional infarct contractility, ↓MACE	Schächinger et al. (2006)
ASTAMI	97	i/c	6 months	Lack of LVEF changes, ↑ exercise capacity	Lunde et al. (2006)
FINCELL	80	i/c	6 month	↑ LVEF	Huikuri et al. (2008)
MYSTAR	60	i/c and transendocardial	12 months	↑ LVEF, viability, perfusion and ↓ infarct size	Gyöngyösi et al. (2009)
REGENT	200	i/c	6 months	Lack of LVEF changes	Tendera et al. (2009)
BONAMI	101	i/c	3 months	Lack of LVEF changes	Roncalli et al. (2011)
HEBE	200	i/c	12 months	Lack of LVEF changes	Hirsch et al. (2011)
LATE-TIME	87	i/c	6 months	Lack of LV global and local contractility function changes	Traverse et al. (2011)
TIME	120	i/c	12 months	Lack of LVEF changes and clinical improvement	Traverse et al. (2012)
SWISS-AMI	192	i/c	12 months	Lack of LVEF changes and clinical improvement	Sürder et al. (2013)
TECAM	120	i/c	12 months	Lack of LVEF changes and clinical improvement	San Roman et al. (2015)
Engrafted cell types: human ATDRCs					
APOLLO	14	i/c	6 months	↑ LVEF, perfusion, scar formation	Houtgraaf et al. (2012)
Engrafted cell types: human MSCs					
Safety Study of Adult MSC to Treat AMI; NCT00114452	53	i/v	6 months	↑ LVEF and ↓ symptoms	Hare et al. (2009)
POSEIDON	30	transendocardial	1 year	↓ SAEs, ↑ functional capacity, quality of life, ↓ of LV remodelling	Hare et al. (2012)
TAC-HFT	65	transendocardial	1 year	↑ regionar LV myocardial function at the site of MSCs injection. Lack of changes in LV chamber volume and global LVEF	Heldman et al. (2014)
PROMETHEUS	6	Intramyocardial injection	18 months	↑ LVEF, ↓ scar mass, ↑ perfusion	Karantalis et al. (2014)
Engrafted cell types: human autologous myoblast cells					
MAGIC	67	Intramyocardial injection in post MI scars	6 months	↓ LVEDV	Menasché et al. (2008)
Engrafted cell types: human cardiosphere-derived cells					
CADUCEUS	25	i/c	6 months	Lack of global LVEF changes, ↓ scar, ↑ viable mass and regional contractility	Makkar et al. (2012)
Engrafted cell types: human peripheral CE34+ pre-treated with G-CSF					
ACT34-CMI	167	trans-endocardial	6 months	↑ physical exercise	Losordo et al. (2011)
PreSERVE-AMI	161	i/c	12 months	Lack of differences in myocardial perfusion or AE, ↓ LVEF and MI size	Quyyumi et al. (2017)

Abbreviations: *AE* adverse effect, *RCT* randomized clinical trial, *BMMNCs* bone-marrow-derived mononuclear cells, *i/c* intracoronary, *LVEF* left ventricular ejection fraction, *G-CSF* granulocyte-colony stimulating factor, *t/e* transendocardial, *i/m* intramyocardial, ↑ increase, ↓ decrease

was associated with a significant increase in global cardiac function ($p = 0.003$), improved regional LV contractility ($p < 0.001$), and significantly reduced LVESV during 4-month follow-up. However, there were no significant changes in these parameters in the nonrandomized matching reference group. The coronary blood flow reserve in the culprit artery and myocardial viability in the infarcted area were significantly improved in the randomized group (Britten et al. 2003; Tendera and Wojakowski 2005). Investigators also emphasized the absence of any clinically significant inflammatory response and malignant arrhythmias in the cell therapy recipients (Obradovic et al. 2004). One-year follow-up using contrast-enhanced MRI showed significantly increased LVEF ($p < 0.001$), reduced LV area of dyskinesia ($p < 0.001$), and absence of reactive LV hypertrophy, which were suggestive of functional myocardial regeneration (Schächinger et al. 2004). In the BOOST RCT, 60 STEMI patients were randomized into control group ($n = 30$) or autologous BMMNC group ($n = 30$) after PCI. All patients received optimal medical treatment. Over 6 months, mean global LVEF had significantly increased ($p = 0.0026$) in autologous BMMNCs treatment group as compared to the control group. Nevertheless, stem-cell delivery did not increase the risk of adverse clinical events, in-stent stenosis, or fatal arrhythmias (Wollert et al. 2004).

The autologous BM-derived stem cells infused into culprit coronary artery after PCI in STEMI patients were therapeutically superior to the placebo treatment in increasing global LVEF, reduction in the infarct size, and recovery of regional contractility function. However, myocardial perfusion and myocardial oxidative metabolism, which were assessed by serial 1-[(Beltrami et al. 2003)C]acetate cardiac positron emission tomography, exhibited strict similarity in both groups over 4-month follow-up (Janssens et al. 2006). Thus, optimal PCI was not found to turn worse global LVEF when compared with the transfer of autologous BMMNCs (Penn 2006).

REPAIR-AMI RCT has shown that intracoronary infusion of autologous BMMNCs was strongly associated with sufficient reduction in the combined clinical endpoints of cardiovascular-related deaths, recurrence of MI, and need for any revascularization procedure ($p = 0.01$), besides improved global LVEF as compared to the placebo group (Schächinger et al. 2006). In ASTAMI (autologous stem cell transplantation in AMI) RCT, no significant difference was observed between autologous BMMNC group and the placebo group in terms of change in LVEDV and infarct size (Lunde et al. 2006). Additionally, investigators have reported similarities in adverse events rate in both groups. The FINCELL RCT enrolled 80 STEMI patients (40 patients were allocated in the placebo group and 40 patients were included in BMMNC group). The patients received thrombolytic therapy followed by PCI 2–6 days after STEMI (Huikuri et al. 2008). The authors reported that cell-based therapy was associated with significant improvement of global LVEF and neutral effects on risks of fatal arrhythmia and restenosis of the culprit coronary artery. However, there was nonsignificant impact of autologous BMMNCs on the frequency of nonsustained and sustained ventricular tachycardia episodes (Trzos et al. 2009).

Combined intracoronary and intramyocardial administration of autologous BMMNCs among post-STEMI patients with LVEF ≤45% were performed in the MYSTAR study (Gyöngyösi et al. 2009). BMMNCs were administered early (early group; 3–6 weeks) and late (late group; 3–4 months) post-STEMI period. Over 3 months after initiating the therapy, infarct size and LVEF remained significantly higher than at baseline in both groups. However, the late group had better improved LV function as compared to the early group. The authors also concluded that a higher number of BMMNCs was required to achieve significant improvement in the primary endpoints. Nevertheless, there are many RCTs such as REGENT (Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in AMI), BONAMI (BOne Marrow in AMI), HEBE trial, LATE-TIME, TIME, SWISS-AMI (SWiss Multicenter Intracoronary Stem Cells Study in Acute Myocardial Infarction), and TECAM (Trial of Hematopoietic Stem Cells in Acute Myocardial Infarction), wherein no positive changes were observed in regional and global LVEF and LVEDV after implantation of BMMNCs (Tendera et al. 2009; Roncalli et al. 2011; Hirsch et al. 2011; Traverse et al. 2012; Sürder et al. 2013; San Roman et al. 2015).

RCTs involving intracoronary infusion of BM-derived MSCs in patients after STEMI have shown contradictory results. A meta-analysis of seven trials ($n = 660$) has shown that transfer of BMSCs at 4–7 days in post-STEMI patients significantly improved LVEF, reduced LV end-systolic dimensions, decreased the incidences of recurrent revascularization and the cumulative clinical events of death or MI, and lowered the rate of culprit artery restenosis or ventricular arrhythmias (Zhang et al. 2009). Similarly, meta-analysis of 16 RCTs ($n = 1641$, 984 patients in cell therapy groups, 657 patients in placebo groups) has revealed that BMMC-based therapy was associated with more pronounced absolute improvement in the global LVEF and reduction of LVEDV when compared with the placebo-treated patients ($p < 0.001$) (Delewi et al. 2014). Interestingly, the therapy was more beneficial in younger patients (age <55 years) as compared with the older patients (age ≥55 years) and individuals with baseline LVEF <40% versus those who had LVEF ≥40%. Nevertheless, authors of the meta-analysis emphasized that there was heterogeneity in the treatment effects with respect to the reduction in LVEDV and LVESV (Delewi et al. 2014). The majority of RCTs for BMMC-based therapy were designed with primary endpoints that focused on the changes

in LVEF and MI size from baseline to follow-up. The surrogate endpoints instead of hard points, that is, mortality rate and MACEs, as well as high heterogeneity of clinical studies included in the meta-analysis were the factors that possibly led to statistical bias during the evaluation of clinical efficacy and safety of the RCTs. A recent meta-analysis has shown a significant difference between the efficacy of BMMC-based therapy among patients with ischemia-induced cardiomyopathy and STEMI (Yang et al. 2020). Indeed, implantation of BMMCs corresponded to a sustained increase in LVEF in the ischemic cardiomyopathy patients, but STEMI patients exhibited minimal and clinically insignificant improvement in LVEF, thus rendering the significance of BMMCs therapy as uncertain for post-STEMI patients.

On the other hand, a wide spectrum of various limitations in BMMC therapy led to difficulties in interpretations of findings obtained. First of all, most patients received a heterogeneous mixture of subsets of BM cells. The BM obtained from these patients after MI had reduced viability, angiopoetic, and vasculogenic properties. Besides, STEMI patients with the highest levels of circulating CD34+ EPCs showed the best increase in LVEF after transplantation of BMMNCs. Although these studies support an expert opinion that heterogeneity of cell numbers and viability can affect the therapeutic potential of BMMNCs, the relative ease of availability of stem cells in BM, the low cost of their aspiration, a well-developed program for their selection and growth, and ease of acquisition have led to the widespread adoption of these cells in a huge number of preclinical and clinical studies.

8.5.1 Human Allogeneic MSCs

There are several high-quality RCTs that allow unveiling the potency of human allogeneic MSCs for post-STEMI patients or patients with ischemia-induced cardiomyopathy. The Safety Study of Adult MSCs to Treat AMI (NCT00114452) was double-blind, placebo-controlled, dose-ranging (0.5, 1.6, and 5 million cells/kg) safety trial involving intravenous injection of human allogeneic MSCs to the STEMI patients who had previously undergone successful reperfusion (n = 53) (Hare et al. 2009). This study provided evidence regarding the safety of the human allogeneic MSCs. However, no significant changes in LVEF were observed in the cell therapy patients as compared with the placebo group. The POSEIDON (Phase I/II, Randomized Pilot Study of the Comparative Safety and Efficacy of Transendocardial Injection of Autologous MSCs versus Allogeneic MSCs in Patients with Chronic Ischemic Left Ventricular Dysfunction Secondary to MI) enrolled 30 patients with LV dysfunction. The enrolled patients were randomized to receive various doses (20, 100, and 200 million cells) of allogeneic or autologous BM-derived MSCs (Hare et al. 2012). Although this trial included patients with mild-to-moderate LV dysfunction, the design of the trial allowed a non-HF study, but adverse cardiac remodeling trial. The authors concluded that transendocardial injection of allogeneic and autologous MSCs without a placebo control were both strongly associated with significantly low rate of treatment-emerging SAEs including immunologic reactions. Additionally, treatment with MSCs led to increasing functional capacity, quality of life, and attenuation of LV-remodeling among the patients with ischemic cardiomyopathy.

The TAC-HFT (Transendocardial Autologous MSCs and BMMNCs in Ischemic HF Trial) included 65 patients having ischemic cardiomyopathy with LVEF <50% (Heldman et al. 2014). The main aim of the study was to compare the safety and effectiveness of transendocardial injection of MSCs (n = 19) with placebo (n = 11) and BMCs (n = 19) with placebo (n = 10) with 1-year follow-up. The authors reported that regional LV function at the site of MSCs injection was significantly improved, but BMCs or placebo did not increase regional contractility. Therefore, LV-chamber volume and global LVEF did not change in any of the groups. In the PROMETHEUS (The Prospective Randomized Study of MSCs Therapy in Patients Undergoing Cardiac Surgery) RCT, six patients were eligible to participate in the treatment arm (injected with autologous MSCs into akinetic/hypokinetic myocardial territories) or placebo. The enrolled patients had not undergone coronary artery bypass graft (CABG) for clinical reasons (Karantalis et al. 2014). Over 18 months, the patients who received MSCs injection exhibited a significant increase in LVEF (p = 0.0002) and a decrease in scar mass (p < 0.0001) when compared with their respective baseline. Moreover, there was no LV-functional restitution in the placebo group.

Interestingly, post-hoc analyses of ambulatory ECGs collected from the POSEIDON and the TAC-HFT trials have shown that there was no significant difference in ventricular pro-arrhythmia, manifested by sustained or nonsustained ventricular ectopy or worsened HRV in the MSCs treatment and placebo groups (Ramireddy et al. 2017). Human allogeneic BM-derived MSCs are more attractive to treat patients after STEMI due to their homogeneous constitution as well as based on the encouraging data from preclinical animal studies. Additionally, the number of MSCs in freshly isolated adipose tissue is greater than that of actively aspirated from BM, because this procedure makes the cell culture unnecessary to generate therapeutically sufficient cells. However, it remains unclear whether allogeneic MSCs aspirated from different tissues and collected with standard procedures will be biologically similar and demonstrate strict similarities in terms of their angiopoetic and vasculogeneic abilities after digestion, expansion, and purification. On the other hand, engineering MSCs is economically more expending process as compared to BMMNCs' preparation.

8.5.2 Other Types of Cells

Besides MSCs, there have been several attempts to use ATDRCs, human autologous myoblast cells, and human cardiosphere-derived cells for myocardial cell therapy. The APOLLO (NCT00442806) RCT was a randomized, double-blind, placebo-controlled, phase I/IIa study. The main purpose of study was to evaluate the clinical efficacy and tolerability of ATDRCs that included a mixture of various immune-competent cells, i.e., EPCs and MSCs (Houtgraaf et al. 2012). The study enrolled 14 STEMI patients and randomized them 3:1 to receive an intracoronary infusion of either 20 million ATDRCs ($n = 10$) or placebo ($n = 4$) directly into the culprit artery. The intracoronary infusion was well tolerated and there were no reports for SAE related to ATDRC implantation including alteration of coronary blood flow or microvascular obstruction. During a 6-month follow-up, significant improvement in global LVEF and attenuated MI size were observed in ATDRC-treated group as compared to the placebo group.

The MAGIC (Myoblast Autologous Grafting in Ischemic Cardiomyopathy) trial was the first investigation of clinical efficacy and safety of autologous myoblasts in 67 post-MI patients with global LVEF ≤35% (Menasché et al. 2008). Patients received injections of myoblasts in low (400 million; $n = 33$) or high (800 million; $n = 34$) daily doses or the placebo treatment ($n = 30$), respectively. The primary efficacy endpoints were the 6-month changes in global and regional LVEF evaluated by transthoracic echocardiography. Besides an absolute increase in global LVEF, attenuated LV volumes were observed in patients who received high dose of autologous myoblasts as compared to the low-dose cells or placebo-treated patients. However, there were significantly increased events of early postoperative arrhythmias after myoblast transplantation. The 6-month follow-up showed that the rate of MACE and nonsustained/sustained ventricular arrhythmias did not significantly differ between the treatment groups and the placebo group. In a prospective, double-blind, randomized, phase II study (ClinicalTrials.gov identifier: NCT00300053), a significant improvement of myocardial perfusion and LV function was observed in 167 patients with refractory angina and ischemia-induced cardiomyopathy. The patients received one of the two cell doses (1×10^5 or 5×10^5 cells/kg) of mobilized autologous CD34+ cells or placebo (Losordo et al. 2011).

The PreSERVE-AMI (phase II, randomized, double-blind, placebo-controlled trial) is one of the largest cell-based therapy trials for STEMI completed in the USA and provided strong evidence supporting the high safety and potential efficacy in post-STEMI patients with LV dysfunction who were at risk for death and comorbidity (Quyyumi et al. 2017). The study enrolled 161 STEMI patients with LVEF ≤48% who underwent successful PCI and were randomized in 1:1 ratio to receive either autologous CD34+ cells (minimum 10 mol/L ± 20% of CD34+ cells; $n = 78$) or diluent alone as placebo ($n = 83$) through an intracoronary infusion. The results of the RCT showed no significant differences in myocardial perfusion or adverse events between treatment groups and the control group during the 6-month follow-up. However, CD34+ cell dose-dependent effect resulted in a significant change in LVEF and MI size. Over 12 months, 3.6% ($n = 3$) and 0% of deaths were observed in the placebo control and treatment groups, respectively.

The CADUCEUS (intracoronary cardiosphere-derived cells for heart regeneration after MI) RCT was the first study to unveil that intracoronary infusion of autologous CDCs after STEMI was safe and effective to significantly reduce the scar area, increase the viable myocardial mass, and improve regional LV contractility when compared with the placebo group (Makkar et al. 2012). Allogeneic CSCs (AlloCSC-01) were tested in the CAREMI (Safety and Efficacy of Intracoronary Infusion of Allogeneic Human CSCs in Patients with STEMI and Left Ventricular Dysfunction) was a phase I/II multicenter, randomized, double-blind, placebo-controlled trial in 49 STEMI patients. The patients included in the study had LVEF ≤45% and MI size ≥25% of LV mass evaluated by cardiac magnetic resonance tomography. Patients were randomized (2:1) to receive AlloCSC-01 ($n = 33$) or placebo ($n = 16$) via the intracoronary injection at days 5–7 after STEMI (Fernández-Avilés et al. 2018). The investigators did not observe any death or MACE over 12-month follow-up in both groups. No immune-related AEs were reported, and no significant difference between groups was observed in magnetic resonance-based MI size and LVEF over a 12-month follow-up. The authors concluded that AlloCSC-01 can be safely administered in STEMI patients, but CSCs did not significantly improve scar size and LV function during 1-year follow-up.

Despite promising results, widespread clinical acceptance of CSCs, CDCs, EPCs, and autologous myoblast cells for routine clinical practice remains uncertain due to several challenges. These challenges include intensive nature of the protocols for the isolation of CSCs and CDCs and the requirement of sophisticated methodology of harvesting cardiac tissue through specially designed probe for percutaneous endomyocardial biopsies or direct surgical extraction (Kawamoto and Asahara 2007; Bianconi et al. 2018; Qiu et al. 2018; Fernández-Avilés et al. 2018). Additionally, digestion, expansion, and purification of these cells to isolate desired cell type are time-consuming and expensive processes that shape serious limitation to achieve the required number of cells for their subsequent use (Torella et al. 2006; Chong et al. 2016). Finally, stem/progenitor cell-based therapy has exhibited protective paracrine effects in the majority of studies in STEMI patients as well as in patients with ischemic cardiomyopathy. Although various cells have demon-

strated difference in their ability to ensure cardiac repair and regeneration and attenuate adverse cardiac remodeling, more and larger RCTs are required for clearer understanding of the relevant paracrine molecular mechanisms contributing to the reversal of impaired cardiac function and structure, and this could be a target for future investigations.

The FOCUS-CCTRN (FOCUS-Cardiovascular Cell Therapy Research Network) study was a phase-II randomized double-blind, placebo-controlled trial, which enrolled 92 HF patients (average age was 63 years) with LVEF <50%. The patients were allocated into two treatment arms for transendocardial injection of 100 million BMMNCs ($n = 61$) or placebo treatment ($n = 31$) for a 6-month follow-up (Perin et al. 2012). After completion of the study, the authors did not find any significant difference in any of the outcomes, including change in LVESV index, maximal oxygen consumption, percent myocardial defect, total defect size, fixed defect size, regional wall motion, and clinical improvement.

The CELLWAVE (effect of shock wave-facilitated intracoronary cell therapy on LVEF in patients with chronic HF) trial is a double-blind, randomized, placebo-controlled study conducted in post-STEMI chronic HF patients treated with intracoronary administration of autologous BMMNCs (Assmus et al. 2013). Patients were randomized at low-dose ($n = 42$), high-dose ($n = 40$), or placebo ($n = 21$) groups.

The primary endpoint (change in LVEF from baseline to 4 months in the pooled groups shock wave + placebo infusion vs shock wave + BMMNCs) was significantly improved in the shock wave + BMMNC group as compared with the shock wave + placebo infusion group. Regional wall thickness was significantly improved in the shock wave + BMMNC group, but not in the shock wave + placebo infusion group. Occurrence of MACE was significantly less frequent in the shock wave + BMMNC group ($n = 32$ events) as compared with the placebo shock wave + BMMNCs ($n = 18$) and shock wave + placebo infusion ($n = 61$) groups (hazard ratio = 0.58; 95% confidence interval = 0.40–0.85; $p = 0.02$) (Table 8.2).

The investigators concluded that a relationship between improved LVEF and reduction in MACE after transplantation of BMMNCs required concise confirmation in larger clinical trials.

The TOPCARE-CHD (Transplantation of Progenitor Cells and Regeneration Enhancement in Chronic Post-infarction HF) trial showed small but significant increase in LVEF after BMMNCs treatment besides sufficient decline in the serum levels of N-terminal pro-brain natriuretic peptide (NT-proBNP). However, scar size was not reduced when compared to placebo group (Assmus et al. 2007). Unfortunately, the results of the RCT SCIPIO (CSCs in patients with ischemic cardiomyopathy) toward efficacy and tolerability of CSCs in HF patients with ischemic cardiomyopathy undergoing CABG have been retracted from the *Lancet* journal after the publication of an expression of concern (Bolli et al. 2011; The Lancet Editors 2014). The preliminary findings of the positive effect of CSCs transplantation on LV structure and function were not considered as reliable. However, cardiac magnetic resonance imaging showed that treatment with CSCs was associated with good tolerability and resulted in sufficient improvement in both global and regional LV function. Therefore, the authors found a significant reduction in the infarct area and an increase in viable myocardial tissue over 1-year follow-up (Chugh et al. 2012).

One of the earliest face-to-face comparisons of BMMNCs with MSCs in HF patients was the TAC-HFT (The Transendocardial Autologous Cells [hMSC or hBMC] in Ischemic HF Trial) (Heldman et al. 2014). The results of the study were intriguing and unveiled no benefits in BMMNCs when compared to MSCs in terms of LVEF improvement, but both groups showed significant improvement in the quality of life evaluated by the Minnesota Living with HF Questionnaire score. Of note, physical exercise tolerance measured by the 6-min walk distance was improved in the MSC treatment group, but not in the BMMNC treatment group. Myocardial infarct size was reduced in the MSC group, but in the BMMNC group and placebo group, this parameter did not show significant change.

In the POSEIDON trial (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis), study investigators compared allogeneic MSCs with autologous MSCs and did not report any benefit for one group over another in reducing MI scar size (Hare et al. 2012). Interestingly, improving quality of life has been observed beyond positive changes in LVEF. The C-CURE (Cardiopoietic stem Cell therapy in heart failURE) was a prospective, multicenter, randomized trial, which was conducted in HF patients. The patients received either standard of care alone or standard of care plus lineage-specified stem cells (Bartunek et al. 2013). Over 2-year follow-up, LVEF was improved by cell therapy as compared to the standard of care alone with a concomitant reduction in LVESV. Cell therapy led to significant improvement in 6-min walk distance and provided a superior composite clinical score encompassing cardiac parameters in tandem with NYHA class, quality of life, clinical condition, hospitalization, and event-free survival.

The MSC-HF (BM-derived MSCs treatment in patients with severe ischemic HF) was a randomized, double-blind, placebo-controlled trial in which clinical efficacy of intramyocardial injections of MSCs versus placebo was assessed in 60 patients with severe ischemic HF (aged 30–80 years and having New York Heart Association (NYHA) classes II–III and LVEF <45%) (Mathiasen et al. 2015). Over 6 months, patients enrolled in the MSC group have demonstrated a significant reduction in LVESV, but in the placebo group, the increase of it was also observed. The difference between groups was significant ($p = 0.001$). Compared with the pla-

Table 8.2 Stem and progenitor cells clinical trials in HF patients.

RCT name	n	Delivery	Duration	Outcomes	References
Engrafted cell types: autologous BMMNCs					
FOCUS-CCTRN	92	transendocardial	6 months	Lack of improvement in improve LVESV, maximal oxygen consumption, or reversibility on SPECT	Perin et al. (2012)
CELLWAVE	103	i/c	4 months	↑ regional contractility	Assmus et al. (2013)
TOPCARE-AMI	31	i/c	4 month	↑ LVEF, ↓ NT-proBNP	Assmus et al. (2007)
Engrafted cell types: autologous CSCs					
SCIPIO	56	i/c	12 months	↑ regional and global LVEF, ↓area of MI	Bolli et al. (2011) and Chugh et al. (2012)
Engrafted cell types: autologous MSCs					
MSC-HF	60	intra-myocardial injection	6 months	↓ LVESV, ↑ LVEF and myocardial mass	Mathiasen et al. (2015)
TRIDENT	30	transendocardial injection	12 months	↓ scar size and serum levels of NT-proBNP, ↑ LVEF	Florea et al. (2017)
Engrafted cell types: ATDSCs					
PRECISE	27	transendocardial injection	36 months	↑ LV mass and regional contractility	Perin et al. (2014)

Abbreviations: *LVEF* **left ventricular ejection fraction,** *LVESV* **left ventricular end-systolic volume,** *NT-proBNP* **N-terminal pro-brain natriuretic peptide,** *MI* **myocardial infarction,** *RCT* **randomized clinical trial,** *SPECT* **single-photon emission computerized tomography,** *t/e* **transendocardial,** *i/m* **intramyocardial**

cebo group, there was a significant improvement in LVEF ($p < 0.0001$) and myocardial mass ($p = 0.001$), while there was no difference between the treatment groups in NYHA class, 6-min walking test, and Kansas City cardiomyopathy questionnaire (Mathiasen et al. 2015). Therefore, investigators have found a strong correlation between MSCs' dose and improvements in cardiac performances.

The randomized, double-blinded TRIDENT trial (Dose Comparison Study of Allogeneic MSCs in Patients with Ischemic Cardiomyopathy) tested the hypothesis that allogeneic MSC dose and concentration could play crucial role in phenotypic responses in HF patients (Florea et al. 2017). Thirty HF patients received, in a blinded manner, either 20 million ($n = 15$) or 100 million ($n = 15$) allogeneic human MSCs through transendocardial injection (0.5 cm^3 per injection × 10 injections per patient). Investigators did not observe treatment-emergent AEs during 12-month follow-up after implantation. Therefore, no difference in MACE and worsening HF readmission between groups was observed, but a significant reduction in scar size and serum levels of NT-proBNP, and an increase in LVEF was observed in high-dose MSC group as compared with a low dose of cells.

The efficacy of ATDRCs in HF patients had been investigated in the PRECISE (ATDRCs in patients with ischemic cardiomyopathy) trial (Perin et al. 2014). It has been enrolled in 21 ATDRC-treated and 6 control patients. The results have revealed a significant difference in change in maximal oxygen consumption from baseline to 6 and 18 months in ATDRC-treated patients when compared with controls. The ATDRC-treated patients showed significant improvement in total LV mass by magnetic resonance tomography and regional contractility index. Single-photon emission computed tomography (SPECT) results suggested a significant reduction in inducible ischemia in ATDRC-treated patients over 18 months (Perin et al. 2014). In fact, the results of this small study suggest that ATDRCs can maintain LV function, myocardial perfusion, and exercise capacity. Cochrane systematic review published in 2016, including 38 RCTs ($n = 1907$) with findings of 1114 cell-therapy-treated patients and 793 participants who received a placebo treatment. The data showed that cell therapy reduced long-term mortality (1 year and more) (risk ratio = 0.42, 95% confidence interval = 0.21–0.87; $n = 491$; RCTs = 9; $I^2 = 0\%$; low-quality evidence) (Fisher et al. 2016). Periprocedural AE rates were similar in both cell therapy and placebo groups. Additionally, cell therapy exhibited a significant long-term reduction in the incidence of nonfatal MI and arrhythmias, but it did not relate to risk reduction in HF rehospitalization or composite incidence of mortality, nonfatal MI, and/or HF rehospitalization, or long-term LVEF when compared with the placebo group (Fisher et al. 2016).

There are sufficient differences in the efficacy of cell therapy in RCTs and the main cause of it remains unclear that requires a concise explanation. Perhaps, some variations in RCTs design, cell source, the method of cell aspiration, isolation, dosing, route of delivery, and clinical

performances of participants can be causative factors that contributed toward variability in the studies' results. Additionally, the majority of RCTs used surrogate endpoints instead of clinical hard endpoints. Finally, a small sample size of the trials and a personified approach to deliver the cells were reasons to refuse to collect clinical events to evaluate them further.

8.6 Ongoing Cell-Based Therapy Clinical Trials Among HF Patients

The CONCERT-HF Trial (Combination of MSCs and c-kit+ CSCs As Regenerative Therapy for HF) was a phase II trial aimed at addressing these issues by assessing the feasibility, safety, and efficacy of transendocardial delivery of autologous MSCs and CPCs in 162 HF patients (Bolli et al. 2018). The four-arm design would enable a direct comparison of MSCs alone with CPCs alone and with their combination. CONCERT-HF is underway in the USA and consists of 18 patients included in a safety lead-in phase (stage 1) and 144 patients enrolled in the main trial (stage 2). The results of the study are not yet available.

The BAMI trial (The effect of intracoronary infusion of BMMNCs on all-cause mortality in AMI) was designed to demonstrate that intracoronary infusion of BMMNCs was safe and would significantly reduce the time to the first occurrence of all-cause death in patients with reduced LVEF after successful PCI for STEMI. The BAMI is the large RCT, in which 3000 HF patients in 11 European countries with at least 17 different sites should have been enrolled, but so far only 375 patients have been randomized and the number is unacceptable to evaluate clinical outcomes (Mathur et al. 2017). The DREAM-HF trial (Double-Blind Randomized Assessment of Clinical Events with Allogeneic MSCs in Advanced HF) is an ongoing, randomized, sham-controlled phase III study to ascertain the safety and efficacy of MSCs in severe chronic HFrEF patients (Borow et al. 2019). Investigators have declared that the main aim of the trial is to confirm earlier phase II results and evaluate whether MSCs will reduce the rate of nonfatal recurrent HF-related MACEs while delaying or preventing HF progression to terminal cardiac events. The results of these ongoing trials are anticipated to determine future findings and interest in stem/progenitor cell therapy trials for HF.

Additionally, there are several small clinical trials in which both safety and efficacy of a patch with 100 million reprogramed iPSC cardiomyocytes in HF patients are being evaluated (Cyranoski 2018). So far, there are no reports from these studies. Continuing studies are required to investigate with clarity whether cell therapy is suitable for routine clinical application.

8.7 Conclusions

Despite more than a decade of research, further investigations are still needed to determine whether stem cell regenerative therapy is an effective treatment strategy and can be routinely used in clinical practice. Although there is a wide range of cell-type variants to be implanted, the majority of RCTs were based on transfer autologous BMMNCs, while MSCs, CSCs, CDCs, and myoblast cells have revealed high ability to rejuvenate and regenerate injured myocardium in patients with post-STEMI adverse cardiac remodeling, ischemia cardiomyopathy, and advance chronic HF. Other cells, such as ESCs and iPSCs, having powerful regenerative potencies and the greatest multiple lineage capability, require to be thoroughly evaluated in large clinical trials due to the highest potential risks including arrhythmias, tumorigenesis, and rejection. Several preclinical studies have confirmed the cardioprotective effects of these cell lines, but the clinical studies are ongoing. It does not seem to be obvious that only cell lines are the best option for transplantation because a combination of several cell lines including MSCs and CD34+ EPCs provided promising clinical findings. However, stem-cell therapy in HF is an unauthorized activity, because it is not approved by the US Food and Drug Administration and European Medical Agency, and so far, clinical experiment is required for thorough investigation in large clinical trials in future.

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9 Dental Mesenchymal Stem/Progenitor Cells: A New Prospect in Regenerative Medicine

Aiah A. El-Rashidy, Israa Ahmed Radwan, Dina Rady, Sara El Moshy, Marwa M. S. Abbass, Khadiga M. Sadek, Azza Ezz El-Arab, and Karim M. Fawzy El-Sayed

Abbreviations

ABMSCs	Alveolar bone proper-derived stem/progenitor cells
ARDS	Acute respiratory distress syndrome
BM	Bone marrow
BDNF	Brain-derived neurotrophic factor
BMP-2	Bone morphogenetic protein
BM-MSCs	Bone marrow mesenchymal stem cells
CM	Conditioned medium
CSF	colony-stimulating factor
DFSCs	Dental follicle stem/progenitor cells
DMP-1	dentin matrix phosphoprotein 1
DMSCs	Dental mesenchymal stem/progenitor cells
DPSCs	Dental pulp stem cells
DSPP	Dentin sialophosphoprotein
ESCs	Embryonic stem cells
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FGF-2	Fibroblast growth factor 2
FGF-R	Fibroblast growth factor-receptor
GDNF	Glial cell line-derived neurotrophic factor
GMCSF	Granulocyte-macrophage colony stimulating factor
GMSCs	Gingival mesenchymal stem/progenitor cells
HLA-DR	Human Leukocyte Antigen – DR
HSCs	Hematopoietic stem cells
IFNγ	Interferon gamma
IGF-1	Insulin-like growth factor-1
JNK	c-Jun N-terminal kinase
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
MAP 2	Microtubule-associated protein 2
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MSCs	Mesenchymal stem/progenitor cells
NFKb	Nuclear factor-kappa B pathway
NGF	Neuronal growth factor
NF-M	Neuronal specific intermediate filament
NT-3	Neurotrophin-3
PDL	Periodontal ligament
PDLSCs	Periodontal Ligament Stem/progenitor Cells
PPARγ2	Peroxisome proliferator-activated receptor γ2
Runx 2	Runt-related transcription factor 2
Sca-1	Stem cell antigen
SCAPs	Stem/progenitor Cells from Apical Dental Papilla
SHEDs	Stem cells from human exfoliated deciduous teeth
SSEA-4	Stage-specific embryonic antigen 4
Oct4	Octamer-binding transcription factor
TERM	Tissue engineering and regenerative medicine
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor-α

A. A. El-Rashidy · K. M. Sadek
Biomaterials Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Stem cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

I. A. Radwan · D. Rady · S. El Moshy · M. M. S. Abbass
Stem cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

A. Ezz El-Arab
Stem cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

K. M. Fawzy El-Sayed (✉)
Stem cells and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian Albrechts University, Kiel, Germany

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TRA Tumor recognition antigens
VEGF Vascular endothelial growth factor

9.1 Introduction

Tissue engineering and regenerative medicine (TERM) is an interdisciplinary emerging field offering innovative solutions for most human body damaged/lost tissues. It is based on material science, cellular, molecular biology, and stem/progenitor cell engineering. TERM solutions in the dental practice depend on scaffolds combined with mesenchymal stem/progenitor cells (MSCs), precisely, dental mesenchymal stem/progenitor cells (DMSCs), with the use of specific growth factors and/or signaling molecules (Amrollahi et al. 2016).

Generally, stem/progenitor cells may be classified on different bases. According to their differentiation potential, they could be classified as totipotent, multipotent, and pluripotent. Their source of origin has two main divisions; the embryonic stem/progenitor cells derived from the blastocyst of 4–5 days old embryo and the adult stem/progenitor cells obtained from any postnatal adult tissue or organ in the human body. The adult stem/progenitors are multipotent cells that can expand rapidly, causing minimal immunological responses, besides the absence of any ethical or legal concerns about their clinical use. Among the adult stem/progenitor cells, DMSCs are included; their presence in the human dental pulp was first reported by Yamamura in 1985, while their identification was performed in 2000 by Gronthos (Goswami et al. 2020).

DMSCs include dental pulp stem/progenitor cells (DPSCs) isolated from dental pulpal tissues of permanent teeth and those isolated from pulpal tissues of human shed deciduous teeth (SHED) (Gronthos et al. 2000; Miura et al. 2003; Stanko et al. 2018). They also include periodontal ligament stem/progenitor cells (PDLSCs) isolated from the periodontal ligament (Seo et al. 2004; Jo et al. 2007), gingival stem/progenitor cells (GMSCs) isolated from gingival tissues (Palmer and Lubbock 1995; Fawzy El-Sayed et al. 2016; Fawzy El-Sayed et al. 2019a, b; Fawzy El-Sayed and Dorfer 2016; Fawzy El-Sayed et al. 2015), alveolar bone proper-derived stem/progenitor cells (ABMSCs) (Fawzy El-Sayed et al. 2012; Fawzy El-Sayed et al. 2017; Fawzy El-Sayed et al. 2014) and dental follicle stem/progenitor cells (DFSCs), derived from the dental follicle surrounding the third molar in most cases (Morsczeck et al. 2005). Additionally, stem/progenitor cells are isolated from apical papilla (SCAP) of immature permanent teeth, (Jo et al. 2007; Sonoyama et al. 2006), while the tooth germ progenitor cells are isolated from the late bell-stage of the third molar's tooth germs, (Ikeda et al. 2008). Furthermore, stem/progenitor cells could be isolated from diseased periapical cysts (Marrelli et al. 2013; Tatullo et al. 2017) or even from inflamed pulp tissue (Alongi et al. 2010; Malekfar et al. 2016) (Fig. 9.1).

As DMSCs are derived from the ectomesenchyme's neural cells, they are characterized by unique biological criteria based on gene- and protein-expression profile. Moreover, they possess self-renewal ability and undergo multiple cycles of undifferentiated cell division. They are characterized by their immunomodulatory properties in addition to the ability to obtain them with a minimally invasive painless procedure. They could be easily derived multiple times throughout the individual's life during simple dental procedures/surgery, such as tooth extraction or cyst removal (Goswami et al. 2020).

DMSCs can differentiate into multiple cell lineages forming different tissues; dental and non-dental. For instance, osteogenic (Kumar et al. 2018; Davies et al. 2015), hepatogenic (Kumar et al. 2017b), and neurogenic (Isobe et al. 2016; Kumar et al. 2017a).

Regarding their surface markers, DMSCs express most MSCs' surface markers, including CD90, CD73, and CD105, with a lack of expression of CD14, CD34, CD45, CD19, CD79a, CD11b and human leukocyte antigen-DR isotype (HLA-DR) (Huang et al. 2009). They also express Stro-1, CD106, CD 44, and CD146, in addition to Nanog, stage-specific embryonic antigen 4 (SSEA-4), octamer-binding transcription factor (Oct)-4 and tumor recognition antigens (TRA)-1–60 which designate their pluripotency (Aydin and Şahin 2011). The direct cellular activity of DMSCs and its positive effect in tissue regeneration occurring after its engraftment is indirectly mediated through paracrine effects (El Moshy et al. 2020). This effect is induced by the release of trophic and modulatory bioactive factors (secretome) into the adjoining environment, influencing tissue homeostasis, and stimulating tissue regeneration (Li et al. 2014b; Ranganath et al. 2012). Secretome can induce cellular migration, proliferation, immunomodulation, and tissue regeneration, offering a novel concept of cell-free regenerative medicine solutions as an alternative to cell-based approaches (El Moshy et al. 2020).

This chapter briefly presents different dental stem/progenitor cells, specific criteria of their characterization; advantages in addition to the limitations encountered their use. Furthermore, this chapter displays the signaling molecules involved in dental stem/progenitor cells and their effect on the differentiation and regeneration potential of such cells, offering a concise brief review about them and the possible ways for their clinical translation.

9.2 Types of Dental Mesenchymal Stem/Progenitor Cells

9.2.1 Dental Pulp Stem/Progenitor Cells (DPSCs)

The dental pulp is a delicate connective tissue composed of odontoblasts on its periphery, fibroblasts, immune cells, and

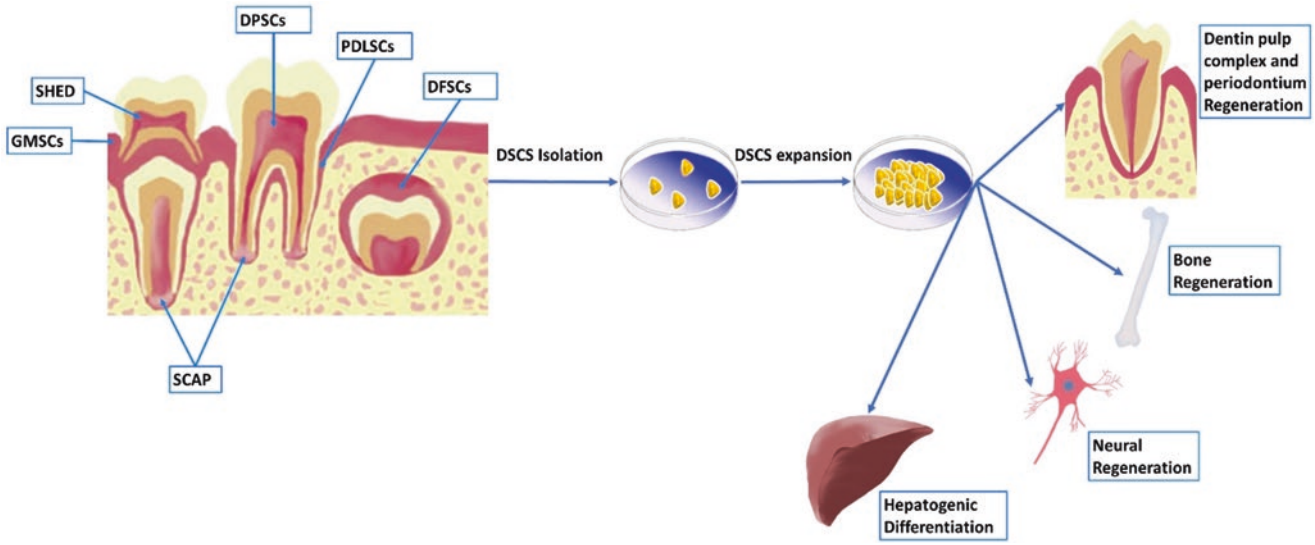

Fig. 9.1 Illustrative diagram showing sources of DMSCs and their possible differentiation potential and angiogenic tissues (Murakami et al. 2015; Song et al. 2017); in addition to their potential to regenerate dentin, pulp, cementum and periodontal ligament (Goswami et al. 2020)

stem/progenitor cells embedded in the extracellular matrix in addition to vascular, neural, and lymphatic elements (Nanci 2017). The DPSCs were the first dental MSCs population isolated and identified from impacted third molars' dental pulp tissue (Gronthos et al. 2000). Moreover, DPSCs were isolated from teeth removed due to orthodontic extraction and during routine surgical practice. Interestingly, DPSCs can be passaged for more than 80 passages without losing their differentiation capacity (Laino et al. 2005; Laino et al. 2006). DPSCs are a diverse population of cells that demonstrate different proliferation rates and differentiation potential within the individually isolated clones (Gronthos et al. 2000; Huang et al. 2009; Aurrekoetxea et al. 2015; Alraies et al. 2017). This heterogeneity is attributed to different telomere lengths and CD271 expression among DPSCs populations (Alraies et al. 2017).

9.2.2 Properties and Differentiation Ability of DPSCs

DPSCs are ectodermal-derived stem/progenitor cells originating from migrating neural crest cells. These cells possess the typical characteristics of MSCs, as fibroblast-like morphology, plastic adherence, high proliferative potential, multi-lineage differentiation potential, colony-formation upon in vitro culture, and immunomodulatory properties (Mortada and Mortada 2018). DPSCs express several surface markers, including CD9, CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD106, CD146, CD166, and CD271, but lack the expression of CD14, CD19, CD24, CD31, CD34, CD45, CD117, and CD133 (Gronthos et al. 2002). DPSCs showed a pronounced differentiation potential into a wide variety of cells, making them an excellent cell source for tissue regeneration. In addition to their odontogenic potential, DPSCs can differentiate into adipocytes, osteoblasts, neurons, endothelial cells, chondroblasts, and myocytes (Zhang et al. 2006). Interestingly, DPSCs maintain their differentiation towards odontogenic, osteogenic, adipogenic, and chondrogenic lineages after 2 years of cryopreservation (Alsulaimani et al. 2016). Hence, further attention is focused on the DPSCs' clinical application in regenerative medicine.

Different proteins involved in mineralization, such as dentin matrix phosphoprotein 1 (DMP-1) and dentin sialophosphoprotein (DSPP) (specific markers for odontoblasts) were upregulated in DPSCs when cultured in osteogenic and odontogenic media (Chen et al. 2005; Siew Ching et al. 2017), in addition to osteopontin, alkaline phosphatase, osteocalcin, type I collagen and osterix (markers for osteoblastic proliferation and differentiation) (Siew Ching et al. 2017). Owing to its origin, when cultured in a neuroinductive environment for an extended period, DPSCs expressed neural markers (β-III tubulin, nestin, and neurofilament-M). They acquired a neural morphology (Park et al. 2019). DPSCs cultivated three-dimensionally in a chondrogenic medium revealed highly sulfated glycosaminoglycans in the center of the culture pellet, denoting chondrogenic differentiation of DPSCs (Almeida et al. 2018).

DPSCs demonstrated the ability to differentiate in vitro into an odontoblastic phenotype, characterized by being polarized with mineralized nodules (Jo et al. 2007; About et al. 2000). DPSCs from impacted third molars have been differentiated into adipocytes and expressed adipocyte-specific genes like PPAR-A2 and ap2 (Xing et al. 2015). DPSCs demonstrated the ability to differentiate into hepatocytes expressing specific hepatic markers, including α-fetoprotein, albumin, and hepatic nuclear factor 4α, besides

storing glycogen and producing urea (Ishkitiev et al. 2010). Also, DPSCs were able to differentiate into a pancreatic cell lineage similar to islet-like cell aggregates and release insulin in a glucose-dependent manner (Carnevale et al. 2013). Moreover, DPSCs may serve as a source for salivary gland cells (Yamamura et al. 2013). Interestingly, DPSCs displayed the potential to differentiate into mature melanocytes without stimulation by specific culture media in vitro (Paino et al. 2010). DPSCs can also differentiate into osteoblasts (d'Aquino et al. 2007) as well as endothelial cells forming capillary-like structures upon culturing with vascular endothelial growth factor (VEGF) (Marchionni et al. 2009). Owing to their availability and differentiation potential, DPSCs are considered an ideal cell source for tissue regeneration.

9.2.3 Immunomodulatory Properties of DPSCs

Immunomodulatory properties of DPSCs were revealed by their ability to suppress T-cell proliferation, which opens the door for treating T-cell alloreactivity associated with solid organ or allogeneic hematopoietic transplantation (Pierdomenico et al. 2005). Moreover, interferon-gamma (IFN-γ)-primed DPSCs inhibited T cell proliferation, reduced interleukin (IL)-17 production, and stimulated regulatory T cell differentiation (Özdemir et al. 2016). Besides, coculturing DPSCs with anti-CD3/CD28 antibody-activated peripheral blood mononuclear cells resulted in inhibition of $CD8^+$ T cell proliferation and B cell immunoglobulin production (Kwack et al. 2017). This inhibitory effect was mediated by transforming growth factor-β (TGF-β) and enhanced by IFN-γ (Kwack et al. 2017). Moreover, knocking down of Fas ligand expression reduced DPSCs immunomodulatory properties explaining its role in activating T-cell apoptosis in vitro and improved tissue inflammation in mice with colitis (Zhao et al. 2012). Interestingly, osteo-differentiated DPSCs inhibited the proliferation of phytohemagglutinin-activated peripheral blood mononuclear cells (Hossein-Khannazer et al. 2019). Furthermore, DPSCs triggered macrophage M2 polarization when transplanted into unilateral hindlimb skeletal muscle and suppressed sciatic nerve inflammation (Omi et al. 2016). Additionally, the complement system could influence DPSC proliferation and mobilization by activating C3a and C5a complement system receptors expressed on DPSCs (Cardoso et al. 2008; Rufas et al. 2016).

9.2.4 Regulation of DPSCs' Behaviors

To enhance the tissue regeneration efficiency of DPSCs, it is essential to understand the regulatory mechanisms controlling the behavior of DPSCs to exploit their optimal regeneration efficiency. Different oxygen levels affected DPSCs proliferation where the normal physiologic range of oxygen level (between 3 and 6%) kept the DPSCs in a quiescent state. In comparison, ambient oxygen tensions in the culture (21%) made DPSCs exhibited high proliferation rates (El Alami et al. 2014). Moreover, mechanical stimuli play an essential role in the regulation of DPSCs behavior. The application of an appropriate level of mechanical tension effectively modulated DPSCs proliferation, differentiation, and extracellular matrix deposition (Han et al. 2008). Additionally, signaling molecules may contribute to controlling DPSCs differentiation. For example, bone morphogenetic protein (BMP)-2 induced in vitro osteogenic differentiation of DPSCs (Tóth et al. 2020). The use of recombinant human BMP-2 and BMP-4 in combination with inactivated dentin matrix on amputated pulp, formed tubular dentin and osteodentin after 2 months while the amount of dentin was markedly decreased in response to dentin matrix alone, implying the role of BMP in the differentiation of DPSCs into odontoblasts (Nakashima 1994). Fibroblast growth factor 2 (FGF-2)-induced neurogenic (Zhang et al. 2017) and osteogenic (Qian et al. 2015) differentiation of DPSCs. Additionally, there are intrinsic mechanisms that regulate DPSCs' behavior in response to extrinsic factors. Wnt signaling pathway plays a significant role in maintaining DPSCs' stemness and regulate their differentiation (Scheller et al. 2008; Zhong et al. 2019). Lipopolysaccharide (LPS) or tumor necrosis factor-α (TNF-α) in the inflammatory microenvironments alter the DPSCs functions through the nuclear factor-kappa B pathway and the mitogen-activated protein kinase (MAPK) pathway (Chang et al. 2005; Botero et al. 2010).

9.2.5 DPSCs Versus Other MSCs

Researchers have been trying to point out the differences between DPSCs and other MSCs. Although DPSCs required long time to reach confluence after isolation compared to bone marrow mesenchymal stem/progenitor cells (BM-MSCs) and adipose-derived stem/progenitor cells, they displayed higher viability after 14 days of cryopreservation than BM-MSCs, higher colony-formation, and mineralization ability (Demirci et al. 2016; Nuti et al. 2016). Comparing the proliferative capacity of DPSCs and BM-MSCs, DPSCs revealed a more significant proliferative potential (Tamaki et al. 2013) and a remarkable odontogenic capability than BM-MSCs. This renders DPSCs a more appropriate cell source for tooth regeneration (Yu et al. 2007). DPSCs transplanted into immunocompromised mice formed dentin-like tissue while BM-MSCs formed bone (Shen et al. 2019). Additionally, the secretome of DPSCs demonstrated a significant increase in neural genes expression, that is,

microtubule-associated protein-2 (MAP-2), β-tubulin III, Nestin, and SOX-1. Also, growth factors and cytokines involved in neural regeneration, that is, colony-stimulating factor (CSF), IFNγ, TGF-β, neuronal growth factor (NGF), neurotrophin-3 (NT-3), and brain-derived neurotrophic factor (BDNF) were upregulated in DPSCs secretome as compared to the BM-MSCs secretome. These data show that DPSCs are a better candidate-cell source in neural lineage differentiation (Kumar et al. 2017a). Gene expression profiling of DPSCs and BM-MSCs revealed differential upregulated and downregulated profile in DPSCs compared to BM-MSCs (Kim et al. 2011). Although DPSCs demonstrated better odontogenic and neurogenic differentiation potential, it showed lower chondrogenic potential in comparison to BM-MSCs (Fabre et al. 2019).

9.2.6 Preclinical and Clinical Applications of DPSCs

Based on the remarkable differentiation potential, attempts to achieve various tissue regeneration using DPSCs have been extensively investigated in preclinical and clinical studies. DPSCs' ability to regenerate dentin-pulp-like complex combined with Puramatrix hydrogel in human tooth slice was evaluated (Cavalcanti et al. 2013). After 21 days, DPSCs expressed DMP-1 and DSPP during odontoblastic differentiation. DPSCs pretreated with granulocyte-CSF (having anti-inflammatory, antiapoptotic, neurogenic, and angiogenic effects (Solaroglu et al. 2006)) were used to treat patients with irreversible pulpitis (Nakashima et al. 2017). Electrical pulp test revealed a positive response similar to a normal pulp after 24 weeks, while cone-beam computed tomography displayed dentin formation in only three of the five patients. Despite the small sample of this study, it revealed the safety and efficacy of DPSCs for complete pulp regeneration in humans without toxicity. Moreover, DPSCs transplanted into immunodeficient rats showed the ability to differentiate into cementoblast-like cells, collagen-forming cells, adipocytes, and generating periodontal-like tissues (Kinaia et al. 2012). The differentiated cells expressed STRO-1, CD146, and CD44. Furthermore, autologous DPSCs isolated from inflammatory dental pulp tissues, loaded on β-tricalcium phosphate scaffold, and engrafted into the periodontal defect in humans' root furcation area enhanced the regeneration of the periodontal bony defects after 9 months from the surgical reconstruction (Li et al. 2016). The combination of DPSCs with collagen gel-scaffold showed bone regeneration in a rat critical-size calvarial defect model (Chamieh et al. 2016). In addition to the increased bone mineral density and improved bone micro-architectural parameters, there was significant increase in the fibrous connective and mineralized tissue volume. The DPSCs also expressed type I collagen, alkaline phosphatase, and tartrate-resistant acid phosphatase. Furthermore, the follow up for 3 years of DPSCs with collagen-scaffold engrafted in human extraction defects revealed the formation of entirely compact bone that differed from the normal alveolar bone (Giuliani et al. 2013). These results may help create steadier mandibles and increase implant stability. Additionally, DPSCs affect bone formation around dental implants, where its combination with platelet-rich plasma led to osseointegration of hydroxyapatite-coated dental implants with a well-formed mature bone (Yamada et al. 2010).

The therapeutic potential of DPSCs to treat myocardial infarction (MI) in rats was evaluated (Gandia et al. 2008). DPSCs were injected intra-myocardially on day 7 after MI. Four weeks later, the DPSCs-treated rats showed improvement in the cardiac function, decreased infarct size, and left ventricular anterior wall thickness. These results were attributed to the angiogenic effect of DPSCs due to their paracrine activity, since the expression of angiogenic factors such as VEGF, platelet-derived growth factor, matrix metallopeptidase 9, insulin-like growth factor-1 (IGF-1), and TGF-β from DPSCs has been proved in literature (Matsushita et al. 2000; Tran-Hung et al. 2006; Aranha et al. 2010; Nakashima et al. 2009). These data propose that DPSCs may be an alternative therapy for cardiovascular diseases. The myogenic differentiation ability of DPSCs was explored in treating the Duchenne muscular dystrophy rat model (Pisciotta et al. 2015). DPSCs engrafted into the rat's muscle, promoted angiogenesis, reduced fibrosis, and improved the dystrophic muscle histopathology. Additionally, cryopreserved DPSCs for 6 years demonstrated renotropic and pericyte-like properties contributing to accelerated renal tubule structure regeneration post-engraftment in an immunocompetent rat model with acute renal failure (Barros et al. 2015). Remarkably, a tissue-engineered DPSCs sheet transplanted on a rabbit corneal bed successfully reconstructed the corneal epithelium (Gomes et al. 2010).

Labeled pre-differentiated neuronal DPSCs were injected into the cerebrospinal fluid of injured newborn rats' brains (Király et al. 2011). Four weeks later, the labeled DPSCs migrated into various brain areas and expressed the early neuronal marker, N-tubulin, neuronal-specific intermediate filament protein (NF-M), the postmitotic neuronal marker (neuronal nuclei), and glial fibrillary acidic protein, besides displaying voltage-dependent sodium and potassium channels. DPSCs transplantation in unilateral hind-limb skeletal muscles on diabetic polyneuropathy rat model decreased monocytes/macrophages and TNF-α mRNA expression, besides increasing CD206 and the M2 macrophage marker, thus demonstrating the immunomodulatory properties of DPSCs (Omi et al. 2016). The in vitro part of the previous study presented that DPSC-conditioned media (CM) significantly increased the gene expressions of IL-10 and CD206 in LPS-stimulated RAW264.7 cells. Since macrophage activation plays a significant role in the

pathophysiology of acute respiratory distress syndrome (ARDS) (Herold et al. 2013), and as the intravenous injected BM-MSCs were found to be mostly homed in the lungs, the most affected tissue in COVID-19 patients (Barbash et al. 2003), these findings could open the door for applying DPSCs as an optional treatment to COVID-19. Nevertheless, several clinical trials using MSCs to treat COVID-19 have been conducted, and one of these clinical trials has reported the safety and efficacy of allogenic DPSCs in treating severe cases of COVID-19. Still, the specific underlying mechanisms of this clinical trial remains unclear and needs further investigations to adjust the appropriate dose and concentration of DPSCs in treating COVID-19 (Ye et al. 2020). New strategies based on good manufacturing practice (GMP) are required to exploit optimal therapeutic potential of DPSCs (La Noce et al. 2014) (Fig. 9.2**).**

9.3 Stem/Progenitor Cells from Exfoliated Deciduous Teeth (SHEDs)

Analogous to DPSCs, stem/progenitor cells isolated from the human pulp of exfoliated deciduous teeth (SHEDs) are easily available with little or no trauma to the patient and minimal ethical considerations. Also, the dental pulp of deciduous teeth develops before birth, thereby maintain an active niche rich in stem/progenitor cells, which are not yet intensely affected by the cumulative effect of genetic and/or environmental factors (Kerkis and Caplan 2012). Interestingly, SHEDs can be isolated from carious deciduous teeth (Werle et al. 2016). Hence, this increases the interest in SHEDs for tissue engineering research.

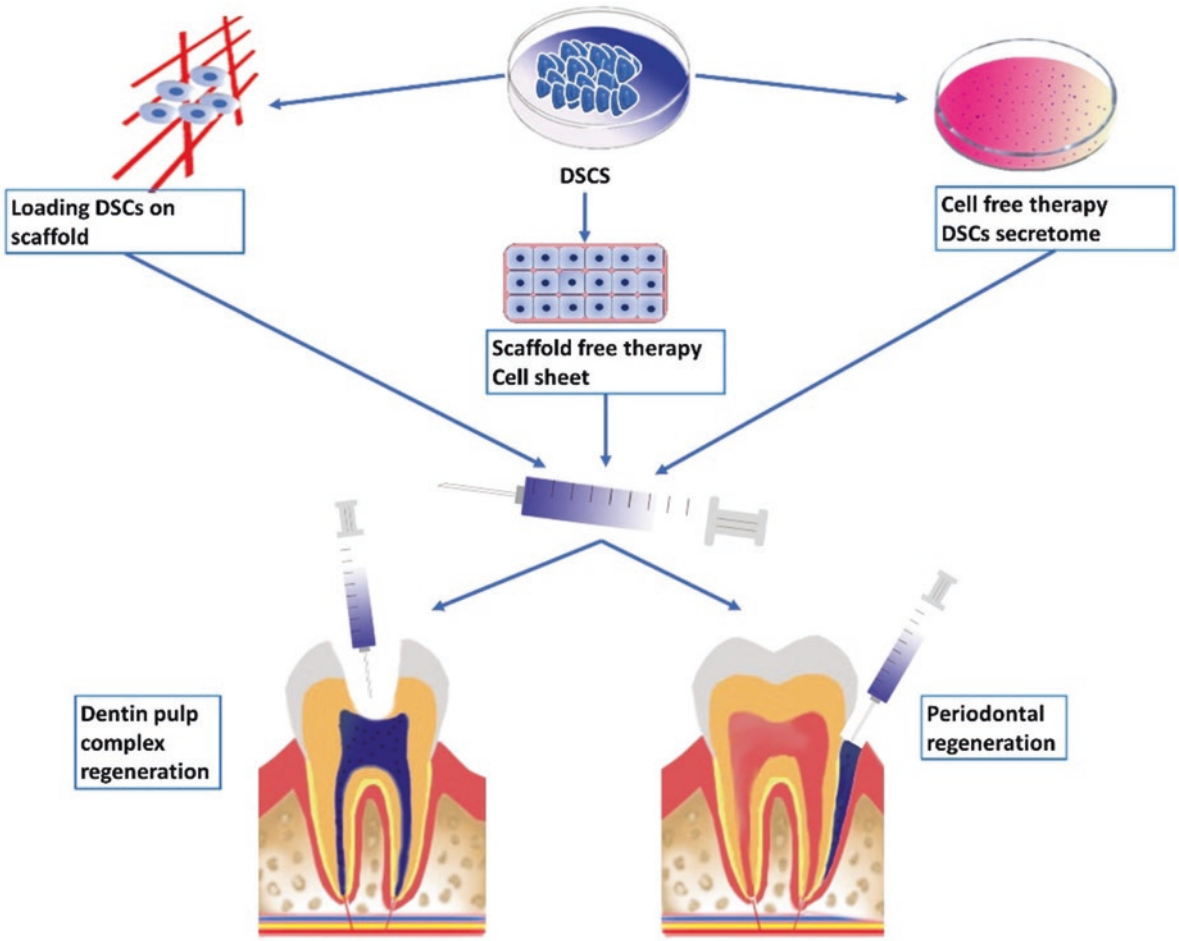

Fig. 9.2 Illustrative diagram showing techniques used for dentin-pulp complex and periodontium regeneration using DPSCs

9.3.1 Properties and Differentiation Ability of SHEDs

SHEDs were first isolated by Miura et al. in 2003 from exfoliated human deciduous incisors (Miura et al. 2003). Compared to DPSCs, SHEDs displayed a higher proliferation rate, a higher number of cell-population doublings, and a higher number of colony-forming cells (Miura et al. 2003; Suchánek et al. 2010; Annibali et al. 2014). Phenotypic analysis revealed early expression of MSCs markers (STRO-1 and CD146) in addition to multiple conventional MSCs cell surface markers. However, they lack the expression of HSCs-specific markers (CD34 and CD45) and immune cell markers (HLA-DR and CD7). SHEDs positively expressed CD117 (receptor for stem cell factor I, typical for pluripotent cells) and negatively expressed CD31 and CD106 markers of endothelial differentiation (Kerkis and Caplan 2012; Suchánek et al. 2010; Saito et al. 2015; Nourbakhsh et al. 2011; Vishwanath et al. 2013; Annibali et al. 2014; Kashyap 2015). Also, SHEDs showed higher expression ESCs' markers as Oct4, Nanog, SSEA-3, SSEA-4, and TRA-1-60 & TRA-1-81 than DPSCs, signifying their more immature state (Saito et al. 2015).

Gene expression profiles presented 4386 genes expressed differentially between DPSCs and SHEDs by two folds or more. SHEDs revealed higher expression of genes contributing to cell proliferation and extracellular matrix formation pathways, including several growth factors such as FGF and TGF-ß (Nakamura et al. 2009). SHEDs are characterized by their high plasticity. They can undergo multi-lineage differentiation such as osteogenic /odontogenic, adipogenic, and neural cells in vitro and in vivo, granting enormous promises for tissue repair and regeneration (Miura et al. 2003).

9.3.2 Immunomodulatory Properties of SHEDs

SHEDs possess immunomodulatory effects that might correct the immune imbalance, thus becoming a promising cellular therapy in autoimmune diseases. SHEDs suppress the $CD4^+$ T cell-driven responses by inhibiting T lymphocytes proliferation and the upregulated ratio of Th1/Th2 by inducing the expansion of T regulatory cells (Dai et al. 2019). Intravenous administration of SHEDs resulted in a significant reduction in serum antibody levels, trabecular bone reconstruction, and regulation of Th17 cells in treating a murine systemic lupus erythematosus model (Yamaza et al. 2010). Besides, local injection of SHEDs increased the number of anti-inflammatory $CD206^+$ M2 macrophages and altered the cytokine expression profiles in inflamed periodontal tissues, reduced gum bleeding, increased new attachment of periodontal ligament, and decreased osteoclast differentiation (Gao et al. 2018).

9.3.3 Preclinical and Clinical Applications of SHEDs and their Secretome

The regenerative and therapeutic potentials of SHEDs have been widely investigated in multiple animal disease models with desirable effects, proposing an encouraging insight for treatment in clinical trials. SHEDs revealed distinctive osteoinductive capacity; unlike DPSCs, SHEDs were capable of inducing recipient murine cells to differentiate into bone-forming cells following transplantation in vivo. Single-colony-derived SHEDs clones transplantation into immunocompromised mice-induced bone formation by recruiting host osteogenic cells rather than differentiating themselves into osteoblasts (Miura et al. 2003). SHEDs were able to repair critical-sized calvarial defects in mice (Seo et al. 2008) and critical-sized mandibular defects in swine (Zheng et al. 2009) with substantial bone formation.

SHEDs' odontoblastic differentiation ability displayed noticeable defects in forming a complete dentin/pulp-like complex in vivo. However, SHEDs could form dentin-like tissue or pulp-like tissue instead of complete dentin–pulp-like complex (Miura et al. 2003; Cordeiro et al. 2008). In a porcine model, SHEDs and β-tricalcium phosphate scaffold composite were used in direct pulp capping on the pulp chamber roof. Complete dentin regeneration and restoration of the defect was obtained (Zheng et al. 2012). Moreover, SHEDs expressed BMP receptors and odontoblastic differentiation markers (DSPP, DMP-1, and matrix extracellular phosphoglycoprotein). Hence, by blocking BMP-2 signaling, these markers' expression was inhibited in SHEDs cultured in tooth slices/scaffolds (Casagrande et al. 2010). Remarkably, SHEDs regenerated 3D whole-dental pulp accompanied by blood vessels and nerves in both animal models and patients with tooth trauma (Xuan et al. 2018).

The adipogenic differentiation capacity of SHEDs was not as strong as BM-MSCs. Yamaza et al. (Yamaza et al. 2010) demonstrated that SHEDs showed impaired adipogenic differentiation and reduced expression of adipocyte-specific molecules, Peroxisome proliferator-activated receptor γ2 (PPARγ2), and Lipoprotein lipase (LPL) compared to BM-MSCs. SHEDs also developed multiple cytoplasmic processes in neurogenic medium and formed a sphere-like cluster, suggesting its neural crest origin (Miura et al. 2003). Furthermore, the neurogenic differentiation potential of SHEDs was confirmed by the upregulation of neuronal and glial cell markers as β-III-tubulin, tyrosine-hydroxylase, MAP-2, and Nestin. Various growth factors and cytokines secreted by SHEDs play an essential role in SHEDs neurogenesis, including FGF-8, sonic hedgehog, FGF-2, and GDNF (Nourbakhsh et al. 2011; Wang et al. 2010; Fujii et al. 2015).

Several preclinical studies showed that SHEDs successfully recovered rat spinal cord injuries with marked anti-

inflammatory action, decreased myelin degeneration, neuronal and oligodendrocytic differentiation, locomotor recovery, and inhibition of glial scar formation (Sakai et al. 2012; Yang et al. 2017a; Nicola et al. 2016; Nicola et al. 2019). Besides, SHEDs survived for more than 10 days in the mouse brain microenvironment, expressed neural markers like neurofilament M, and promoted neural development in immunocompromised mice (Miura et al. 2003). Also, under optimal conditions, SHEDs differentiated into dopaminergic neuron-like spheres, which partially improved the apomorphine-evoked rotation of behavioral disorders in Parkinsonian rats (Wang et al. 2010).

SHEDs also express HLA-A, HLA-B, HLA-C, human hepatocyte-specific antigen (hepatocyte paraffin-1), and human albumin. Upon transplantation, SHEDs promoted hepatic regeneration and improved renal function (Hattori et al. 2015). Islet-like cell clusters derived from either human DPSCs or SHEDs could restore normoglycemia in diabetic mice, whereas SHEDs proved to be superior to DPSCs (Kanafi et al. 2013). Furthermore, SHEDs could alleviate hyposalivation caused by Sjogren's syndrome (Du et al. 2019b) and reconstruct corneal epithelium in an animal model of total limbal stem/progenitor cell deficiency (Gomes et al. 2010).

An alternative approach to SHEDs transplantation, SHED-CM has been suggested to possess therapeutic potential for a variety of diseases such as, Alzheimer's disease (Mita et al. 2015), encephalomyelitis (Shimojima et al. 2016), cerebral ischemia (Inoue et al. 2013), diabetes (Izumoto-Akita et al. 2015), and autoimmune encephalomyelitis (Yamaza et al. 2010). Comparing the therapeutic potential of intravenous transplantation of SHEDs and SHED-CM in bleomycin-induced acute lung injury mice showed that both decreased the lung injury and improved the survival rate through the intense M2-inducing activity of SHEDs and SHED-CM (Wakayama et al. 2015), besides both remedies promoted the recovery of neonatal hypoxia-ischemia brain injury (Yamagata et al. 2013). In addition to soluble factors, exosomes derived from SHED-CM promoted functional recovery of diabetes (Izumoto-Akita et al. 2015), traumatic brain injury (Li et al. 2017), and acute inflammation (Pivoraitė et al. 2015) in animal models.

Stem/progenitor cell banking creates the opportunity to recover and store this convenient source of young stem/progenitor cells as teeth are lost naturally during childhood. Yet, SHEDs' isolation is impractical as exfoliation is unpredictable (Kerkis and Caplan 2012). Ma et al. confirmed that cryopreserved SHEDs maintained the same proliferation analyses, expression of MSCs markers, adipogenic and osteogenic differentiation capabilities, and immunomodulatory properties similar to naive SHEDs (Ma et al. 2012). Concomitantly, 5 years long cryopreserved SHEDs were capable of proliferation and bone formation in a dog mandibular bone defect with no immune response for 3 months, thus verifying that the isolation time didn't affect cells' immunomodulatory properties (Behnia et al. 2014). On the other hand, Ji et al. presented that cryopreserved SHEDs for more than 3 months negatively affected their viability (Ji et al. 2014). Despite these contradictions, stem cell banking validates SHEDs as a promising option for regenerative medicine and cell-based therapies.

9.4 Gingival Mesenchymal Stem/Progenitor Cells (GMSCs)

Gingiva is a pink-colored keratinized mucosa among the components of the periodontium, which surrounds and protects the teeth and it plays a crucial role in supporting and maintaining healthy teeth (Xu et al. 2013). In the spinous layer of the human gingiva, an easily accessible tissue during routine dental procedures, a population of gingival mesenchymal stem/progenitor cells (GMSCs) have been identified (Gan et al. 2020). In the clinic, the collection of gingival tissues by biopsy is a minimally invasive procedure to the patient or even from discarded tissues during routine dental procedures (Xu et al. 2013; Stefańska et al. 2020). GMSCs have a high proliferation rate facilitating their expansion after isolation from the gingival tissues (Xu et al. 2013). GMSCs can be readily used for autologous transplantation based on their ease of collection and isolation (Stefańska et al. 2020).

Also, the fast regeneration of gingival tissues following injury with minimal or no scar formation, as compared to skin, makes them an attractive source of stem/progenitor cells. Healing of gingival wounds without scar formation was suggested to result from the persistent expression of αv-β6 integrin and the higher local accumulation of TGF-β3 in the basal epithelium in the later stages of the gingival wound healing in the gingival wound basal epithelium (Eslami et al. 2009).

The gingiva has an ectomesenchymal origin, arising from the neural crest cells, as most periodontal tissues (Stefańska et al. 2020). However, Xu et al. reported that GMSCs from the cranial neural crest cells constitute about 90% of GMSCs, while 10% arise from the mesoderm (Xu et al. 2013). Both cranial neural crest cells-derived GMSCs (N-GMSCs) and mesoderm-derived GMSCs (M-GMSCs) showed identical stem/progenitor cell properties, including expression of MSCs surface markers and multipotent differentiation. However, N-GMSCs, when compared with M-GMSCs, were reported to exhibit a higher differentiation potential into neural cells when cultured under neural differentiation conditions, making them a promising candidate for use in neural

tissue regeneration. They also showed a higher chondrogenic differentiation potential and immunomodulatory capacity by elevated expression of Fas ligand, through inducing activated T-cell apoptosis in vitro in comparison with M-GMSCs (Xu et al. 2013).

In a study conducted by Li et al., GMSCs from human inflamed gingival tissues showed a higher proliferation rate in vitro than GMSCs isolated from normal human gingival tissues (Li et al. 2013). Besides, increased proliferation of normal-derived GMSCs was evident following in vitro culturing in IL-1β (5 ng/ml) and TNF-α (10 ng/ml), the main inflammatory cytokines that simulate the in vivo inflammatory environment. On the other hand, the osteogenic and adipogenic differentiation potential of inflamed tissue-derived GMSCs was lower than normal-derived GMSCs. These data suggest that the inflammatory environment directs the GMSCs to differentiate towards a pro-fibrotic lineage and lose stem/progenitor cell characteristics, as reflected by suppressed osteogenic/adipogenic differentiation potential, but maintain an evident proliferative potential (Li et al. 2013).

9.4.1 Properties and Differentiation Ability of GMSCs

GMSCs, in comparison to other MSCs, are easily obtained with a high proliferation rate without the need for any external growth factors; they are abundant and homogenous (Fawzy El-Sayed and Dorfer 2016; El Moshy et al. 2020). The GMSCs' primary culture has a uniformly homogenous population of spindle-shaped cells, while for a homogenous culture of BM-MSCs, two to three passages are required to achieve the same uniformity and confluence (Tomar et al. 2010). In addition, GMSCs are genetically more stable than BM-MSCs, preserving normal karyotyping and stable morphology in both early and late passages (El Moshy et al. 2020; Stefańska et al. 2020). Besides MSC surface markers, GMSCs express CD13, CD38, CD44, CD54, CD117, CD144, CD146, CD166, Sca-1 (stem cells antigen-1), Oct-3/4, Nestin, integrin β1, and vimentin (El Moshy et al. 2020). They also express proteins regarded as pluripotency markers or embryonic stem/progenitor cell markers, namely, Oct-4, STRO-1, SSEA-4, and Nanog (Stefańska et al. 2020). GMSCs have the ability to differentiate into lineages derived from all three primary germ layers, showing osteogenic, chondrogenic, adipogenic, and myogenic differentiation potential. In addition, they can differentiate into neurons and endothelial cells (Stefańska et al. 2020).

9.4.2 Immunomodulatory Properties of GMSCs

Oral-derived MSCs exhibit a broad range of immunomodulatory properties, exerted either by direct cell-to-cell contact or through paracrine release of soluble factors such as IL-1, IL-6, IL-10, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), TGF-β1, and prostaglandin E2 (PGE2) (Zhou et al. 2020). GMSCs also have distinctive immunomodulatory functions similar to BM-MSCs. They can suppress peripheral blood mononuclear cells and upregulate IFN-γ-induced IDO and IL-10 (Zhang et al. 2009). Spheroid-derived GMSCs can enhance the secretion of several chemokines, cytokines, and improve the resistance to oxidative stress-induced apoptosis. They can also reduce the severity of chemotherapy-induced oral mucositis (Zhang et al. 2011).

9.4.3 Preclinical and Clinical Applications of GMSCs and their Secretome

Preclinical studies with GMSCs in anti-cancer therapies are limited and mostly targeting oral carcinomas, that is, tongue squamous cell carcinoma (Stefańska et al. 2020). The antitumorigenic ability of GMSCs has been reported in vitro through direct and indirect coculture of GMSCs with two human oral cancer cell lines CAL27 and WSU-HN6 (Ji et al. 2016). They inhibited oral cancer cell growth in both direct and indirect cocultures in vitro. These data show the anticancer potential of GMSCs-CM and support the hypothesis that cytokines secreted by GMSCs (including IL-6, IL-8, and granulocyte-macrophage (GM-CSF etc.) may be responsible for the anti-proliferative potential. The suppression of oral cancer cells' growth by GMSCs activates c-Jun N-terminal kinase (JNK) signaling pathway. Western blotting showed induction of pro-apoptotic genes, that is, Bax, p-JNK, cleaved Poly (ADP-ribose) polymerase, and cleaved caspase-3) and a concomitant downregulation of pro-proliferation and antiapoptotic genes, that is, p-ERK1/2, CDK4, cyclin D1, proliferating cell nuclear antigen, Bcl-2, and survivin. GMSCs were also able to inhibit the growth of CAL27 cells in vivo (Ji et al. 2016).

Several methods have been investigated to engineer GMSCs for enhanced anti-cancer properties as depicted in Fig. 9.3 by Stefańska et al. (Stefańska et al. 2020). Methods investigated included transfection of GMSCs with lentiviral vectors incorporating anticancer genes. For example, TNF-related apoptosis-inducing ligand (TRAIL) (Xia et al. 2015), IFN-β (Du et al. 2019a) or loading with antineoplastic drugs (Paclitaxel, Doxorubicin, and Gemcitabine) (Coccè et al. 2017).

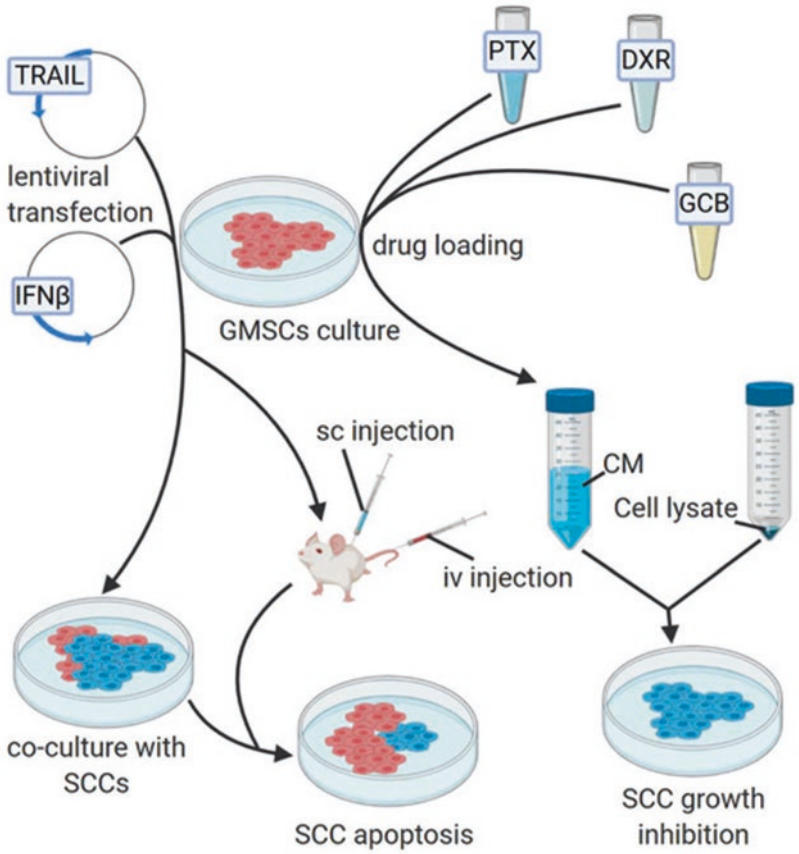

Fig. 9.3 Overview of GMSCs engineering for enhanced anti-cancer properties. Abbreviations: *TRAIL* tumor necrosis factor-related apoptosis-inducing ligand, *IFNβ* interferon β, *SCC* squamous carcinoma cell, *CM* culture medium, *sc* subcutaneous, *iv* intravenous, *PTX* paclitaxel, *DXR* doxorubicin, *GCB* gemcitabine. (Reproduced from Stefańska et al., Creative Commons Attribution License (CC BY).

Based on the gingival tissues' rapid healing potential after injury, GMSCs represent a promising potential in various tissue regenerative applications. Preclinical investigations include cutaneous wound healing, elicited by M2 polarization of macrophages (Zhang et al. 2010), muscle tissue regeneration (Ansari et al. 2016), and bone tissue regeneration (Al-Qadhi et al. 2020; Kandalam et al. 2020; Xu et al. 2014; Wang et al. 2011a). Yet, GMSCs were shown to have lower osteogenic differentiation capability than PDLSCs (Moshaverinia et al. 2014). Systemically transplanted GMSCs were established to promote periodontal tissue regeneration (Huang et al. 2008; Sun et al. 2019). GMSCs were able to undergo neurogenic differentiation in vivo (Ansari et al. 2017) and also showed great potential in the regeneration of peripheral nerve defect/injury (Zhang et al. 2018), or spinal cord injury (Mammana et al. 2019). Clinical studies investigating the regenerative potential of GMSCs in association with gingival fibroblasts loaded on beta-tricalcium phosphate scaffold in the treatment of intraosseous periodontal defects showed promising results (Abdal-Wahab et al. 2020). It was observed that extracting the gingival tissue and its implantation for the treatment of periodontal defect without their associated extracellular matrix and exposing them to periodontal tissue mediators promoted tissue regeneration (Abdal-Wahab et al. 2020).

GMSCs release a wide array of secretome with diverse biological and therapeutic actions (El Moshy et al. 2020). Various investigations suggested that GMSC-derived exosomes, extracellular vesicles, or CM provide promising novel therapeutic alternatives to cell-based therapy approach to treat peripheral nerve injury (Mao et al. 2019; Rao et al. 2019), motor neuron injury (Rajan et al. 2017), skin repair (Shi et al. 2017), and regenerating bone defects (Diomede et al. 2018b).

9.5 Periodontal Ligament Stem/Progenitor Cells (PDLSCs)

The PDL (periodontal ligament) is the soft connective tissue attaching the cementum to the alveolar bone of the socket, with a specific function of sustaining and supporting the teeth within the jaw. Additionally, PDL contributes to the regeneration of the injured tissue through the resident progenitor cells and the residual epithelial sheets of Malassez (Seo et al. 2004).

PDLSCs can be easily isolated noninvasively from periodontal tissue throughout dental scaling and root planning (Trubiani et al. 2015). Although periodontal tissues originate from migrated neural crest cells (Chai et al. 2000), PDLSCs possess stem/progenitor cell properties similar to the MSCs rather than neural crest cells (Kaku et al. 2012). More precisely, PDLSCs express MSCs-specific surface markers CD90, CD105 (Wang et al. 2011b), and CD73 (Iwasaki et al. 2014), but lack the expression of CD34, CD45, CD14 or CD79a, CD11b, CD19, and HLA class II (Zhu et al. 2013). Surprisingly, PDLSCs situated in the periodontal ligaments' perivascular wall are comparable to pericytes in their differentiation potential, morphology, cell phenotype (expression of pericyte-associated markers, neural/glial antigen-2, CD146 and CD140B), and potential to constitute blood vessel-like structures in vitro (Iwasaki et al. 2013). Additionally, PDLSCs isolated from the PDL of the extracted third molar expressed the early MSCs-specific markers STRO-1,CD146/MUC18, and exhibited higher levels of scleraxis, a protein implicated in cementum-periodontal ligament complex formation compared to DPSCs (Seo et al. 2004). Since PDLSCs are a subpopulation of MSCs, using MSCs identification criteria (Dominici et al. 2006) for PDLSCs may be helpful in the absence of a standard criterion specific for PDLSCs (Zhu and Liang 2015).

Ex vivo expanded human PDLSCs have a phenotypic profile similar to BM-MSCs, but with a higher proliferation rate (Eleuterio et al. 2013) and immunomodulatory functions. Interestingly, the PDLSCs cultured until the 15 passages did not show signs of senescence (Diomede et al. 2017). The unique criteria of periodontal ligament's MSCs resides in the expression of proteins, that is, NQO1, CLPP, SCOT1, a new isoform of DDAH1 and TBB5 that are not exhibited by BM-MSCs (Eleuterio et al. 2013). These proteins are involved in cell cycle regulation, stress reaction, homing, and detoxification (Morsczeck et al. 2005).

9.5.1 Differentiation Ability of PDLSCs

PDLSCs have the potential to differentiate into several cells under-identified culture conditions. In particular, osteoblast/cementoblast-like cells, adipocytes, chondrogenic cells (Trubiani et al. 2015), neurogenic cell lineages (El Moshy et al. 2020) and endothelial cells (Zhu and Liang 2015; Okubo et al. 2010). They constitute the most favorable stem/progenitor cell population used in periodontal regeneration (El Moshy et al. 2020), owing to high scleraxis expression (Seo et al. 2004). PDLSCs are the key regulator of osteogenic differentiation (Diomede et al. 2018a). In addition, PDLSCs could differentiate into Schwann cells through the ERK1/2 signaling pathway (Osathanon et al. 2013) and retinal ganglion-like cells (Ng et al. 2015). PDLSCs can be differentiated into cardiomyocytes expressing cardiac cell markers, that is, sarcomeric actin and cardiac troponin T (Pelaez et al. 2017), besides their ability to generate islet-like cells expressing endoderm- and pancreas-related genes (Lee et al. 2014).

9.5.2 Immunomodulatory Properties of PDLSCs

Due to difficulty in the engraftment of large numbers of stem/progenitor cells in regenerative applications, immunomodulation of the milieu in situ is of great significance for the therapeutic application of MSCs (Trubiani et al. 2019). In this context, PDLSCs possess low immunogenicity owing to the absence of HLAII DR or T cell costimulatory molecules (CD80 and CD86) (Ding et al. 2010b). Moreover, PDLSCs inhibit allogeneic T cells propagation of by increasing PGE-2 and cyclooxygenase-2 (COX- 2) expression (Ding et al. 2010b). This inhibitory effect continues after osteogenic induction (Tang et al. 2014). Additionally, PDLSCs downregulated the proliferation, differentiation, and migration of B cells via cell-to-cell contact mediated programmed cell death protein-1 (Liu et al. 2013). The low immunosuppressive potential and immunogenicity on T and B cells support the utility of allogeneic PDLSCs in the regeneration of periodontal tissue. This has been substantiated in a sheep (Mrozik et al. 2013), and a swine (Ding et al. 2010b) model as the therapeutic potential of allogeneic PDLSCs is comparable to autologous PDLSCs.

9.5.3 Factors that Regulate the Differentiation and Therapeutic Potential of PDLSCs

MSCs harvested from inflamed periodontal tissue have increased proliferative capacity, together with higher collagen content, whereas diminished osteogenic differentiation (Trubiani et al. 2008) and downregulated immunosuppressive ability (Liu et al. 2012). This could be attributed to the

significantly diminished inhibitory effects on the proliferation of T cells as compared to those of healthy cells (Shinagawa-Ohama et al. 2017), a finding that has directed the attention toward immunomodulation in the therapeutic attempts for periodontitis. On the contrary, FGF-2 enhances the immunosuppressive potential of MSCs in vivo (Sotiropoulou et al. 2006). The tissue origin also was reported to influence PDLSCs criteria; PDLSCs harvested from the alveolar socket had a higher proliferative ability, and more substantial adipogenic and osteogenic differentiation potential compared to the conventional PDLSCs from the mid-third root surface (Wang et al. 2011b).

Moreover, whether PDLSCs isolated from deciduous teeth differ from those gained from permanent teeth is still questionable. Permanent PDLSCs induced the expression of more cementum/PDL-related genes (CP23 and collagen XII) and revealed a more typical cementum/PDL-like tissue than did deciduous PDLSCs transplants (Song et al. 2012). On the contrary, no significant differences were documented between deciduous PDLSCs and permanent PDLSCs in terms of proliferation rate, expression of stem/progenitor cell markers, or in vitro differentiation potential (Zhu and Liang 2015). Ultimately, PDLSCs derived from shed primary teeth expressed upregulated levels of runt-related transcription factor 2 (RUNX-2), which subsequently increased receptor activator of NF-kB ligand. There was a concomitant decrease in osteoprotegerin expressions at both gene and protein levels that finally induced osteoclastic differentiation and root absorption (Li et al. 2014a).

Growth factors application to support the proliferation or differentiation of stem/progenitor cells at different stages is crucial. Sequential use of growth factors is promising and effective in improving stem/progenitor cell regeneration. However, the interaction between various growth factors requires further clarification. For example, VEGF, BMP-2, and − 7 upregulate osteogenic differentiation of PDLSCs and enhance the regeneration of bony defects in animal models (Hakki et al. 2014; Oortgiesen et al. 2014; Lee et al. 2012; Maegawa et al. 2007). On the contrary, TGF-β1 and its downstream connective tissue growth factors promoted fibroblastic differentiation of PDLSCs by upregulating α-SMA, type-I collagen, and periostin (Fujii et al. 2010; Kono et al. 2013; Yuda et al. 2015). Furthermore, FGF-2 enhanced the proliferation of PDLSCs, while reversed the positive effects of VEGF and BMP-2 on osteogenic differentiation (Lee et al. 2012). Interestingly, consecutive use of FGF-2 followed by BMP-2 induced more osteogenesis than using either of them alone (Maegawa et al. 2007). Likewise, consecutive use of FGF-2 followed by TGF-β1 also upregulated fibroblastic differentiation of PDLSCs (Zhu and Liang 2015). Aspirin incubation also modulates the osteogenic potential of PDLSCs through the upregulation of several growth factor genes (Liu et al. 2012).

9.5.4 Clinical Applications of PDLSCs and their Secretome

Most of the clinical trials using PDLSCs were conducted for periodontal disease treatment. In two clinical studies (the first one including 3 participants with 16 defects (Feng et al. 2010) and the second one on 10 participants with 14 defects (Iwata et al. 2018)), improvement of the periodontal index (periodontal probing depth, clinical attachment level, and radiographic bone height) following treatment with PDLSCs has been reported. In a randomized clinical trial involving 30 participants with 41 intra-bony defects treated with autologous PDLSCs obtained from the third molars. The data showed insignificant increase in the alveolar bone height in the cell-treated group as compared to the control group (Chen et al. 2016). This could be referred to the different cell processing procedures and components of the transplanted products. Therefore, clinical translation of PDLSCs must establish proper methods to isolate and culture PDLSCs and set up the appropriate scaffolds (Yamada et al. 2020).

Like other MSCs, the therapeutic potential of human PDLSCs and their crucial role in periodontal tissue regeneration is mediated by paracrine release of bioactive molecules (Rajan et al. 2016)[44]. Human PDLSCs regulated the osteogenic and adipogenic differentiation of alveolar bone MSCs and inhibited alveolar bone MSCs-induced osteoclastogenic differentiation of mononuclear cells (Park et al. 2012b). Additionally, periodontal ligament cell-CM can regulate the expression of genes responsible for cell proliferation and bone homeostasis from MSCs upon coculturing with BMP-2 (Mizuno et al. 2008). The cytokine analysis of deciduous and permanent periodontal ligament cells revealed that immune response-related proteins and their degradation were markedly expressed in deciduous periodontal ligament-CM. On the contrary, the cytokines related to angiogenesis (epidermal growth factor (EGF) and IGF-1) and neurogenesis (NT-3 and NT-4) were resident in permanent periodontal ligament-CM making them a potential candidate for tissue regeneration (Kim et al. 2016). Human PDLSCs-CM loaded on collagen sponge for 4 weeks were transplanted in rat model of periodontal defect. The results showed induced alveolar bone regeneration, decreased exposed-root surface area and a concomitant formation of new periodontal tissue (Stuepp et al. 2019).

Moreover, the analysis of cytokines expressed by epithelial cell rests of Malassez, concealed within the periodontal ligament, revealed the expression of upregulated amounts of chemokines (IL-1, IL-6, IL-8, and IL-10), growth factors,

and related proteins (monocyte chemoattractant protein (MCP)-1, 2, 3, GM-CSF, VEGF, amphiregulin, GDNF, and IGF-binding protein 2) (Ohshima et al. 2008). The multilineage differentiation potential of PDLSCs and their paracrine activity (Eleuterio et al. 2013), and extracellular microvesicles with a high content of anti-inflammatory mediators (Yeo et al. 2013), render these cells and/or their products a novel therapeutic option in the clinical perspective.

9.6 Stem/Progenitor Cells from Apical Dental Papilla (SCAPs)

SCAPs are apical dental papilla derived-MSCs related to apices of developing roots (Sonoyama et al. 2008). They are positive for STRO-1, CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166, and alkaline phosphatase and negative for CD34, CD45, CD18, and CD150. CD24 is a unique surface marker for SCAPs, which is not shared by other MSCs including DPSCs and SHED. Therefore, CD24 is used as a specific marker to identify SCAPs (Sonoyama et al. 2006). CD24 expression decrease upon SCAP differentiation (Sonoyama et al. 2006).

SCAPs are characterized by their high proliferative potential, which is higher than that of DPSCs (Bakopoulou et al. 2011) and PDLSCs (Han et al. 2010). This can be referred to their longer telomere length and more significant telomerase activity than DPSCs (Jeon et al. 2011), whereas possessing a lower proliferation rate than DFSCs (Patil et al. 2014). SCAPs being a derivative of a developing tissue, is characterized by higher plasticity, remarkable migrational ability, and higher surviving expression than DPSCs and other DMSCs (Sonoyama et al. 2006). Additionally, the apical papilla is characterized by higher stem/progenitor cell yield than mature dental pulp (Sonoyama et al. 2006).

9.6.1 Immunomodulatory Properties of SCAPs

SCAPs have low immunogenicity and immunomodulatory properties. Swine derived SCAPs displayed low expression of swine leukocyte antigen class I and negative expression of swine leukocyte antigen class II DR molecule in a mini-pig model (Ding et al. 2010a). SCAPs inhibited effectively the autologous T-cell proliferation in vitro (Ding et al. 2010a) and can also reduce TNF-α expression in inflamed spinal cord tissues (De Berdt et al. 2018). The immunomodulatory properties of SCAPs were attributed to their ability to promote regulatory T-cells in vivo in dogs (Liu et al. 2019).

9.6.2 Differentiation Potential of SCAPs

SCAPs possess odontogenic/osteogenic and adipogenic differentiation potential (Bakopoulou et al. 2011; Abe et al. 2007; Sonoyama et al. 2006), with higher mineralization potential than DPSCs (Bakopoulou et al. 2011). They also express neurogenic markers in vitro without any neurogenic induction (Abe et al. 2007), in addition to their ability to differentiate into odontoblasts (Huang et al. 2008). It is noteworthy that SCAPs constitute the primary source of odontoblasts in the apical part of the root (Huang et al. 2008). They also have hepatogenic differentiation potential comparable to the BM-MSCs and superior neurogenic differentiation potential compared to the BM-MSCs. (Rao et al. 2019; Kumar et al. 2017a). Additionally, SCAPs have chondrogenic differentiation potential (Dong et al. 2013; Patil et al. 2014).

9.6.3 Potential Application of SCAPs

SCAPs can have a potential application in bone tissue regeneration. Hydroxyapatite loaded with SCAPs was associated with mineralized dentin/bone-like structure upon subcutaneous implantation in immune-compromised mice (Abe et al. 2008). It also showed promising results in dentin-pulp complex and periodontium regeneration. SCAPs and PDLSCs were successfully used for the generation of bio-root. Following in vivo implantation in a swine model, bio-root showed root/periodontal structure regeneration and support for porcelain crown (Sonoyama et al. 2006).

Furthermore, SCAPs and DPSCs implanted on a root fragment, they successfully regenerated dentin-pulp complex following ectopic subcutaneous implantation in rats (Li et al. 2018). SCAP-based scaffold loaded on treated dentine matrix fragments and implanted subcutaneously in immunodeficient mice promoted pulp regeneration and deposition of a layer of dentin-like tissue (Na et al. 2016). SCAPs promoted periodontal regeneration in miniature pigs with induced periodontitis (Li et al. 2018). Clinically, SCAPs loaded on polylactic polyglycolic acid and polyethylene glycol hydrogel were also successfully used for apexogenesis of lower left second premolar with immature apex and thin radicular dentinal walls in a 20-year old patient (Holiel et al. 2020). Growth factors as BMP and IGF-1 can enhance SCAPs odontogenic differentiation (Diao et al. 2020; Wang et al. 2016).

SCAPs also promoted neurite outgrowth (De Almeida et al. 2014), and demonstrated a neuroprotective effect in vitro through modulation of neuro-inflammation and upregulation of oligodendrocyte progenitor cell differentiation. This offers a potential application for treatment of spinal cord injury repair (De Berdt et al. 2018). Additionally, SCAPs showed promising results upon implantation in spi-

nal cord lesions in a rat model (De Berdt et al. 2015; Yang et al. 2017a). It also showed superior peripheral nerve repair in a rat sciatic nerve injury model compared to DPSCs and PDLSCs (Kolar et al. 2017). Further, SCAPs were used for in vitro generation of three-dimensional cell-based nerve-like tissue under EGF and basic FGF (Kim et al. 2017).

The regenerative potential of SCAPs is due to the secreted bioactive molecules. SCAPs express into their CM bioactive molecules, including chemokines, proteins responsible for angiogenesis, immunomodulation, chemotaxis, neuroprotection, anti-apoptosis, and extracellular matrix formation. SCAPs also express growth factors and cytokines involved in neural regeneration, that is, CSF, IFNγ, TGF-β, NGF, NT-3, and BDNF (Kumar et al. 2017a). They also express hepatic lineage proteins essential for hepatic differentiation (Kumar et al. 2017b) and osteogenic lineage proteins implicated in osteoblastic maturation, BMPs activation, and osteocytes differentiation (Kumar et al. 2018). SCAPs also have a pro-angiogenic effect in vitro and in vivo (Bakopoulou et al. 2015; Hilkens et al. 2014). Compared to BM-MSCs, SCAPs showed upregulation in the expression of proteins related to the metabolic processes and transcription, in addition to chemokines, and neurotrophins with lower levels of proteins responsible for adhesion, immunomodulation, angiogenesis, and extracellular matrix proteins (Yu et al. 2016).

9.7 Dental Follicle Stem/Progenitor Cells

DFSCs are MSCs derived from dental follicle surrounding the crown of the developing tooth; usually the third molar (Morsczeck et al. 2005). They express Nestin, Notch-1, (Morsczeck et al. 2005) STRO-1, CD29, CD44, CD90, and CD146 but negative for hematopoietic and angiogenic lineage-specific markers including CD31, CD34, and CD45 (Chen et al. 2017; Guo et al. 2013). They also express FGF-receptor (FGF-R)1-IIIC (Morsczeck et al. 2005). The characteristics of DFSCs remain unchanged during cryopreservation and, hence, they offer a valuable source for stem/progenitor cell banking (Yang et al. 2017b).

9.7.1 Immunomodulatory Properties of DFSCs

Like other DMSCs, DFSCs have immunomodulatory properties (Kang et al. 2015). DFSCs effectively suppressed the proliferation of T-helper cells and upregulated T-regulatory cells in vitro (Genç et al. 2018). DFSCs also down regulated pro-inflammatory cytokines (MCP-1), IL-1, IL-6, and TNF-α, as well as upregulated anti-inflammatory cytokine IL-10 in a rat acute lung injury model. They were also associated with upregulation in macrophage anti-inflammatory M2 phenotype in vitro via expression of TGF-β3 and thrombospondin-1 (Chen et al. 2018). DFSCs immunosuppressive properties can be employed to manage autoimmune diseases as they showed promising results in reducing the inflammation associated with Myasthenia gravis in a mouse model (Ulusoy et al. 2015).

9.7.2 DFSCs Differentiation Potential

DFSCs demonstrated osteogenic and cementogenic differentiation capacity (Kemoun et al. 2007; Morsczeck et al. 2008; Morsczeck et al. 2005) and odontogenic differentiation potential in vitro and in vivo (Guo et al. 2013). DFSC revealed comparable hepatogenic potential and superior neurogenic ability to BM-MSCs (Rao et al. 2019; Kumar et al. 2017a). They also displayed the ability to differentiate into cardiomyocytes (Sung et al. 2016).

9.7.3 Potential Application of DFSCs

DFSCs can be used in bone regeneration with promising results. DFSCs loaded on scaffold revealed bone formation in an experimental mice model (Park et al. 2012a). DFSCs loaded into a polycaprolactone scaffold enhanced bone regeneration in critical-size cranial defects in rats (Rezai-Rad et al. 2015). Additionally, DFSCs can be used on the surface of the titanium implant to improve bone regeneration (Lucaciu et al. 2015).

Dentin-pulp complex and periodontium regeneration can be achieved using DFSCs. DFSCs combined with treated dentin matrix and implanted subcutaneously in mice, formed pulp-dentin/cementum periodontium like tissues (Guo et al. 2013). Further, DFSCs loaded on scaffold successfully regenerated root-like tissues upon implantation in rats' alveolar fossa (Guo et al. 2012) and in jaws of miniature swine (Chen et al. 2015). Moreover, DFSCs cell sheet combined with treated dentin matrix scaffold and implanted subcutaneously in rats successfully produced dentin-pulp-like tissues and cementum-periodontal complexes (Yang et al. 2012).

DFSCs have also been used for salivary gland regeneration. Human epithelial stem-like cells isolated from human dental follicle tissues loaded on decellularized rat parotid gland scaffolds and implanted into the renal capsule of nude mice, were successfully able to differentiate into salivary gland-like cells (Xu et al. 2017). DFSCs secretome can also be potentially applied in tissue regeneration as a cell-free regenerative approach, as DFSCs express numerous bioactive molecules. DFSCs express hepatic lineage proteins; oncostatin M and hepatocyte growth factor receptor, which are important inducers of hepatic lineage differentia-

tion (Kumar et al. 2017b). They also express BDNF, NT-3, and NGF promoting neural regeneration (Kumar et al. 2017a). Additionally, DFSCs secretome contains osteogenic lineage proteins, including proteins for regulating endochondral ossification (MINPP1), for bone turnover (WISP2), and for mineralization (enamelin) (Kumar et al. 2018).They also express collagen type I, bone sialoprotein, and osteocalcin (Morsczeck et al. 2005).

9.8 Limitations Associated with Employing DMSCs in Tissue Regeneration

Despite advancements in TERM, reliance on DMSCs has revolutionized tissue regeneration in dentistry. There are some limitations that should be resolved before their clinical translation. Among the obstacles encountered in the use of DMSCs; the control of their differentiation, the difficulty of selection, and the delivery of the proper growth factors (Amrollahi et al. 2016).

Another challenge concerning the DMSCs is their low survival rate after transplantation and the possible risk of malignant transformation. The tendency of malignant transformation was primarily observed during in vitro expansion to achieve adequate cell number needed for clinical usage (Baglio et al. 2012; Rubio et al. 2008).

MSCs are promising tools in anti-cancer therapy as they have a preferential tendency to migrate towards tumors. This was mainly attributed to the enhanced inflammatory tumor microenvironment attracting the MSCs (Kidd et al. 2009). However, their potential for tumor formation, cancer progression, and metastasis were reported in the literature (Liu et al. 2017; Albarenque et al. 2011). The enhancement of cancer metastasis, for example, in breast cancer, was linked with the increased level of lysyl oxidase in MSCs (El-Haibi et al. 2012). The extended immunomodulatory properties of MSCs could be considered not beneficial in some cancer cases. This is explained by the fact that the MSCs may protect cancer cells from the immune clearance due to their ability to inhibit the natural killer cells and the cytotoxic T-lymphocytes, in addition to increasing the T-regulatory cells level (François et al. 2019). It is important to mention that MSCs used for anti-cancer therapy should be better derived from the same tissue/organ origin to decrease such undesirable effects (Ji et al. 2016).

Another limitation associated with the clinical application of DMSCs in the case of employing stem/progenitor cells derived from inflamed tissue; for example inflamed periodontal and pulp tissues; has been reported (Liu et al. 2012; Zhang et al. 2014). These cells show reduced immunomodulatory properties, downregulation in some osteogenesis-related genes and reduced ability to inhibit T-cell proliferation, T-helper differentiation, and IL-17 compared with MSCs derived from healthy tissues (Tang et al. 2016; Fawzy El-Sayed et al. 2019a, b). In addition to high amounts of IL-2, TNF-β, and TNF-α and low expression of CD90, CD166, and CD73 surface markers involved in immunomodulation (Yazid et al. 2014).

Furthermore, the variable isolation protocols of MSCs (that is, the concentration, method, and duration of enzymatic digestion) may significantly affect the surface antigens of the MSCs and change their surface topography (Furcht and Wendelschafer-Crabb 1978) (Rady et al. 2020). Additionally, their presence in very minute concentration in their particular tissue sources form an obstacle for the isolation of DMSCs in sufficient amounts; namely for SHED, SCAPs, and DPSCs (Rouabhia 2015). Overcoming and resolving these limitations are considered a great challenge in an attempt to reach successful and efficient DMSCs-based therapeutic approach.

9.9 Conclusion

Many studies have proved the regenerative capacity of DMSCs and their ability to form dental and non-dental tissues, offering novel approaches for treating damaged tissues and even organs in the human body (Fau and Park 2015). Although DMSCs are considered a magical tool in TERM with their simple, painless, non-invasive retrieval process, significant limitations still exist for their routine clinical use (Rady et al. 2020). The need for more research and clinical trials focusing on the immunology of DMSCs, the problems encountered with their isolation and transplantation are mandatory before their licensed clinical application. Simultaneously, combining the different emerging concepts in TERM, especially the novel cell-free therapeutic approach relying on the MSCs secretome, will offer a new perspective in the clinical translation of stem/progenitor cells in the medical and dental fields.

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Stem Cell-Based Tissue Engineering for Functional Enamel and Dentin/Pulp Complex: A Potential Alternative to the Restorative Therapies

10

Geraldine M. Ahmed, Eman A. Abouauf, Nermeen AbuBakr, Azza Ezz Elarab, and Karim Fawzy El-Sayed

Abbreviations

ACP	Amorphous calcium phosphate
ADSCs	Adipose-derived stem/progenitor cells
ALN	Alendronate
BD	Biodentin
bFGF	basic fibroblast growth factor
BM-MSCs	Bone marrow mesenchymal stem cells
BMP	Bone morphogenetic protein
BMP-2	Bone morphogenetic protein-2
BMP-4	Bone morphogenetic protein-4
CH	Calcium hydroxide
CMC	Carboxymethyl chitosan
DE	Dental epithelium
DFSCs	Dental follicle stem cells
DL	Dental lamina
DPSCs	Dental pulp stem cells
EB	Evoked bleeding
ECM	Extracellular matrix
ED	Embryonic day
DDGF	Dentin-derived growth factor
EOE	Enamel organ epithelial
ERM	Epithelial cell rest of Malassez
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
FGF8	Fibroblast growth factor 8
GC	Gubernacular cord
G-CSF	Granulocyte-colony stimulating factor
Gly	L-$_1$ glycine
GSK-3	Glycogen synthase kinase 3
H2S	Hydrogen sulfide
HA	Hydroxyapatite
HA/TCP	Hydroxyapatite/tricalcium phosphate
HAP	Hydroxyapatite
HERS	Hertwig's epithelial root sheath
hESCs	Human embryonic stem cells
hKSCs	Human keratinocyte stem cells
hTD	human treated dentin
iPSCs	Induced pluripotent stem cells
JE	Junctional epithelium
MSCs	Mesenchymal stem cells
MTA	Mineral trioxide aggregate
NaClO	Sodium hypochlorite
NF-SMS	Nanofibrous spongy microspheres
NFκB	Nuclear factor-κB
OEpSCs	Odontogenic epithelial stem cells
PCL	Polycapro lactone
PDGF	Platelet-derived growth factor
PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem cells

G. M. Ahmed
Stem Cell and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Department of Endodontics, Faculty of Dentistry, Cairo University, Cairo, Egypt

E. A. Abouauf
Stem Cell and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Department of Conservative Dentistry, Faculty of Dentistry, Cairo University, Cairo, Egypt

N. AbuBakr
Stem Cell and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Biology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

A. E. Elarab
Stem Cell and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

K. Fawzy El-Sayed (✉)
Stem Cell and Tissue Engineering Research Group, Faculty of Dentistry, Cairo University, Cairo, Egypt

Oral Medicine and Periodontology Department, Faculty of Dentistry, Cairo University, Cairo, Egypt

Clinic for Conservative Dentistry and Periodontology, School of Dental Medicine, Christian Albrechts University, Kiel, Germany

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PET	Polyethylene terephthalate
PEVA	Polyethylene-*co*-vinyl acetate
PGA	Polyglycolic acid
PLA	Polylactic acid
PLGA	Polylactic-*co*-glycolic acid
PLLA	Poly-L-lactic acid
PMNs	Polymorphonuclear neutrophils
PRF	Platelet-rich fibrin
PRP	Platelet-rich plasma
REE	Reduced enamel epithelium
SCAP	Stem cells from apical papilla
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor-1
Sema 3A	Smaphorin 3A
SHED	Stem cells from human exfoliated deciduous teeth
Shh	Sonic hedgehog
SIM	Simvastatin
TCP	Tricalcium phosphate
TDM	Treated dentin matrix
TGF-β	Transforming growth factor-β
UCMSCs	Umbilical cord mesenchymal stem cells
VEGF	Vascular endothelial growth factor

10.1 Introduction

Tissue engineering is an emerging field with the potential to impart various up-to-date medical and dental solutions. It employs the usage of cells, scaffolds, or signaling molecules in addition to their combination to produce tissue-like structures. This in turn helps to enhance or substitute the function of damaged cells, tissues, and organs of the body. Therefore, creating a successful tissue engineering assembly must be guided by the presence and the compatible interaction of these three vital constituents, which are identified as the tissue engineering triad.

Stem cells, either embryonic or adult, are considered the key tool in the field of regenerative medicine. Stem cells are defined as undifferentiated cells which possess unique capabilities of self-renewal. Moreover, stem cells gained their popularity in tissue engineering research because of their capability to proliferate and differentiate into a wide variety of different lineages, being accessible, easy to be isolated and expandable. All these unique properties make stem cells an extremely preferable source to promote a specific tissue regeneration, plus the fact of being heterogenous both in vitro and in vivo.

For efficient usage of cells in tissue engineering research, it is doubtless that there should be an adequate source of oxygen and nutrition provided to the transplanted cells to maintain their survival, vitality, and functionality. Hence, the importance of utilizing well-designed scaffolds in the field of regenerative medicine emerged. There are several types of scaffolds either natural or synthetic. Moreover, decellularized tissues and bioresorbable materials can be counted as scaffolding materials, because of their enhanced properties. Scaffolds are constructed to regulate the biological, physical, and chemical microenvironment encompassing a particular population of cells. Scaffolds provide the mechanical support required for cells to attach and tissues to grow and develop. Additionally, scaffolds have a crucial role in delivering signaling molecules either growth factors, cytokines, or chemokines to the transplanted cells. Therefore, the function of scaffolds can be classified under two main categories, either as a structural support of cells, on which cells can be cultured in vitro and can produce extracellular matrix, or as a delivery tool for growth factors and signaling molecules.

Finally, to accomplish the perfect tissue engineering triad, growth factors are the molecules required to enhance various cellular activities as migration, proliferation, and differentiation. Recently, numerous growth factors proved to have a vital therapeutic role in the regeneration and repair of various damaged organs and tissues in the body. Growth factors involved in tissue regeneration include, transforming growth factor-β (TGF-β), bone morphogenetic proteins (BMPs), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and nerve growth factor. These factors can amend cells–scaffold constructs in vitro and enhance their function and efficacy in vivo. However, translational therapy using growth factors is usually restricted by their quick dissemination from delivery site and their short half-life.

10.2 Cells

10.2.1 Sources of Cells for Dental Tissue Engineering Strategies

The formation of dental tissue involves two major cell types: ameloblasts, which are of epithelial origin and produce enamel, and odontoblasts, which are of mesenchymal origin and form dentin/pulp complex. In tooth development, ameloblasts are the solitary cells of ectodermal origin derived from epithelial stem/progenitor cells. Ameloblasts undergo apoptosis just after tooth eruption. Therefore, they don't exist in permanent teeth and cannot be stimulated in vivo to reform enamel (Lymperi et al. 2013). On the contrary, odontoblasts exist throughout life. After dentin formation, odontoblasts continue to produce dentin throughout life. They might also produce a type of reparative/reactionary dentin in response to caries and other external stimuli that might affect the tooth (Arana-Chavez and Massa 2004).

Regeneration of dental tissues necessitates special cells that can produce the extracellular matrix specific for each

tissue (Zaky and Cancedda 2009). In this regard, stem/progenitor cells are used in regenerative medicine, being nonspecialized and having the ability to self-renew and differentiate (Conrad and Huss 2005). Embryonic and adult stem cells are the two primary sources of stem/progenitor cells existing in humans.

Embryonic stem cells (ESCs) originate from the blastocyst's undifferentiated inner cell mass (Thomson et al. 1998). They are pluripotent cells that can differentiate into the three germinative layers (ectoderm, mesoderm, and endoderm), thus differentiating into any cell in the body (Rao and Zandstra 2005). However, their application is limited by legal and ethical considerations, restricting their usage (Hemmat et al. 2010). Another source of stem/progenitor cells is the umbilical cord, where blood is extracted via the umbilical cord containing stem cells that are genetically similar to those of the newborn baby. These cells are multipotent and are capable of differentiating into certain types of cells (Arien-Zakay et al. 2010). Oppositely, adult stem/progenitor cells are more readily applied in regenerative medicine, as they are more frequently available and can be easily harvested without damaging an embryo in addition to their ability of differentiation into numerous cell types (Bossù et al. 2014; Conrad and Huss 2005; Rai et al. 2015; Zaky and Cancedda 2009). Mesenchymal stem cells (MSCs) are multipotent, nonhematopoietic cells that are capable of proliferation and differentiation into various cell types (Friedenstein et al. 1976). They express specific protein markers as CD105, CD44, CD90, and CD73 and are negative for CD31, CD45, and CD34 (Dominici et al. 2006). Besides bone marrow, mesenchymal stem/progenitor cells are also found in skeletal muscle, adipose tissue, amniotic fluid, joint fluid, and umbilical cord. Numerous populations of MSCs are present in teeth and have the same properties as those present in the bone marrow. According to their location in the tooth, they can be categorized as dental pulp stem/progenitor cells (DPSCs) (Gronthos et al. 2000), stem/progenitor cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003), periodontal ligament stem/progenitor cells (PDLSCs) (Seo et al. 2004), dental follicle stem/progenitor cells (DFSCs) (Morsczeck et al., 2005), and stem/progenitor cells from apical papilla (SCAP) (Sonoyama et al. 2006).

10.2.2 Cells Used in Enamel Regeneration (Fig. 10.1 and Table 10.1)

10.2.2.1 Differentiated Cells

Differentiated Cells of Dental Origin

Dental Lamina and Gubernacular Cord The remnants of the dental lamina (DL), known as the "epithelial rests of Serres" in addition to the gubernacular cord (GC), both reported to contain remnants of the DL and may represent a new source of odontogenic epithelial stem/progenitor cells (OEpSCs) (Padma Priya et al. 2015). They have the potential for odontogenic epithelial cell proliferation. The pericoronal tissues of the third molar region are considered an essential source for the GC (Adeyemo, 2006). However, the isolation of odontogenic epithelial stem/progenitor cells from the active DL (from young children) raises ethical concerns. Moreover, tracing the "cell rests of Serres" is difficult because their existence is questionable, but the GC can be easily located on the gingiva over the erupting tooth (Ferreira et al. 2013; Padma Priya et al. 2015).

Epithelial Cell Rests of Malassez Epithelial cell rest of Malassez (ERM) originates from Hertwig's epithelial root sheath (HERS). It was proved that HERS contains OEpSCs, and these can differentiate into enamel-forming cells and regenerate dental composites when cocultured with DPSCs in the dental pulp core (Shinmura et al. 2008). ERM can be collected from the root surface of any extracted tooth or the tooth socket lining (Nam et al. 2014). Interestingly, the ability of the ERM as ectoderm-derived epithelial cells to undergo epithelial–mesenchymal transition has been studied by Xiong et al. (2012).

Reduced Enamel Epithelium and Junctional Epithelium The junctional epithelium (JE) proved to be odontogenic in origin and to have epithelial stem/progenitor cell potential. Moreover, it was demonstrated that the reduced enamel epithelium (REE) contains OEpSCs (Yajima-Himuro et al. 2014). Luckily, the REE can be obtained from the tissue covering any erupting crown and from the JE of erupting or recently erupted teeth (Priya et al. 2015).

Differentiated Cells of Nondental Origin

Nondental epithelial cells are supposed to be a new source of cells used in enamel tissue engineering technology, where postnatal nondental oral epithelial cells can regenerate into enamel-forming ameloblasts (Jayasudha et al. 2014).

Bone Marrow Cells Bone marrow cells and single-cell suspension of dental epithelial cells were cocultured with dental mesenchymal cells from a tooth germ. Twenty days later, constructs of enamel and dentin were regenerated (Hu et al. 2006).

Skin Epithelial Cells Skin epithelial cells were proved to regenerate into enamel forming cells. Thus, skin epithelial cells can function as an ideal replacement for ameloblasts under appropriate inductive methods (Liu et al. 2013).

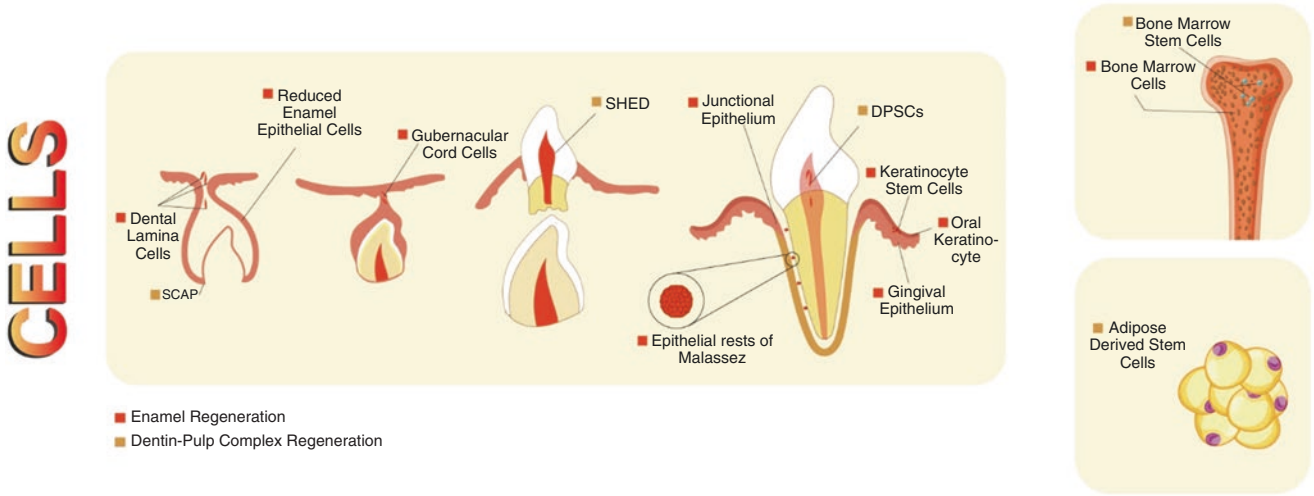

Fig. 10.1 Cells used in enamel, dentin, and pulp regeneration

Human Gingival Epithelial Cells When human gingival epithelial cells were combined with mice dental embryonic mesenchymal cells and transferred into kidney capsules for 6 weeks, micro-CT examination demonstrated the formation of tooth-like structures. Moreover, histological sections demonstrated the existence of enamel-like spaces, dentin, and well-vascularized pulp. Cells with an epithelial appearance and cuboidal shape were present on the outer-surface of the enamel space, indicating an ameloblast-like cell population at the post-secretory stage (Volponi et al. 2013).

Oral Keratinocytes Postnatal oral mucosal keratinocytes can provide an alternative for embryonic dental epithelium since cells obtained from young animals, cultured as cell sheets and recombined with embryonic dental mesenchyme, proved to form complex tooth-like structures (Nakagawa et al. 2009). Moreover, when oral epithelial lines were combined with fetal molar mesenchymal tissues and transplanted for 2–3 weeks, tooth formation was observed (Takahashi et al. 2010).

10.2.2.2 Stem/Progenitor Cells

Stem/Progenitor Cells of Dental Origin

Dental epithelial cells of early-stage embryos were proved to induce odontogenesis. The main limitation of these epithelial cells is that they remain in an immature form (Volponi et al. 2010). The first epithelial stem/progenitor cell niche was discovered in the cervical loop of rodents' incisors. These dental epithelial stem/progenitor cells are capable of differentiating into ameloblasts and producing enamel. This niche is a characteristic feature of rodents, since their incisors are erupting continuously throughout life (Smith and Warshawsky 1975).

Stem/Progenitor Cells of Nondental Origin

Human Embryonic Stem Cell-Derived Epithelial Cells As enamel organ epithelial (EOE) cells don't exist in adult teeth after tooth eruption, ESCs were used as an alternative cell source for ameloblast regeneration (Zheng et al. 2013). It was demonstrated that transplantation of EOE cells associated with DPSCs in scaffolds resulted in several processes related to amelogenesis. Also, enamel was produced in most mature transplants. Moreover, amelogenin immunoreactivity was distinguished in the epithelial cells on enamel or dentin surfaces, demonstrating that the tissue-engineered enamel contains well-developed ameloblasts. Together, these results demonstrated that cultured EOE cells could produce enamel (Honda et al. 2009).

Human Keratinocyte Stem/Progenitor Cells Human keratinocyte stem/progenitor cells (hKDCs) were reported to differentiate into enamel-forming ameloblasts with enamel deposition when associated with the mouse or human embryonic dental mesenchyme in the presence of proper growth factors and transplanted into mice renal capsules (Hu et al. 2018; Hu et al. 2014; Wang et al. 2010a).

Induced Pluripotent Stem Cells Mice induced pluripotent stem cells (iPSCs) cocultured with epithelial cell rests of Malassez conditioned medium regenerated into ameloblast-like cells and showed upregulation in the expression of various enamel proteins as ameloblastins, keratin 14, and amelogenins (Yoshida et al. 2015).

Table 10.1 Summary of studies on enamel, dentin, and pulp regeneration

Author	Cells	Scaffolds	Signaling molecules	Outcome
Gronthos et al. (2000)	Human DPSCs	Hydroxyapatite/ Tricalcium phosphate (HA/TCP) ceramic powder	–	Formation of dentin-like structures lined with odontoblast-like cells and surrounding a pulp-like tissue
Gronthos et al. (2002)	Human DPSCs	HA/TCP ceramic powder	–	Dentin-pulp-like tissue was formed
Batouli et al. (2003)	Human DPSCs	HA/TCP ceramic powder	–	Dentin/pulp-like complexes were formed with pulp-like tissues in addition to the expression of dentin sialoprotein (DSP)
Miura et al. (2003)	Stem/Progenitor cells from human exfoliated deciduous teeth (SHED)	HA/TCP ceramic powder	–	Odontoblasts were formed with dentin-like structure immunoreactive to dentin sialophosphoprotein (DSPP) antibody
Duailibi et al. (2004)	Rat tooth bud cells	Polyglycolic acid (PGA) and polylactic-*co*-glycolide copolymer (PLGA)	–	Successful bioengineering of mature tooth structures
Honda et al. (2006)	Porcine odontogenic epithelial cells	PGA fiber mesh (PGA scaffold)	Epidermal growth factor	Multiplication of odontogenic epithelial cells and the characteristics of differentiated ameloblasts
Hu et al. (2006)	c-Kit$^+$-enriched bone marrow cells (BMCs) with embryonic dental epithelial cells cultured in re-association with dental mesenchyme	–	–	Ameloblast-like cells were formed with the expression of amelogenin and ameloblastin
Cordeiro et al. (2008)	SHED	Biodegradable scaffolds	–	SHED differentiated into odontoblasts and endothelial-like cells and dental pulp-like tissue was formed
Shinmura et al. (2008)	Porcine epithelial cell rest of Malassez (ERM) obtained from periodontal ligament tissue subcultured with dental pulp cells	Collagen sponge	–	Cytokeratin-14 and amelogenin proteins were expressed in vitro in addition to the formation of enamel-like tissues in vivo
Honda et al. (2009)	Subcultured enamel organ epithelial cells in combination with primary dental pulp cells	Collagen sponge	–	Enamel–dentin structures were formed
Nakagawa et al. (2009)	Mice palatal epithelium-embryonic mandibular molar mesenchyme re-associations	–	–	Calcified teeth with molar structures were formed. Immunohistochemical staining revealed the expression of amelogenin
Okamoto et al. (2009)	hDPSCs	HA/TCP ceramic powder	Simvastatin	Osteocalcin and dentin sialophosphoprotein were upregulated in vitro in addition to mineralized tissue formation in vivo
Huang et al. (2010)	DPSCs and SCAP	Poly-D,L-lactide/ glycolide	–	Pulp and dentin-like tissues were formed with odontoblast-like cells expressing dentin sialophosphoprotein, bone sialoprotein (BSP), alkaline phosphatase (ALP) and CD105
Sakai et al. (2010)	SHED	Poly-L-lactic acid (PLLA)	–	SHED differentiated into angiogenic endothelial cells and odontoblasts capable of generating tubular dentin

(continued)

Table 10.1 (continued)

Author	Cells	Scaffolds	Signaling molecules	Outcome
Takahashi et al. (2010)	Clonal cell lines from the oral epithelium of p-53 deficient fetal mice cultured with fetal molar mesenchymal tissues	–	–	Ameloblasts were polarized and regularly lined up along with calcified enamel with the expression of amelogenin
Wang et al. (2010a)	Human keratinocytes recombined with mouse embryonic dental mesenchyme	–	Fibroblast growth factor 8 (FGF8)	Human keratinocytes expressed the dental epithelial marker *PITX2* and differentiated into enamel-secreting ameloblasts
Wang et al. (2010b)	hDPSCs	Nanofibrous (NF)-poly(L-lactic acid) (PLLA) scaffolds	Control medium, DXM medium or BMP7 + DXM medium	NF-PLLA scaffold supported odontogenic differentiation of hDPSCs and formation of dentin-like tissue BMP-7 and DXM better promoted the odontogenic differentiation and dentin-like tissue formation both in vitro and in vivo
Bakopoulou et al. (2011)	DPSCs and SCAP	–	–	3D mineralized structures were formed and expressed DSPP, BSP, osteocalcin, ALP
Hung et al. (2011)	ADSCs	Collagen gel	BMP-2	Tooth-like structures were formed with a dental pulp embedded in the regenerated dentin
Volponi et al. (2013)	Human gingival epithelial cells cocultured with embryonic mouse molar mesenchyme tissues	–	–	Micro-CT analysis revealed the formation of tooth-like structures and histological sections confirmed the presence of teeth-like structures with enamel spaces, dentin, and pulp tissues
Lei et al. (2013)	Bone marrow mesenchymal stem cells (BM-MSCs)	Dentin matrix	–	BM-MSCs were polarized into odontoblast-like cells with processes penetrating into dentinal tubules and with expression of DSP
Liu et al. (2013)	Rat skin epithelial cells cocultured with rat dental papillae mesenchymal cells	–	–	Enamel–dentin-like structures were formed with the expression of CK14, a specific marker for ameloblast-lineage cells
Nowicka et al. (2013)	Direct pulp capping	Bio-dentin and MTA	–	Layers of well-arranged odontoblast and odontoblast-like cells were found to form
Rosa et al. (2013)	SHED	Puramatrix™ (peptide hydrogel), or human collagen (rhCollagen) type I	–	Pulp-like tissues were formed with odontoblasts capable of generating new tubular dentin
Yamamoto (2013)		Flexible double-layered sheet of hydroxyapatite (HAp) coated with a tricalcium phosphate	–	HAp and HAp/TCP sheets were structurally unified in part to tooth enamel, offering an ideal method for enamel repair
Zheng et al. (2013)	hESCs line	–	BMP4 and α-retinoic acid (RA) and lithium chloride	hESC-derived epithelial cells were induced and expressed amelogenesis-associated genes in addition to cytokeratin 76
Cao et al. (2014)	–	Amorphous calcium phosphate hydrogel mats	Fluoride	Enamel prism-like layers were generated with well-formed hydroxyapatite crystals
Chen et al. (2015a)	DPSCs	Fragments of platelet-rich fibrin (PRF) and DPSCs	–	Pulpal and dental-like tissues were formed in the root canal, both subcutaneously and in canines' roots
Chen et al. (2015b)	Human UCMSCs	Human tooth dentin matrix (hTDM)	–	Human UCMSCs differentiated into odontoblast-like cells and expressed (DSP and DMP-1)
Chrepa et al. (2015)	MSCs	Evoked bleeding and blood clot	–	Evoked-bleeding technique delivers undifferentiated MSCs into the root canal systems of mature teeth

(continued)

Table 10.1 (continued)

Author	Cells	Scaffolds	Signaling molecules	Outcome
Kuang et al. (2015)	hDPSCs	Nanofibrous spongy microspheres (NF-SMS) from poly(L-lactic acid) -block-poly(L-lysine)	–	Dentin-like tissue formation
Murakami et al. (2015)	ADSCs	Atelocollagen scaffold	–	Pulp-like tissues were regenerated in addition to collagenous matrix containing *DSPP*-positive odontoblast-like cells
Seki et al. (2015)	Mouse iPSCs	–	Pax9 and BMP-4	iPSC-derived neural crest-like cells were differentiated into odontoblast-like cells
Tran and Doan (2015)	HDPSCs	Dentin-derived scaffold	–	Regeneration of dentin-like tissues
Yoshida et al. (2015)	Mouse iPSCs	–	–	Differentiation of ameloblast-like cells in addition to expression of high levels of amelogenin and ameloblastin
Zhang et al. (2015)	BM-MSCs	–	SDF-1	Dental-pulp-like tissue and newly formed vessels were regenerated
Yoshida et al. (2016)	DPSCs	Nano-β-tricalcium phosphate collagen scaffold	Semaphorin 3A	Induced cell migration, chemotaxis, proliferation, and odontoblastic differentiation of DPSCs in vitro in addition to reparative dentin formation in vivo
Neves et al. (2017)	Mice-resident pulp stem cells	Collagen sponge	GSK-3 antagonists	Reparative dentin was formed
Ruangsawasdi et al. (2017)	hMSCs	Fibrin gels	SCF	Accelerated cell homing and maturation of dental–pulpal composites in human immature teeth
Wang et al. (2017)	Remineralization of enamel	Carboxymethyl chitosan conjugated with alendronate guided by glycine	Glycine	HAP/amorphous calcium phosphate nanoparticles were organized in rod-like apatite crystals
Xie et al. (2018)	DPSCs	Dentin discs with PLA scaffolds	–	Pulpal-like tissues were produced in addition to dentin
Hu et al. (2018)	Cultured human keratinocyte stem cells (hKSCs) combined with embryonic mouse dental mesenchyme	–	FGF8 and SHH	Histological analysis revealed differentiation of ameloblasts and formation of tooth-like structures. Scanning electron microscope revealed the similarity between regenerated enamel and that of human enamel
Yang et al. (2018)	hDPSCs		Cystathionine β-synthase and cystathionine γ-lyase siRNA treatment	Increased DPSCs proliferation and decreased DPSC dentinogenic differentiation and decreased expression of RUNX2, ALP and DSPP in vitro in addition to decreased dentin-like hard tissue formation of DPSCs in vivo
Li et al. (2019)	hESCs in vitro. The generated dental epithelium mixed with mouse embryonic dental mesenchyme in vivo	–	Different concentrations of BMP4	Differentiation of oral ectoderm and dental epithelium in vitro Tooth-like structures were formed in vivo
Chang et al. (2020)	DPSCs	Allogenous-treated dentin matrix	–	New dentin pulp-like tissues, enamel dental pulp complexes were developed

10.2.3 Cells Used in Dentin–Pulp Complex Regeneration (Fig. 10.1 and Table 10.1)

10.2.3.1 Stem/Progenitor Cells

Stem/Progenitor Cells of Dental Origin

All dental stem/progenitor cells have the same origin, being derived from cranial neural crest ectomesenchyme, and thus developmentally and functionally could appear identical. Yet, studies have proved that they have different gene expression profiles (Nakamura et al. 2009).

Stem/Progenitor Cells Derived from Human Adult Teeth

Dental Pulp Stem/Progenitor Cells The presence of stem/progenitor cells in the dental pulp of adults was first discovered in 2000 (Gronthos et al. 2000). DPSCs are located at the peri-odontoblastic and perivascular chambers inside the tooth pulp (Janebodin et al. 2011; Shi and Gronthos 2003). They are best obtained from third molars (Gronthos et al. 2002). Their shape is similar to the pericyte-like smooth-muscle-actin-expressing cells (Zhao et al. 2012). DPSCs can differentiate into cells from the three embryonic layers, endoderm, mesoderm, and ectoderm, and are thus comparable to ESCs and iPSCs (Atari et al. 2011; Janebodin et al. 2011). DPSCs express pluripotency markers as Lin-28, Oct-4, Nanog, and Sox-2 (Yu et al. 2007). These cells have an elevated rate of proliferation compared to bone marrow MSCs (BM-MSCs) (Gronthos et al. 2000; Huang et al. 2009) and can differentiate into multiple cell lineages, as dentin forming cells, cartilage cells, fat cells, muscle cells, and nerve cells (Gronthos et al. 2002; Yang et al. 2007).

Several studies demonstrated that DPSCs have an essential role in dentin–pulp tissue regeneration (Batouli et al. 2003; Gronthos et al. 2002; Gronthos et al. 2000). When DPSCs were combined with hydroxyapatite/tricalcium phosphate (HA/TCP) scaffolding materials and transplanted in immunocompromised mice, dentin–pulp complex containing odontoblastic cells was generated (Batouli et al. 2003; Gronthos et al. 2000; Batouli et al. 2003; Gronthos et al. 2002). It was reported that DPSCs and SCAP are able to differentiate into dentin–pulp-like complexes with well-formed vascularity when transplanted into an empty root canal space (Huang et al. 2010). A further study demonstrated that DPSCs obtained from impacted third molars at the stage of root development could regenerate into dentin forming-like cells with strong mineralization potential, leading to well-organized three-dimensional dentin-like structures in vitro (Bakopoulou et al. 2011).

Stem/Progenitor Cells Derived from Developing Teeth

Stem Progenitor Cells from Human Exfoliated Deciduous Teeth The existence of multipotent MSCs within the dental pulp of deciduous teeth was first reported in 2003 (Miura et al. 2003). Fortunately, SHED expressed numerous pluripotency markers (Kerkis et al. 2006). They can differentiate into odontoblasts, neuro-like cells, adipocytes, and osteoblasts (Miura et al. 2003). When SHED were transferred in the subcutaneous tissue of immunodeficient mice, an effective dental pulp was produced in the roots of human premolars (Rosa et al. 2013). It was also reported that when SHED were seeded on a poly-L-lactic acid (PLLA) scaffold assembled inside slices of teeth, and these scaffolds were transplanted subcutaneously in immunodeficient mice, SHED differentiated into angiogenic endothelial cells and functional odontoblasts capable of producing tubular dentin. Studies using SHED in dental pulp tissue engineering in vivo reported that the formed tissue had architecture and cellularity resembling dental pulp (Cordeiro et al. 2008). Also, SHED have significantly higher proliferation rates compared with DPSCs and BM-MSCs (Nakamura et al. 2009). Comparing the profiles of genes expression, it was revealed that 4386 genes are expressed at different levels in DPSCs and SHED by twofold or more. SHED expressed upregulated levels of genes involved in cell proliferation pathways and synthesis of ECM, inclusive of numerous growth factors as FGF and TGF-β (Nakamura et al. 2009).

Stem/Progenitor Cells from Apical Papilla A distinctive category of dental stem cells. They are found at the ends of developing tooth roots, where the apical papilla tissue exists only during formation of roots ahead of tooth eruption in the oral cavity (Huang et al. 2008). Like DPSCs and SHED, stem/progenitor cells from apical papilla (SCAP) are positive for CD146 and STRO-1 and negative for CD34 and CD45. However, CD24 is only found on SCAP and not on DPSCs and SHED. The expression level of CD24 was downregulated when SHED were differentiated into odontoblasts in vitro coinciding with alkaline phosphatase upregulation (Bakopoulou et al., 2011). An important source of SCAP is the third molars and teeth with open apices. It was documented that human SCAP had a higher differentiation rate than DPSCs, with higher proliferative capacity and mineralization potential (Bakopoulou et al. 2011; Sonoyama et al. 2006).

Furthermore, recent data have reported that SCAP displayed unique "embryonic" characteristics (Jo et al. 2007). These data showed that SCAP could be differentiated into

osteoblasts, odontoblasts, and adipocytes, in vitro, whereas in vivo they can produce osteoblasts and odontoblasts (Ikeda et al. 2006; Kikuchi et al. 2004). Moreover, transplantation of SCAP along with HA/TCP (hydroxyapatite/tricalcium phosphate) as a carrier on mice resulted in dentin production (Bakopoulou et al. 2011). Therefore, SCAP are considered a good source of primary odontoblasts when forming root dentin. Oppositely, DPSCs are the main source of secondary odontoblasts when forming reparative dentin (Han et al. 2010). The discovery of SCAP explained a clinical phenomenon observed in many clinical case reports in which apexogenesis occurred in an immature infected permanent tooth, having apical periodontitis or abscess (Chueh and Huang 2006). It showed that SCAP present inside the apical papilla overcame such infection due to their closeness to the periapical tissues and their collateral circulation (Volponi et al., 2010).

Induced Pluripotent Stem Cells

When iPSCs reprogrammed from DPSCs were cocultured with PLLA scaffolds on dentin discs and then, transplanted subcutaneously in mice, it was found that iPSCs produced a tissue resembling pulp with tubular dentin (Xie et al. 2018). Moreover, it was demonstrated that iPSCs transfected with Pax9 and BMP-4 genes possessed an enhanced potential to form dentin forming-like cells and express upregulated levels of dentin matrix protein 1 and dentin sialophosphoprotein, which are usually accompanied with odontoblastic differentiation of iPSCs (Seki et al. 2015).

Stem/Progenitor Cells of Nondental Origin

Bone Marrow-Derived Mesenchymal Stem/Progenitor Cells (BM-MSCs) When BM-MSCs were cultured on a dentin matrix scaffold, they differentiated into dentin forming-like cells with numerous processes extending into dentinal tubules (Lei et al. 2013). Meanwhile, a study (Zhang et al. 2015) reported that endogenous BM-MSCs have a powerful systemic homing effect to root canals. This effect was induced by stromal cell-derived factor-1 (SDF-1), in a tooth with a root canal which was transplanted subcutaneously.

Adipose-Derived Stem/Progenitor Cells In an earlier study (Hung et al. 2011), adipose-derived stem/progenitor cells (ADSCs) were suggested as alternative source to DPSCs due to their large population in mammals and higher rate of proliferation with similar results to DPSCs in tooth regeneration. A further study (Murakami et al. 2015) demonstrated that even though DPSCs were superior, sufficient amount of ADSCs and BM-MSCs could be used as a substitute for DPSCs.

Umbilical Cord Mesenchymal Stem/Progenitor Cells (UCMSCs) UCMSCs can be available in extensive amounts without the need to traumatizing methods. They can also be preserved in banks of stem cells. It was reported that UCMSCs could be differentiated into odontoblasts and produce hard tissue. Markedly, UCMSCs are protected by the placenta against any viral infection, so they are considered safe, and this granted them a significant clinical importance (Chen et al. 2015b).

10.3 Scaffolds

10.3.1 Definition

The American Society for Testing Materials (ASTM—F2150), defined a scaffold as "the support, delivery vehicle, or matrix for facilitating the migration, binding, or transport of cells or bioactive molecules used to replace, repair, or regenerate tissues." In view of tissue engineering, it is a highly porous artificial extracellular matrix (ECM), which acts as a template for tissue creation (Ahmed et al. 2020; Mathew et al. 2016).

According to diverse conditions and requirements, a variety of scaffolds of different sources and natures could be serviceable. Scaffolds could be natural or synthetic, biodegradable or not. Scaffolds could be classified according to the source as follows.

10.3.2 Natural Sources

Some scaffolds are derived from mammalian tissues, such as acellular natural scaffolds (e.g., decellularized dentin matrix) or natural polymeric materials (collagen, chitosan, hyaluronic acid, silk fibroin, and gelatin). The advantage of the natural polymeric scaffold is the biocompatibility with the surrounding tissues.

10.3.3 Synthetic Sources

Synthetic, fully degradable, and bioresorbable polymers, such as polylactic acid (PLA), polyethylene terephthalate (PET), polycaprolactone (PCL), poly(lactic-*co*-glycolic acid (PLGA), and polyethylene-*co*-vinylacetate (PEVA), have been introduced. However, only five of these polymers have been approved by the FDA: PLA, polyglycolic acid (PGA), PCL, polydioxanone, and poly-PCPP-anhydride. Synthetic polymers offer valuable physicochemical and mechanical properties. Other synthetic sources such as alumina or titania, ceramics, and their composites were also utilized as

scaffolds, especially for bone regeneration and dental purposes.

Other recent scaffolds were engineered using bionanomaterials, including nanochitosan/nanochitin, nanocellulose, carbon-based nanomaterials, calcium phosphate nanoparticles, and, finally, the hydroxyapatite nanoparticles.

10.4 Requirements of an Ideal Scaffold

The biological components and the scaffold material should not initiate damaging effects to the surroundings, neither to the cells nor to the tissues of concern. Accordingly, the materials should not be antigenic, carcinogenic, or toxic to the living tissues. Moreover, some other requirements must be taken into consideration.

10.4.1 Porosity

Sufficient porosities in a scaffold permit the flow of nutrients inside the scaffold to reach the cells at the same time as the flow of the extracellular matrix (ECM) formed by these cells. Also, it allows the dissemination of scaffold degradation products and waste as well.

10.4.2 Mechanical Properties

The mechanical characteristics of the scaffold should match its proposed application and function in a specific environment. Properties like the elastic modulus, yield stress, and ultimate stress determine the stiffness, deformability, and strength of a material, for example, in the case of bone construction. Another property is fatigue, which could be a sign of failure due to cyclic stress, as in prosthetic heart valves.

10.4.3 Biocompatibility

The scaffold should not provoke the tissues to generate neither general nor local toxic actions in the body. The scaffold should be completely biodegradable and fully replaced by the natural tissues at the implant site.

10.4.4 Immune Acceptance

Transplanted cells and implanted scaffolds could initiate a chronic immune response, leading to rejection of the implanted tissues. However, a positive type of limited immune response could be valuable. In response to a foreign body within the hosted tissue, inflammatory cytokine and chemokine production will provoke polymorphonuclear neutrophils, monocytes, and fibroblasts assembly at the injury sites. Macrophages summit at the injury sites secreting reactive oxygen species and cytokines. These macrophages will secrete growth factors and phagocyte the cell debris resulted from the damage promoting healthy tissue regeneration. Therefore, modifications of the biomaterials are being carried on to minimize the host rejection upon implantation.

10.5 Scaffolds Used in Enamel Regeneration (Fig. 10.2 and Table 10.1)

The mature enamel is an acellular structure and cannot regenerate itself. Unlike other biomineralized tissues such as bone and dentin, once the tooth has fully erupted, enamel reformation is not an option to repair any casual damage of the hard-dental structure. Moreover, amelogenesis is a complex and sensitive process of multiple interactions involving various proteins, extracellular matrix components, and a precise mixture of inorganic constituents. Three main proteins are crucial for enamel formation: ameloblastin, enamelin, and amelogenin. They are present in teeth undergoing development. Trials and attempts to create synthetic enamel or regenerate enamel via cell-based strategies were tested but faced many challenges (Moradian-Oldak 2009; Moradian-Oldak and Library 2012).

10.5.1 Synthetic Enamel Fabrication: Regenerating Enamel-Like Hydroxyapatite Microstructures

Attempts to generate enamel via hydroxyapatite (HAP) crystals using immediate hydrogel mats of amorphous calcium phosphate and polymer nano- and microfibers and trials to adhere flexible HAP sheets were tested as a treatment of eroded tooth surface. Also, HAP was structured in an enamel-like configuration using a mediating agent of organic phosphate surfactant and gelatin (Cao et al. 2014; Yamamoto et al. 2013).

Synthetic initial enamel formation in a three step procedure was attempted: first carboxymethyl chitosan (CMC) is conjugated with alendronate (ALN) to stabilize amorphous calcium phosphate (ACP) and produce CMC/ACP. Then sodium hypochlorite (NaClO) degrades the CMC-ALN matrix. Lastly, L-$_1$ glycine (Gly) is used to organize HAP/ACP nanoparticles in rod-like apatite crystals (Wang et al. 2017).

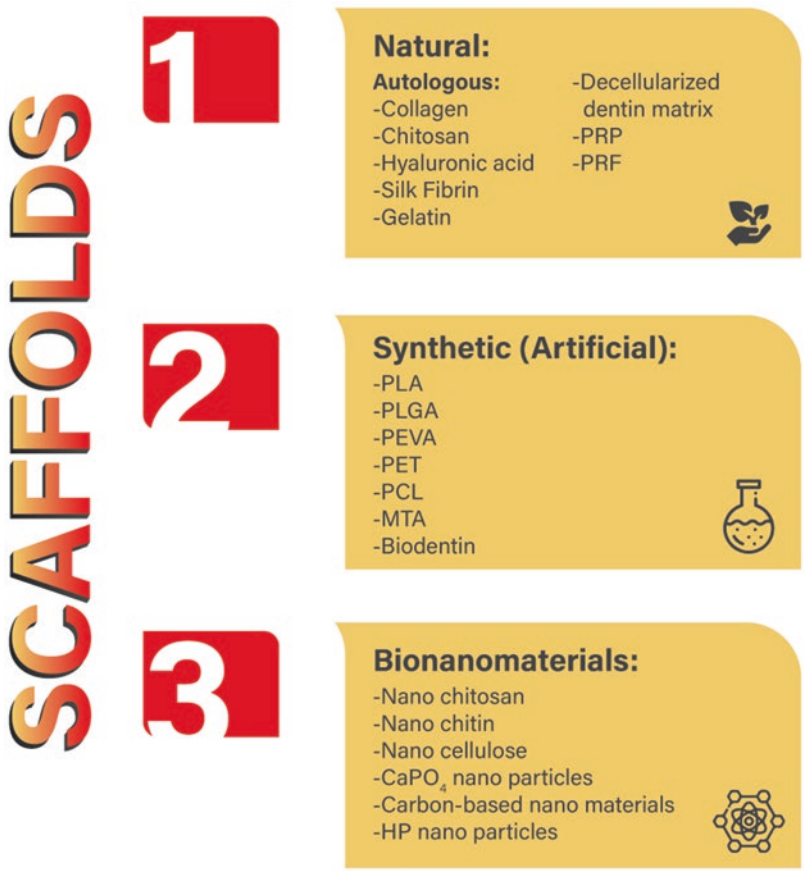

Fig. 10.2 Scaffolds used in enamel, dentin, and pulp regeneration

10.5.2 Enamel Regeneration via Cell-Based Strategies: Regenerative Treatment Requires Stem Cells, Scaffold, and Growth Factors

Enamel tissue engineering faces many challenges, especially that ameloblasts' differentiation (though epithelial in origin) is strictly dependent on odontoblast differentiation. Moreover, the definite mechanism of ameloblast cell accumulation, nucleation, and inorganic crystallization is considered to be a puzzling problem. It is an obstacle to unite the original tooth structure (enamel and/or dentin) with the de novo generated enamel. The strict organization of the morphology and exact shaping of the tissue-engineered enamel to resemble the typical shape or size as required is also problematic goal. It is governed by reciprocal signals between various components of the tooth. Another challenge is that dental epithelial cells are rarely generated through pluripotent cells from somatic origin or alternative source cells.

Innovative tactics and models were carried on to regenerate enamel on the cellular level. Some attempts succeeded in inducing enamel regeneration using a dental epithelium cell line and a three-dimensional biodegradable polymer scaffolds (polyglycolic acid fiber mesh, i.e., PGA scaffold) coated with collagen (Honda et al. 2006). Autoclaved dentin was employed to obtain treated dentin matrix scaffold (TDM) to support DPSCs to build up enamel-like tissue in vivo (Chang et al. 2020).

10.6 Scaffolds and Biodegradable Materials for Dentin and Pulp Regeneration (Fig. 10.2 and Table 10.1)

Both the dentin and pulp originate from the same source (ectomesenchyme embryonic cells). Thus, the attempts to induce pulp healing could directly influence dentin regeneration.

Early in-vitro trials were successful in aiding dentin regeneration and tertiary dentin formation, basically using chitosan/collagen matrices, fibrin, human-derived treated dentin (hTD), and biodegradable collagen sponge. Also, ceramic scaffolds and bioactive glass proved to aid in dentin regeneration, and tissue mineralization when embedded with tricalcium phosphate (TCP) or hydroxyapatite (HA). Moreover, regular pulp capping materials, as mineral trioxide aggregate (MTA), calcium hydroxide (CH), and biodentin (BD), proved to promote tertiary dentin formation (Aliasghari et al. 2016; Nowicka et al. 2013; Tran et al.

2015). Dentin regeneration and mineralization were achieved by using nanofibrous spongy microspheres (NF-SMS), primarily when biodegradable and biocompatible poly(L-lactic acid) block-poly(L-lysine) were constructed into the NF-SMS with interconnected pores (Kuang et al. 2015). In-vitro and in-vivo studies investigated PLA biomaterials and proved to induce differentiation of DPSCs to mature odontoblasts (Wang et al. 2010b).

Early successful attempts of evoked bleeding (EB) were tried, where the blood clot should act as a protein scaffold and interact with endogenous stem/progenitor cells and growth factors, promoting the healing of both immature and mature teeth with peri-apical lesions (Chrepa et al. 2015). Other natural scaffolds were introduced for pulp regeneration to replace conventional endodontic therapy, including platelet-rich fibrin (PRF) and platelet-rich plasma (PRP) derived from the patient's blood samples and introduced inside the root canal. The 3D fibrin network is thereby acting like an autologous scaffold, loaded with growth factors and bioactive molecules, thus decreasing the risk for immunological reactions. Yet, one drawback is that its mechanical characteristics are not comparable to the synthetic biomaterials. Still, it was reported that a transplant of cell-sheet DPSCs and PRF granules regenerated pulp-dentin-like tissues in the roots of experimental canines (Chen et al. 2015a).

10.7 Signaling Molecules

10.7.1 Importance of Growth Factors and Signaling Molecules in Dental Regeneration

During embryo development, tooth organogenesis is synchronized by reciprocal epithelial and mesenchymal interaction that is a principal developmental mechanism involving stem cells, signaling molecules, and transcription factor pathways. Ligand–receptor interactions trigger the transcriptional changes to orchestrate the cellular processes required for the progression of tooth development. The tooth-forming fields in mice during the early development are specified at embryonic day (ED) 10–11 through the expression of homeobox genes such as Msx1, Msx2, Barx1, and Lhx8 and secretory molecules including bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs). At ED 11.5, the oral epithelium invaginates into the mesenchymal region; then, the tooth bud is formed by the aggregation of mesenchyme tissue derived from neural crest cells. The enamel knot, which is a transient epithelial signaling center, expresses numerous signaling molecules, including Shh (sonic hedgehog), Wnt10b (wingless), BMPs, and FGFs, that regulate cell fates and epithelial–mesenchymal interactions at ED 13.5–14.5. The cells in the tooth germ terminally differentiate into the tooth tissue progenitor cells, including ameloblasts, odontoblasts, and dental follicle cells at ED 16.5–18.5. These progenitor cells accumulate the enamel or dentin matrix. The periodontal tissues are derived from dental follicle cells differentiated into cementum, periodontal ligament (PDL), and alveolar bone. Accordingly, tooth root elongation is followed by tooth crown formation, then the mature teeth erupt. After tooth development is complete, it is believed that various immature cells are maintained as adult stem cells acting as a self-repair system for injured dental tissue.

The signaling molecules are considered the third essential aspect in the tissue engineering for regulating the cellular processes for new tissue formation. They are typically released from cells and are directly presented to cell surface receptors through their interaction with the neighboring extracellular matrix. The signaling molecules include growth factors, cytokines, chemokines, proteins, or drugs. To achieve the cell type desired during tissue engineering, growth factors must bind to specific cell-membrane-linked receptors, activating mechanisms and pathways (Kitamura et al. 2012; Werner and Grose 2003). The growth factor-mediated cell responses are crucial for growth, wound healing, and angiogenesis in repair/regeneration processes (Zarei and Soleimaninejad 2018).

Groups of signaling molecules have been used for dentin–pulp regeneration procedures in experimental animals and even human trials. It seems to resemble the in-vivo situation in which the possible interactive synergistic effects of different signaling factors are essential for tissue regeneration. Controversies remain regarding the choice of signaling molecules, their respective concentration, and the stability of the bioactive molecules. The choice to use a single signaling molecule during tissue regeneration seems to be a straightforward approach. However, the choice of a specific signaling molecule, which acts as a catalyst to start more complex signaling cascades that are essential for tissue reformation and information on definite and exact concentrations, is purely empirical. Unfortunately, adverse effects such as increased carcinogenicity or problems related to immune response and the stability of these molecules should also be considered (Schmalz et al. 2017).

Signaling pathways are composed of cell-surface receptors, intracellular molecules, and transcription factors regulating gene expression. Signaling molecules became a part of the dental regeneration to induce the genesis of the required tissue types by mastering the differentiation and proliferation of the stem cells. Examples of signaling pathways involved in dental tissue formation are Notch signaling, Wingless-integration pathways (WNT1, WNT/β catenin), mitogen-activated protein kinases pathways (p38 MAPK pathway, ERK MAPK, JNK MAPK), phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway, Shh pathway, heme oxygenase 1 pathway, and signaling pathways mediated by Ephrin-B1-4.

10.7.2 Signaling Molecules Used in Enamel Regeneration (Fig. 10.3 and Table 10.1)

The enamel is the most highly mineralized tissue in the body, consisting of more than 96% inorganic material as apatite crystals and traces of organic material. The ameloblasts (cells responsible for enamel formation) form a continuous surface layer during tooth formation but undergo apoptosis after tooth eruption. As previously expressed, the death of ameloblasts renders the enamel a nonvital and insensitive structure, which cannot be replaced or regenerated if destroyed by wear or caries. To overcome such inherent deficiency, enamel was created as a highly mineralized complex structure. These architectural and compositional features allow the enamel to endure challenging masticatory forces and continual assaults by acids from food and bacterial sources. The hydroxyapatite crystals in enamel are tightly packed in different orientations to form enamel rods cemented by interrod enamel. Even though enamel is considered biologically as dead tissue, it is semipermeable where ionic exchange can take place between enamel and oral cavity (the saliva).

It should be notified that the oral epithelium-mesenchyme interaction is mediated by gene regulatory networks engaging several diffusible signaling factors. The bone morphogenic protein (BMP) and the wingless (WNT) signaling pathways are considered major mediators of the interaction during early tooth development. WNT signaling is a critical factor for oral and tooth development. Shh (sonic hedgehog) signaling is also an essential component. Shh encodes a signaling peptide that is present in the oral epithelium before invagination and in the tooth epithelium throughout its development. A mouse having *Gas1* mutant (Shh antagonist) shows an increased Shh activity and could result in supernumerary teeth formation (Ohazama et al. 2009). Moreover, knockdown of Shh signaling by applying an anti-Shh antibody shows molar fusion or supernumerary tooth formation in mouse embryos (Cho et al. 2011).

During tooth formation, the signaling molecules suggested to participate in the epithelial–mesenchymal interactions include FGF, Shh, WNT, BMP, and TGF-β (Tummers and Thesleff 2009). BMP signaling was essential for ameloblastic differentiation and enamel regeneration (Wang et al. 2004).

Remarkably, the tooth morphogenesis is characterized by a sequence of events that guide cusp morphogenesis and the histological differentiation of epithelial cells into ameloblasts. During amelogenesis, the homeobox gene Msx2 is

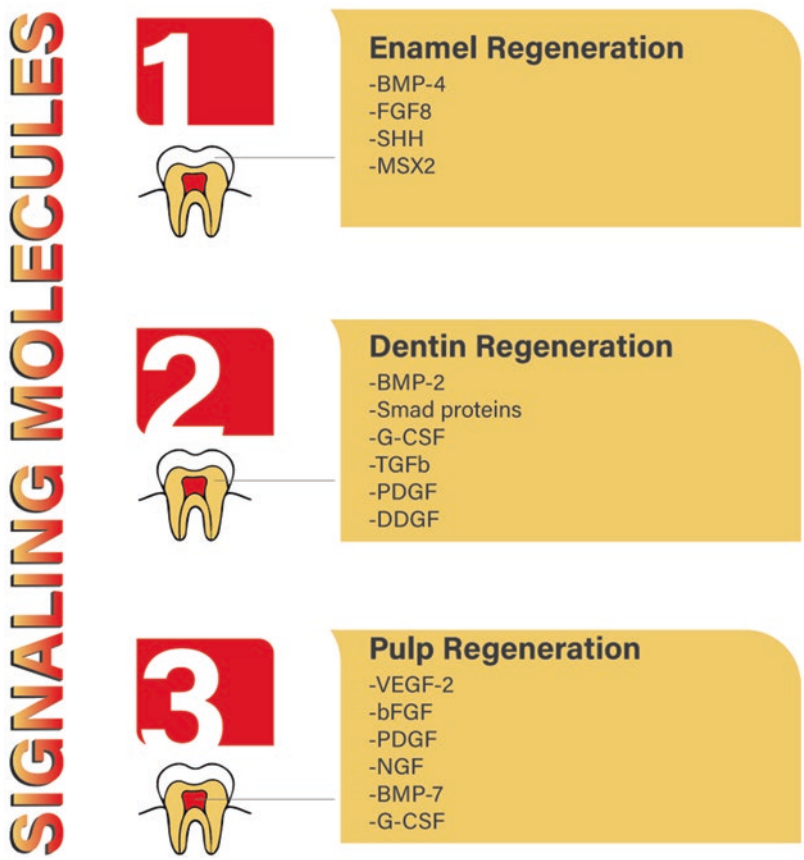

Fig. 10.3 Signaling molecules used in enamel, dentin, and pulp regeneration.

mandatory for Laminin 5 alpha 3 gene expression. This extracellular matrix gene plays an essential role in the differentiation of ameloblasts. Cusp formation is regulated by the enamel knot. BMP-4 was proved to be responsible for mediating termination of enamel knot signaling by regulating apoptosis. The expression of BMP-4 in the enamel knot is Msx2-dependent (Bei et al. 2004). It was reported that human ESCs were differentiated into dental epithelium (DE) after subsequent induction with different concentrations of BMP-4. Newly generated tooth-like structures possessing enamel resembling natural teeth were formed (Li et al. 2019).

Moreover, utilization of Shh and FGF8 molecules in cultured human keratinocyte stem cells (hKSCs), enhanced the capability of ameloblastic differentiation of hKSCs and the production of tooth-like structures (Hu et al. 2018).

10.7.3 Signaling Molecules Used in Dentin Regeneration (Fig. 10.3 and Table 10.1)

Dentin is formed by odontoblasts that differentiate from cells of the dental papilla. Thus, the dental papilla is the shaping organ of dentin and, at the end, it turns into the pulp of the tooth. At the late bell stage, odontoblasts are differentiated. Their main function is to produce the extracellular matrix of dentin, and then, they undergo mineralization, forming the primary dentin and completing root formation. Secondary dentin is continuously deposited as a physiological process throughout life, while tertiary dentin is formed in response to numerous stimuli, such as caries, wear, or restorative dental procedures. The tertiary dentin is generated uniquely by these cells directly insulted by the destructive stimuli. Tertiary dentin could be either reactionary or reparative. Each type develops from two different cells, postmitotic odontoblasts and cells originating from the pulp (DPSCs), respectively (Sloan and Waddington 2009). During normal development, odontoblastic differentiation from the dental papilla is achieved by expressing growth factors and signaling molecules in inner enamel epithelial cells. BMP-2 is the most bioactive molecule known to enhance osteoblast and odontoblast differentiation. BMP-2 guides DPSCs to differentiate into odontoblasts, and TGF-β can stimulate odontoblast-like cell differentiation and DPSC-mediated mineralization. Platelet-derived growth factor (PDGF-BB) and dentin-derived growth factors (eDMP) proved to promote hDPSCs (human dental pulp stem cells) proliferation and odontoblastic differentiation, creating dentin-like mineralized tissues. G-CSF (granulocyte-colony stimulating factor) promoted the migration and proliferation activity of stem/progenitor cells with dentin regeneration. The members of TGF-β including BMPs can activate Smad proteins. The Smad pathway plays a major role in signal interpretation from the ECM during osteogenesis/dentinogenesis.

WNT/β-Catenin signaling was demonstrated as a significant target in tissue regeneration and repair. The activation of WNT/β-catenin signaling is an immediate–early response to tissue damage and appears to be essential for stimulating the cellular-based repair in all tissues (Sun et al. 2014). Axin 2 is a negative regulator and a downstream target of this signaling pathway. A key cytoplasmic component of WNT/β-catenin signal transduction is the enzyme, glycogen synthase kinase 3 (GSK-3), which in the absence of WNT ligand/receptor binding, phosphorylates catenin and Axin leading to degradation. In the presence of WNT ligands, GSK-3 activity is inhibited allowing β-catenin to enter the nucleus and interacts with Lef/Tcf transcription factors to regulate the target genes expression, which include Axin2 (Sun et al. 2014). Small molecule inhibitors of GSK-3 used in clinical trials for treating of neurological disorders such as Alzheimer's disease stimulated reparative dentine formation, with naturally generated new dentin at sites of damage (del Ser et al. 2013; Neves et al. 2017).

Additionally, β-catenin signaling plays a vital role in dentin formation. H_2S (hydrogen sulfide) leads to Ca^{2+} influx into DPSCs. Ca^{2+} influx triggers GSK3β/β-catenin cascade to regulate DPSCs proliferation and differentiation. So, the gasotransmitter H_2S is essential to maintain DPSCs function via TRPV1-mediated Ca^{2+} influx-stimulated glycogen synthase kinase-3β (GSK3β)/β-catenin pathway (Yang et al. 2018). H_2S may trigger the nuclear translocation of nuclear factor-κB (NFκB) and affect the activity of various kinases, including p38 mitogen-activated protein kinase, extracellular signal regulated kinase, and Akt signaling. It was reported that H_2S can enhance vascular remodeling and angiogenesis via the PI3K Akt/survivin axis in endothelial cells by supporting phosphorylation of ERK and p38 (Wang, 2012).

Amazingly, simvastatin (SIM), a drug used to treat hyperlipidemia, augmented odontogenic differentiation and speeded up mineralized tissue formation and de novo dentin formation (Okamoto et al. 2009). When semaphorin 3A (Sema 3A) was applied to exposed pulp in a rat model, it promoted reparative dentin and enhanced the regeneration of an odontoblastic layer, predentin, and dentinal tubules (Yoshida et al. 2016). Sema 3A treatment of DPSCs clones increased nuclear accumulation of β-catenin, expression of the FARP2 gene was upregulated (FERM, RhoGEF, and pleckstrin domain protein 2), and Rac1 in DPSCs clones was activated.

10.7.4 Signaling Molecules Used in Pulp Regeneration (Fig. 10.3 and Table 10.1)

The dental pulp is a soft connective tissue that supports dentin. Histologically, it is composed of four distinct zones:

1. Odontoblastic zone at the pulp periphery
2. Cell-free zone of Weil under the odontoblasts
3. Cell-rich zone
4. Pulp core

It is well known that the pulp's principal cells are odontoblasts, fibroblasts, undifferentiated ectomesenchymal cells, macrophages, and other immunocompetent cells. The tooth pulp has been reported to be a handy source of multipotent stem cells.

The strategies applied for pulp regeneration include cell-based and cell-homing techniques. The cell-based approach is defined by the transplantation of exogenous stem/progenitor cells loaded onto scaffolds included with signaling molecules into the root canal system of the host to permit pulp regeneration, while the cell-homing technique relies on signaling molecules for the migration, proliferation, and differentiation of endogenous stem/progenitor cells.

The cytokines or signaling molecules contribute to pulp regeneration through their ability to mobilize endogenous cells and to control the proliferation and differentiation of the stem/progenitor cells. The inherent healing potential of endogenous cells needs various sorts of signaling molecules, such as platelet-derived growth factor (PDGF), stem cell factor (SCF),stromal cell-derived factor (SDF-1a), basic fibroblast growth factor (bFGF) , vascular endothelial growth factor (VEGF), and granulocyte colony-stimulating factor (G-CSF). They have been investigated in-vitro and in-vivo animal models were tested as well by being added to scaffolds.

The growth factors utilized should possess three functions:

1. Enhance angiogenesis in the root canal
2. Promote migration of endogenous stem cells
3. Enhance mineralization

Therefore, a proper arrangement of scaffold and growth factors should be elected, and the scaffold selected should be easily manipulated in clinical practice. The utilization of biological signaling molecules for cell homing makes pulp regeneration more clinically translatable, as the delivery of growth factors is not as complicated and costly as cell transplantation. The cell-homing technique prevents some of the manufacturing, technical, and safety difficulties associated with cell transplantation. A study revealed that stem/progenitor cell factor (SCF) could hasten cell homing and the maturation of the pulp–dentin complex in human immature teeth, proliferation and odonto/osteogenic differentiation (Ruangsawasdi et al. 2017). Unfortunately, selecting the ideal growth factors for pulpal regeneration remains unknown creating an open gateway for research and investigations towards alternative treatment options for injured and pulpless dentition.

10.8 Conclusion

A vision of replacing regular restorative therapy of the hard dental tissue defects, as well as conventional endodontic pulp therapy by stem/progenitor cell therapy, is no longer impossible. The tissue engineering triad (cells, scaffolds, and signaling molecules) could serve such goal. Specific scaffolds have to be used with an appropriate choice of cells and signaling molecules to trigger specific dental tissues. However, many challenges must be conquered to reach biologically synthesized tissues, which resemble the original models physically, mechanically, and biologically.

First of all, abundant sources of stem/progenitor cells should be provided. Adult stem cells either from bone marrow, skeletal muscles, adipose tissue, and joint fluid or from dental tissue as DPSCs, SCAP, and SHED are frequently used in dentin-pulp tissue regeneration. In addition, scaffolds of different sources and natures are recommended for each specific situation, either natural or synthetic. These scaffolds are responsible for the transport of cells or bioactive molecules used to repair or regenerate tissues. Moreover, signaling molecules play a vital role in the regenerative process. The binding of growth factors to receptors in this context induces numerous pathways implicated in tissue regeneration.

The dentin regeneration and mineralization process and pulp regeneration via tissue engineering proved to be easier than regenerating enamel tissue. As enamel tissue engineering faces an obstacle that enamel is an acellular structure, it cannot regenerate itself once the tooth has fully erupted.

Tissue engineering using stem/progenitor cell therapy could replace the conventional and invasive mechanisms for hard dental tissue defects' therapy and could be an alternative to regular endodontic treatment. Therefore, the numerous remaining challenges must be thoroughly investigated and carefully tested to guarantee the success and reliability of such approaches.

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Cell- and Stem Cell-Based Therapies for Liver Defects: Recent Advances and Future Strategies

11

Mustapha Najimi

Abbreviations

ACLF	Acute on chronic liver failure
ADHLSCs	Adult-derived human liver stem/progenitor cells
ATMPs	Advanced therapy medicinal products
CAT	Committee for advanced therapies
ESCs	Embryonic stem cells
GMP	Good manufacturing practices
iPSCs	Induced pluripotent stem cells
LCT	Liver cell transplantation
MSCs	Mesenchymal stem cells
OLT	Orthotopic liver transplantation

11.1 Introduction

11.1.1 The Liver Is a Key Organ with an Astonishing Ability of Regeneration

For almost 4000 years, the liver has long been considered by mostly all civilizations as the center of the whole body due to its abundance in the blood and the seat of life (Orlandi et al. 2018). It was also considered as the center of human passion and emotions (Orlandi et al. 2018). Such ancient interest toward this accessory digestive organ was confirmed so far by the different current technical and technological advances.

The liver is the largest and heaviest solid organ of the human body after the skin. It is a very specialized tissue able to concomitantly and persistently perform different functions as long as it is healthy (Gumucio et al. 1996). As a unique feature, the liver is supplied by arterial and venous blood, and around 2 liters of blood are consequently crossing it (Karran 1990). The liver also displays a high-level functionality with an ability to perform more than 500 functions and a significant impact on the regular activity of other distant organs (Gebhardt and Matz-Soja 2014). Indeed, by aiding digestion and metabolism, it synergically cooperates with the endocrine and gastrointestinal systems. For instance, the liver produces and excretes bile mainly containing cholesterol and bile acids, which helps break down fats in the small intestine (Boyer 2013). The liver has the potential to sense systemic content and requirements of several nutrients (glucose, vitamins, iron, and copper) and to regulate their concentrations by storing them intrahepatically or providing them when needed (Anderson and Shah 2013).

Furthermore, the liver is the primary site of protein synthesis and hormone metabolism (Marks 2013). Within the hematological system, the liver modulates the synthesis of proteins and clotting factors and clears the blood from exogenous (xenobiotics) and endogenously generated (bilirubin) harmful compounds (Marks 2013). Finally, thanks to its ability to produce immune factors, and to clear foreign bodies from the blood, the liver's own immune cell compartment composed of Kupffer, NK, and Pit cells manages essential roles for the body's immunologic system like resistance to infections (Bogdanos et al. 2014; Slevin et al. 2020).

The complex vital functions that the liver displays, including the regular processing of harmful compounds/microorganisms which damage the hepatic parenchyma, are in line with its ability to quickly regenerate (Preziosi and Monga 2017). Physiological liver regeneration occurs mainly via replication of mature cells in the remnant tissue and is a hyperplastic compensatory response tightly regulated by cytokine-dependent and cytokine-independent pathways (Li et al. 2020). The liver continues to manage its key functions while regeneration is occurring. Once the liver mass recovered, proliferation stops.

The functional complexity of the liver is supported by different epithelial and non-epithelial cell types displaying various embryological origins and synergically composing the

M. Najimi (✉)
Laboratory of Pediatric Hepatology and Cell Therapy, Institut de Recherche Expérimentale et Clinique (IREC), Université catholique de Louvain (UCLouvain), Brussels, Belgium
e-mail: mustapha.najimi@uclouvain.be

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hepatic parenchymal tissue. Hepatocytes represent the major cell population of the parenchymal fraction by number and mass, occupying over 80% of the hepatic parenchyma. Hepatocytes are unique functional entities that perform most of the complex metabolic functions attributed to the liver (Müsch 2014). These cells display very specific features compared to other epithelial cells, including polarity, which allows concomitant interactions with blood and biliary systems (Müsch 2014). Hepatocytes are typically nondividing quiescent cells but can rapidly replicate after liver insults and participate actively in recovering the initial liver mass (Miyaoka et al. 2012). Such high replicative activity allows the recruitment of only a few hepatocytes to offset the lost cells and reestablish the normal liver tissue. Although originating from those epithelial cells' compartment, liver regeneration is a well-coordinated process that also requires functional interactions with all other cellular and noncellular parts of the hepatic tissue (Taub 2004). In humans, liver regeneration was documented in transplantation settings with a most significant activity noticed the first 2 weeks post-transplantation and a fully recovered liver volume after 2 months (Michalopoulos 2017; Kawasaki et al. 1992; Haga et al. 2008). In rodents, most of the liver mass increase happens 3 days after partial hepatectomy, with a full recovery documented after 5–7 days (Andersen et al. 2013). Liver regeneration in humans frequently arises after ischemia or inflammation, while in other conditions like age, cirrhosis, and steatosis, this ability is significantly impaired (Michalopoulos 2017) (Van Haele et al. 2019). In such conditions, hepatocytes were shown to dedifferentiate and to display high plasticity with the acquisition of stem/progenitor cell phenotypes (Chen et al. 2012; Van Haele et al. 2019; Li et al. 2020).

11.1.2 Treatment of Liver Diseases Remains a Wide Unmet Medical Need for Both Medical and Patient Communities

Liver disease may begin at any age, due to genetic abnormalities, infectious or toxic agents, autoimmune alterations, or cancer (Asrani et al. 2019). Whatever the cause, pediatric or adult chronic liver diseases will eventually lead to liver insufficiency and severe complications. Liver diseases represent a substantial public health burden on a global scale. According to the Global Burden of Disease 2010 study, it is estimated that liver diseases affect more than 650 million people worldwide; more than 21 million people live with end-stage chronic liver disease (cirrhosis), while more than one million die annually (Blachier et al. 2013; Onski et al. 2010; Byass 2014). Estimates also suggest a further million deaths due to liver cancer and acute hepatitis, representing 4% of all deaths per year.

The diseases due to inborn errors of metabolism cause symptoms due to metabolic defects manifesting as early as the neonatal stage (Fagiuoli et al. 2013; Hansen and Horslen 2008). Inborn errors of metabolism are not considered as rare diseases as their cumulative incidence is about 1 in every 5000 live births. More than 300 human diseases caused by inborn errors of metabolism are enumerated. Although each metabolic disease displays specific features, these conditions typically manifest at the clinical level as lethargy, decreased feeding, vomiting, seizure, etc. Timely diagnosis and proper treatment are the keys to limit neurological impairment. For instance, Crigler Najjar syndrome is characterized by an impairment of the uridinediphosphoglucuronate glucuronosyltransferase (UGT) activity, which converts unconjugated bilirubin in the liver into water soluble bilirubin glucuronides excretable into bile (Servedio et al. 2005; Bosma 2003). This will lead to an abnormal accumulation of bilirubin associated with severe jaundice and a significant risk of neurological impairment. The management of Crigler Najjar diseased patients primarily consists of phototherapy for 10–12 hours/day. Urea cycle disease patients display impairment of nitrogen detoxification/arginine synthesis due to urea cycle enzyme defects, leading to hyperammonemia and subsequent severe neurological damage and long-term cognitive deficits (Scaglia et al. 2004; Nassogne et al. 2005). The prognosis of urea cycle diseased children has been dramatically improved by prescribing low natural protein diets, along with pharmacological treatments, although they remain at high risk of metabolic decompensation.

The complexity of concomitant tasks performed by the liver to ensure its function and that of other organs has prompted the development of several therapeutic approaches, including chemical drugs, specific diets, and surgery. Orthotopic liver transplantation (OLT) remains the most clinically validated therapeutic approach for liver diseases, mostly when conservative treatments have failed. However, the significantly increased scarcity of donor grafts limits its availability to only a small fraction of patients (Kamath et al. 2001; Struecker et al. 2014). Given the waiting time for a graft may often exceed 2 years, parents and relatives have no other option than to donate part of their liver to their child or even to an adult relative, which enormously increases the health burden to the family. Many other patients are not eligible to be listed for several economic, social, and geographic reasons. Therefore, continuous efforts are made to develop innovative and widely applicable approaches efficiently able to temporarily or definitively support liver function.

11.2 Liver Cell Transplantation Is a New Therapeutic Modality Initially Designed to Substitute OLT

Liver cell transplantation (LCT) was initially proposed to restore impaired liver functions by simply infusing a liver cell suspension via the blood circulation (Smets et al. 2008). The aim for transplanted cells is to reach liver sinusoids, integrate recipient hepatic parenchyma after crossing the altered sinusoidal endothelium, and start achieving metabolic activities (Gupta et al. 1999). The clinical development of LCT was supported by (i) its lesser radicalism and invasiveness as the recipient's liver was preserved, (ii) the simplicity and efficiency of the infusion procedure, and (iii) the potential repetitiveness of cell infusions with no side effects. Therefore, the applicability of this innovative approach was associated with significantly lesser hospitalization time and reduction of morbidity risks.

The proof of concept for LCT was demonstrated both at the preclinical and clinical levels by using isolated hepatocytes, predominant liver cells carrying most of hepatic metabolic functions (Forbes et al. 2015; Najimi et al. 2016). Indeed, LCT has been validated preclinically after confirming the ability of transplanted hepatocytes to integrate into recipient's livers, to correct various enzymatic defects, and to support hepatic functions and even animal survival (Forbes et al. 2015; Tricot et al. 2020). Therefore, LCT gained more interest in being applied for human liver diseases, especially when OLT was not considered the best solution.

The first successful human liver cell isolation has been performed 36 years ago and was shown to recover high yield, structural integrity, viability, and function of human hepatocytes (Strom et al. 1982; Alexandrova et al. 2005). Isolations of human liver cells are generally done using livers from two sources: whole livers unsuitable for transplantation or morphologically normal liver tissue from resection margins. The applied method was an adaptation of the two-step collagenase perfusion previously described by Seglen on rat hepatocytes (Seglen 1976). The liver vasculature is useful in allowing the perfused enzymes to contact a majority of cells and recover a high yield of single-cell suspensions. The isolation procedure took great care over specific parameters to ensure the suspension's quality and yield, such as the donor's medical history, as well as the preservation conditions. The organ/piece should be of acceptable quality with no alteration of Glisson's capsule. Indeed, the quality of cell suspension post-isolation is critical in LCT while the still remained challenged objectives are the preservation of highly viable and metabolically functional hepatocytes post-cryopreservation/thawing when a recipient candidate is available. The clinical implementation of LCT has prompted the development and improvement of clinically compliant cell isolation protocols. Our team at UCLouvain Brussels center has accumulated substantial expertise in (i) isolating GMP compliant human liver cells from more than 164 livers, (ii) cryopreserving more than 100 billion cells, and (iii) transplanting them fresh and/or cryopreserved/thawed to more than 15 patients with IEM (Smets et al. 2008).

Nonetheless, donor liver availability remains a major obstacle for LCT development programs, as is the case for OLT. Heterogeneity of recovered liver cell suspensions and in-depth description of the optimal cell suspension's purity still impede the optimization and standardization of the isolation protocol between LCT centers. Other critical parameters, i.e., identity of liver cell final products, donor selection criteria, and quality control assays, should be deeply defined between LCT centers to establish a consistent correlation between liver cell quality and posttransplantation long-term efficiency.

Optimal freshly isolated or cryopreserved/thawed hepatocytes are selected based on quality control assays targeting viability, sterility, and the metabolic functionality. For liver metabolic defects, 200–400 million hepatocytes/kg body weight is the preferable final dose to achieve 5–10% of the recipient's liver mass and hopefully measure a clinical, biochemical effect. Infusion of 30–100 million cells/kg body weight per session at a rate < 8 mL/kg/hour is the preferable optimal configuration. The cell suspension is delivered through a catheter placed into the portal vein or one of its branches, after surgery or interventional radiology. In newborn infants, the umbilical vein is the preferable catheter placement site. For acquired diseases, lesser doses seem to be needed, and an infusion is preferably made at the peripheral level. LCT efficacy may be indirectly assessed by detecting/measuring the donor-derived protein expression or activity of the related products on liver biopsies, serum, or urine samples taken at different times posttransplantation. Measurement of de novo metabolic activity should be considered a piece of supportive evidence for donor cell engraftment. For instance, this can be evaluated by measuring ^{13}carbon incorporation into the urea for urea cycle diseased patients or into bilirubin mono- and di-conjugate levels in Crigler Najjar patients. Recipient liver chimerism may also be analyzed after monitoring recipient HLA Class I antigen levels with mismatched donor HLA hepatocyte transplants. Finally, gender-mismatched transplantations and engrafted cells were detected using DNA techniques like fluorescent in situ hybridization or digital PCR (Wang et al. 2002; Lombard et al. 2019a).

In general, all LCT trials resulted in any safety issues. Potential major complications likely to occur include portal vein thrombosis, bleeding, or fatal infection. Hence, blood flow, portal pressure, and microbiological quality must be carefully checked (Smets et al. 2008; Najimi et al. 2016). Given the demonstration of tissue factor-dependent

procoagulant activity in isolated liver cells, coagulation parameters are also monitored (Stéphenne et al. 2007). D-dimer level increases were also detected without any associated changes in other coagulation parameters. Supplementation of N-acetylcysteine to the formulation medium is currently applied to avoid such alterations. Careful selection of potential candidates is thus mandatory to prevent possible post-cell infusion perturbations.

Depending on the injection site, the wanted/expected effect (engraftment or paracrine), and hence the dose to be infused, appropriate experimental approaches should be considered to follow transplanted cells' early distribution, mainly in ectopic tissues like the lungs. Lessons learned from several in vivo studies have highlighted the need to use small animals to efficiently investigate the mechanisms governing cell engraftment (Hsu et al. 2017). Large animals should also be considered, as they better mimic clinical conditions by allowing investigations of critical issues, including safety, repeatability, and infusion device optimization.

11.3 Lessons from Clinical Hepatocyte Transplantation Trial Cases

The first human hepatocyte transplantation was conducted 28 years ago by using autologous isolated hepatocytes on patients suffering from liver cirrhosis (Mito et al. 1992). No clear-cut clinical effects were documented in these trials. When applied to patients displaying inborn errors of liver metabolism, hepatocyte transplantation showed significant clinical results that efficacy and sustainability varied depending on the etiology of the disease. Those promising data collected on more than 100 patients were correlated to several relevant factors, including the number of the cells needed to measure a clinical condition, which is quite low compared to an acute liver failure condition, and the single-gene defect parameter to follow. In some of those metabolic diseases, a few percent of metabolic activity could be sufficient to switch the phenotype of the disease from a severe to a moderate one. From the clinical program of our UCLuvain Brussels center, we can highlight the following:

- The first pediatric LCT in Europe was carried out on a 4-year-old girl with Refsum disease displaying altered peroxisomal biogenesis (Sokal et al. 2003). Two billion isolated hepatocytes were transplanted, and immediate decreases in abnormal bile acid and dihydroxycoprostanoïc acid levels were measured, suggesting cholestasis resolution. Donor chromosome sequences were also detected on posttransplant biopsy, suggesting cell engraftment. These positive effects were maintained up to 18 months posttransplantation (Sokal et al. 2003).
- The original demonstration of the strong correlation between the effect posttransplantation and the infused cells (Stéphenne et al. 2006). This was established in a urea cycle diseased patient suffering from argininosuccinate lyase deficiency, with secondary psychomotor retardation due to recurrent hyperammonemia episodes. The girl was treated with freshly isolated and cryopreserved hepatocytes that markedly lowered her ammonia levels up to 18 months posttransplantation. Indeed, concomitant detection of engrafted male donor cells in four successive biopsies taken up to 1 year following the first infusion and remarkable improvement in psychomotor development and clinical status has been reported.
- At a later stage, all patients that have been cell transplanted from our Brussels center underwent OLT, yet with no sensitization, rejection episodes, or specific complications observed, even in cases employing hepatocytes isolated from more than two different donors.

We should admit that all the published LCT cases that have been documented and reviewed so far were under a proof of concept and first in man settings, which illustrates both richness and limitations of such studies. We also have to consider that only successful cases are considered for publication by medical journals. In such first in man trials, efficacy endpoints were not always definitely fixed, and the protocol was often modified based on intermediary data. Adverse events were likewise not systematically collected nor reported in the same rigorous manner as in clinical trials. Therefore, it is challenging to draw any conclusions about the general safety and efficacy of the procedure, which enforces the need to carry out proper clinical trials. As of January 2021, only six results were documented in the clinical trial registry using the search terms "Hepatocyte transplantation and liver diseases." Only one trial has been completed, while the others were suspended or withdrawn.

One major drawback of LCT is the time-limited clinical efficacy, ranging from a few weeks to a maximum of 18 months. The amount of liver tissue required for the expected efficacy of each LCT procedure is another drawback to be underlined. The risk of infectious agent transmission by fresh or even cryopreserved cells is one of the main potential adverse events, although comparable to OLT.

Increased scarcity of donor livers allowing a good quality of hepatocytes post-isolation, unfortunately, restricts the applicability of LCT to only a few patients from selected transplant centers. While cryopreservation is the only way to preserve extra non-transplanted isolated hepatocytes at the long-term level, this step has been reported to significantly damage those cells at the functional and structural levels (Stéphenne et al. 2010). Several strategies have been developed to better understand long-term cell survival mechanisms and thus enhance LCT efficacy, including improvement

of the quality of raw material, as well as the cryopreservation and immunosuppression protocols.

11.4 Stem Cells Are Developed as Second-Generation Cell Products for Liver

11.4.1 Regenerative Medicine

Due to (i) the significant alterations of the quality of isolated hepatocytes that were documented both post-cryopreservation and post-plating, (ii) their inability to proliferate in vitro, and iii) the worldwide increasing liver scarcity, alternative innovative cell products, namely, stem/progenitor cells, have been investigated for their potential to therapeutically target liver defects. The efficiency of stem/progenitor cell therapy in liver diseases depends on the type of hepatic defect, and its severity and chronicity. Thus the potency depends on the selected cells' capacity to compensate for the pathological deficiency of the targeted disease.

Stem/progenitor cells were extensively studied, thanks to their self-renewal, easy access, differentiation potential, as well as immunomodulatory and immunosuppressive properties. Manufacturing stem/progenitor cells in vitro on an almost limitless scale is also a highly promising approach able to overwhelmed the issues of organ shortage and well-suited to be translated for high-scale pharmaceutical production, a real opportunity for bringing cell-based therapy to any patient in need.

11.5 Extrahepatic Stem Cells

11.5.1 Hematopoietic Stem Cells

Bone marrow and its compartments of hematopoietic and mesenchymal stem cells have been clinically evaluated based on data obtained in several preclinical models showing that transplantation of hematopoietic $CD133^+$ and $CD34^+$ progenitors or monocytes stimulates liver regeneration and ameliorates liver functions (Hu et al. 2016; Zhou et al. 2009; Zhai et al. 2018). Indeed, transplantation of hematopoietic progenitors was reported in mostly uncontrolled clinical trials to improve the patient statuses. This was supported by the demonstration that the use of granulocyte colony-stimulating factor to mobilize those BM cells was correlated to a positive histological effect in patients with alcoholic steatohepatitis (Piscaglia et al. 2010). While a beneficial effect was documented in most of the studies in which acquired liver diseases were targeted (Goldman et al. 2016), the mechanisms of action (paracrine effects on regenerating hepatocytes or resorption of abnormal ECM) and sustainability of this effect are not yet deciphered.

11.5.2 Pluripotent Stem Cells

Given that hepatocyte-like cells can be efficiently generated from pluripotent stem cells, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) were also considered as potential alternative cell populations in liver cell-based therapy (Wobus and Boheler 2005; Murry and Keller 2008). Despite their first isolation 40 years ago, their unlimited differentiation potential, and encouraging preclinical data, ESCs are not yet clinically applied for liver diseases due to several practical (large-scale production of mature and functional hepatocytes, purity of the final cell suspension) and to ethical considerations. Nevertheless, preclinical studies on ESC have been instrumental in revealing consistent information related to the pathways governing the hepatocytic lineage as well as the cell replacement and paracrine effects of those cells after in vivo transplantation.

iPSCs were generated 15 years ago after the genetic reprogramming of somatic cells toward an embryonic pluripotency status (Takahashi and Yamanaka 2006). Although with a low hepatogenic differentiation potential compared to ESC, iPSCs have been reported to stimulate liver regeneration and improve liver metabolic functions after transplantation in several animal models of liver defects (Tricot et al. 2020). The iPSC strategy has provided solutions toward the ethical concerns raised with ESC and is practically attractive when targeting genetic defects under autologous cell transplantation settings. Although clinical evaluation of the use of iPSCs in different other indications, including retinal degeneration, amyotrophic lateral sclerosis, Parkinson's disease, and sever heart failure, no clinical trial dealing with iPSC in liver diseases is documented so far. Still, additional information is significantly lacking concerning cell tumorigenicity, immunogenicity, and sustainability of the posttransplantation efficacy. The correlation of those parameters to reprogramming quality is still unknown.

11.5.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) have been initially isolated from bone marrow and thereafter obtained in several other tissues including the umbilical cord, skin, adipose tissue, and liver (Uccelli et al. 2008). The very significant technological and experimental advances currently allow knowing more about those cells, their origin, and tissue-specific features. MSCs are the most applied cell type for treating liver diseases both at the preclinical and clinical levels. Although several modes of action have been reported for MSCs, strong evidence is accumulated on those cells' paracrine effect and their ability to secrete very potent bioactive molecules with immunomodulatory, immunosuppressive, angiogenic, and antibacterial properties. With a deeper understanding of the

expansion and storage of MSCs from other tissues, we are certain to further progress in the development of liver MSC therapy. This will, at last, enable us to overcome the scarcity of donor material. However, more supportive data is now needed concerning the obligatory MSC stability and quality to enable them to be used for large-scale culture.

Most of the MSCs administered to humans did not cause any safety complications. MSCs have been reported to be safe and well-tolerated in numerous different indications, without any report of malignancies from ongoing clinical testing. While their potency was clearly shown in animal models, their clinical investigation is currently ongoing. Based on clinical trials registry, 56 studies related to evaluating the effect of MSC on several liver defects including fibrosis, cirrhosis, acute on chronic liver failure, liver failure hepatitis, and Wilson's disease are reported. Those trials have used allogeneic MSCs from the umbilical cord, bone marrow, and skin, as well as autologous cells from bone marrow, menstrual blood, and adipose tissue.

11.6 Liver Stem Cells

11.6.1 Endogenous Stem Cells

It is widely agreed that adult organs possess their pool of intrinsic stem cells programmed to differentiate into cells of that tissue. The contribution of those resident stem cells to tissue rejuvenation is closely dependent on the physiological or pathological settings. Mature differentiated hepatic cells were seen to dedifferentiate and to acquire stem cell-like features in certain pathological conditions. Other liver stem cells have been reported, such as adult oval cells, and fetal liver stem cells (hepatoblasts). Both cell types are bipotent and able to differentiate into hepatocytes or bile duct cells when the potential of hepatocytes to regenerate liver parenchyma is completely blocked (Li et al. 2020). These cells are characterized by a well-documented proliferation profile, in conjunction with plasticity features.

The existence of oval cells and whether they originated from bone marrow or endogenous liver are still debated. While accumulated evidence is more supported in rodents, their expression profile's similarity with both human liver epithelial cells renders their investigations difficult (Van Haele et al. 2019). Direct evidence showing the ability of oval cells to give rise to mature hepatocytes during liver regeneration in vivo is also not yet shown. On the contrary, reprogramming of resident liver epithelial cells seems to be the most plausible strategy as reported in several human liver diseases including cirrhosis, acute liver failure, and cholestatic diseased livers. Those reported intermediate hepatobiliary cells generated via reprogramming of adult cells display different expression profile than oval cells (Li et al. 2020).

The fetal liver has also been considered for isolating potential stem/progenitor cells that can be applied for liver cell therapy. Initially, livers from fetuses of 18–22 weeks of gestational age have been processed to isolate such cells that are EpCAM+ and significantly different from hepatoblasts as not expressing AFP and CYP3A7 (Lanzoni et al. 2013). Those cells represent 2% of the total fetal liver parenchymal cell suspensions, may expand in vitro under stringent culture conditions, and are be able to differentiate into both hepatocytes and cholangiocytes after transplantation in vivo. Besides long-term self-replication, fetal liver stem cells also display higher resistance to cryopreservation/thawing, low immunogenic profile, and less tumorigenicity. Those cells have been transplanted enriched and non-enriched to treat both congenital and acquired liver diseases. Data available from the clinical trials registry have clearly shown their ability to engraft into the recipient liver parenchyma and improve hepatic functions with no observed immune reactions up to 6 months posttransplantation (Khan et al. 2008, 2010). Non-purified and nonselected fetal liver cells from fetuses aborted between the 16th and 26th week of gestation have been used in another phase I/II clinical trial that has enrolled 25 patients with liver cirrhosis (Pietrosi et al. 2015). The cells were transplanted via the splenic artery route under an immunosuppression regimen, and their efficacy was appreciated up to 1-year posttransplantation. The transplanted patients did show an improvement of hepatic functions from the first month posttransplantation and remain stable up to the end of the follow-up compared to the control group (Pietrosi et al. 2015).

To achieve their potential clinical use, fetal liver stem/progenitor cells should be studied at a long-term level to assess potential tumorigenic risks. Regular dependency on aborted fetuses and suitability of the related livers for cell therapy may increase ethical concerns. Furthermore, the plasticity of fetal liver stem cells should be deeply investigated. Indeed, those cells can also differentiate into myofibroblastic-like cells, a non-negligible risk factor that may accentuate the availability of pro-inflammatory and profibrogenic molecules, and thus worsen the microenvironments of the injured liver (Ji et al. 2011).

11.6.2 Still Intrinsically Non-defined Stem/Progenitor Cells

In parallel to stem/progenitor cells intrinsically detected in the liver parenchyma, other cell populations with stem/progenitor features have been described post-plating liver parenchymal cell suspensions. Indeed, the primary culture of those cells has led to the emergence of different progenitor cell subpopulations (Herrera et al. 2006; Najimi et al. 2007). These liver-derived cells of mesenchymal phenotype emerge

in parallel to hepatocyte death and subsequently expand in culture while retaining their ability to differentiate into hepatocyte-like cells. Mesenchymal stem cells have also been reported to be isolated directly from fetal human livers (El-Kehdy et al. 2016).

Our team has reported the emergence of mesenchymal-like cell population with stem/progenitor features after isolation of adult healthy human livers and subsequent primary culture (ADHLSC) (Najimi et al. 2007). These mesenchymal stem/progenitor cells have been deeply investigated at the preclinical levels both in vitro and in vivo and revealed an advanced ability to differentiate into hepatocyte-like cells and potent immunomodulatory and immunosuppressive properties (Najimi et al. 2007; Khuu et al. 2010, 2013; Sana et al. 2014; Raicevic et al. 2015; El-Kehdy et al. 2016, 2020; Lombard et al. 2019b).

After successful large-scale production under good manufacturing practices in accredited tissue banks, the cells were developed to be used as allogeneic products. Those cells were granted as medicinal products according to the EU regulation on advanced therapies. The safety of using these clinically compliant cells in the clinic has been initially demonstrated at Cliniques Universitaires Saint Luc (Brussels, Belgium) on a female patient suffering from severe ornithine transcarbamylase deficiency with neonatal-onset, protein restriction diet, and scavenger treatment proved unable to control the girl's conditions (Sokal et al, 2013). Following unsuccessful cryopreserved LCT, the girl was administered two infusions of ADHLSCs expanded in vitro under GMP settings. Two liver biopsies taken 100 days post-ADHLSC-transplantation revealed 3% and 5% of male donor cells in her liver mass after the girl had received an ADHLSC cell quantity equivalent to 0.75% of her calculated liver mass. This almost threefold in transplanted cell number provided evidence that the infused cells had been able to engraft and proliferate in the meantime. According to the girl's parents, there was some hint of clinical improvement in her status following ADHLSC infusions. Immunosuppression was discontinued at 6 months, but the girl underwent OLT and died soon after that owing to procedure-related complications (Sokal et al. 2013). In another patient suffering from type 1 glycogen storage disease, indium-labeled cells were infused in the portal vein showing exclusive liver homing up to the fifth day post-infusion (Defresne et al. 2014). Based on 5 years of liver stem/progenitor cell research development at the academic level and on those encouraging clinical first in man data, the cell product/technology has been transferred to a spinoff Biotech company, Promethera Biosciences in 2009 (Mont St Guibert, Belgium). The company was successfully able to (i) get the authorization for the clinical use of the developed stem/progenitor cell therapy product (Hepastem®) for the treatment of Crigler Najjar syndrome and urea cycle disorders in a pediatric setting and (ii) manufacture significant HepaStem® batches dedicated to the first clinical phase I/II trial in Europe for 20 patients with urea cycle defects and Crigler Najjar syndrome. The aim was to assess the safety and dose escalation of HepaStem® at 6- and 12-month post-transplantation as well as its preliminary efficacy. For these first clinical trials, Hepastem® was dispatched to five distant sites as cryopreserved cell suspensions and successfully formulated in a mobile unit (GMP Van) brought near the clinical site. This approach has assured timely and consistent cell delivery within limited shelf-life. The infusion of HepaStem® was well-tolerated without significant adverse events. The safety profile observed in this study was considered in line with expectations for this new cell therapy, considering infusion procedure, underlying disease, as well as concomitant medication (Smets et al. 2019). Preliminary efficacy data revealed an increased de novo urea formation in most urea cycle diseased patients, 6 months post-HepaStem® infusion, while bilirubin level decrease was reported only in some Crigler Najjar patients (Smets et al. 2019).

Besides their safety profile documented in pediatric patients with inborn errors of metabolism (Smets et al. 2019), Hepastem cells have been shown to display liver-specific homing capacity after peripheral intravenous infusion (Sokal et al. 2017), as well as immunomodulatory and anti-fibrotic properties (El-Kehdy et al. 2017) (Najar et al. 2018; Lombard et al. 2019b; Najimi et al. 2017). In line with those properties, a phase II clinical study was conducted on 24 cirrhotic patients with acute on chronic liver failure (ACLF) or with acute decompensation at risk of developing ACLF (Nevens et al, 2021). Data of that study clearly showed that infusion of the patients with up to 2 doses of 1.2 × 106 cells/kg BW seems safe. Follow up investigations also reported an improvement of the survival rate and systemic inflammation as well as a recovery of the altered liver functions.

11.7 Regulatory Framework

From a regulatory perspective, stem cells are considered medicinal products and must be subject to a more rigorous pharmaceutical development compared to proof-of-concept testing. These stem cells are granted as advanced therapy medicinal products (ATMPs), with status defined by a specific European regulatory framework (Sokal 2014). At the European Medical Agency, the Committee for Advanced Therapies (CAT) is supervising their development.

ATMPs' development must follow a strict production plan and GMP. Release criteria must be established beforehand, such as the percentage of viability, stability, impurity levels, precise cell identification markers, and genetic stability, along with the cells' potency to treat the target disease. The process must also adhere to logistical requirements regarding delivery to the patient's bedside, at times far from

the production site. Ideally, the product should be stored in the hospital pharmacy, with drug substance reconstitution carried out at the patient's bedside, as is current practice for vaccines (Sokal 2014).

Cell-based medicinal products are classified as orphan drugs if they target disease with an incidence lower than 1/5000 live births. Before their use in children, a pediatric investigation plan must be submitted to the Pediatric Committee for approval. The European Regulatory Authorities allow hospital-accredited tissue banks to treat a few ATPM patients under the so-called hospital exemption settings (Sokal 2014). This strategy must be maintained, as this enables medical researchers and physicians alike to investigate new targets for cell therapy. Only once the "proof-of-concept" phase is completed can the further clinical development of cell therapy be envisaged and conducted at a pharmaceutical level. This development must imperatively follow all the regulatory steps outlined for medicinal product market authorization. For more information regarding regulatory issues, we wish to refer the reader to the valuable paper written by Bayon et al. (Bayon et al. 2015). In line with the paper's authors, we would like to insist on the decisive role of the industrialization process to allow a successful translation from the academic environment to patient treatment.

11.8 Concluding Remarks

For regenerative medicine, liver cell therapy has been increasingly shifted toward the use of stem cells for restoring normal liver function and structure, consequently to both inherited defect and acquired tissue damage. The stem cells' ability to proliferate and differentiate under in vitro conditions holds great promise for an unlimited production of liver cells intended to manage various liver diseases.

Restoration of the missing enzyme activity is mandatory in metabolic liver diseases, recovery; and regeneration of the injured liver is the primary goal in acute liver failure, while liver wound and function should be repaired and maintained in chronic and acute-on-chronic liver diseases. Therefore, cell sources for LCT should be tailored for each pathological condition based on their more adapted features, doses to be administered, and modes of action. Hepatoblasts, hHpSCs, iPSCs, and ESC are more appropriate sources for tissue replacement, thanks to their ability to provide hepatocyte-like cells. Hematopoietic stem cells and macrophages look more suitable for reducing scarring in liver cirrhosis and to stimulating the liver's regenerative processes. Finally, the potent immunomodulatory and immunosuppressive features of MSCs support the inhibition of immune-mediated liver injury.

On the other hand, numerous issues must still be resolved concerning liver regenerative medicine. Thus, it is crucial to better define the optimal number of stem cells to be infused and required for the repopulation of a defined level of recipient liver mass. To efficiently prepare future commercialization, innovative logistical solutions have been implemented from the early phases. The cell production process has been further upscaled by bioreactor technology, which significantly minimized manual operations and decreased production costs. A unique reconstitution technology is also currently optimized to allow a straightforward preparation of the cell product by the hospital staff and immediately at the patient bedside. More-refined tools to better assess the engraftment levels are also required. Immunosuppressive regimens need to be further standardized, and composite endpoints for efficacy assessments are to be developed. The anticipated benefits also rely on the cells' capacity to survive on a long-term basis, thereby sustaining their metabolic capacities over time. In the forthcoming future, cell transplantation combined with organ engineering techniques may likely provide solutions to compensate for the shortage of livers available for transplantation and the production of high-quality mature liver cells in full compliance with the existing standards of the industry (Heydari et al. 2020). Before their application in man, all these techniques must be validated in small and large animal models, the latter being better response predictors for use in humans than the former. All those extensive studies should provide valuable insights into how to decipher the mysteries of liver regeneration (normal and pathological), and thus being able to design the appropriate treatments to rejuvenate and sustain the functionality of this incredible organ.

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Stem Cells: A Renewable Source of Pancreatic β-Cells and Future for Diabetes Treatment

12

Saima Kh and Khawaja Husnain Haider

Abbreviations

Ang-I	Angiopoietin 1
Ang-II	Angiopoietin 2
ARX	Aristaless-related homeobox
BM	Bone marrow
BMSCs	Bone marrow stem cells
CSCs	Cardiac stem cells
DPP-4	Dipeptidyl peptidase-4
EGF	Epidermal growth factor
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
FGF-2	Fibroblast growth factor-2
G-CSF	Granulocyte colony-stimulating factor
HGF-1	Hepatocyte growth factor-1
HO-1	Hemoxygenase-1
HSCs	Hematopoietic stem cells
hTERT	Human telomerase reverse transcriptase
IGF-1	Insulin-like growth factor
IL-1	Interleukin-1
IPCs	Insulin-secreting β-cells
iPSCs	Induced pluripotent stem cells
IRS-1	Insulin receptor substrate-1
MACE	Major adverse cardiac events
miRNA	MicroRNA
MCP-1	Monocyte chemoattractant protein-1
MSCs	Mesenchymal stem cells
Ngn-3	Neurogenin-3
Pax	Paired box4
PCL/PVA	Polycaprolactone and polyvinyl alcohol
Pdx1	Pancreatic duodenal homoeobox-1
PDGF-BB	Platelet derived growth factor-BB
PGF	Placental growth factor
PHD3	Prolyl hydroxylase domain 3
PLLA/PVA	Poly-L-lactic acid and polyvinyl alcohol
PMPs	Pancreas-derived multipotent precursors
PREPS	Pancreatic resident endocrine progenitors
REST	Repressor element-1 silencing transcription factor
SDF-1α	Stromal cell-derived factor-1α
SkM	Skeletal myoblasts
Shh	Sonic hedgehog
STZ	Streptozotocin
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

Saima Kh · K. H. Haider (✉)
Department of Basic Sciences, Sulaiman AlRajhi Colleges, Al Bukayriyah, Kingdom of Saudi Arabia
e-mail: kh.haider@sr.edu.sa

12.1 Introduction

Diabetes mellitus is a metabolic disorder characterized by elevated glucose levels due to either poor insulin secretion by the pancreatic β-cells or lack of responsiveness of body cells to insulin (Piero et al. 2015). While diabetes type 1 (insulin-dependent diabetes) mostly emanates from progressive autoimmune destruction of the functionally competent β-cells, type 2 (non-insulin-dependent diabetes) mostly involves reduced tissue responsiveness to insulin as well as poor insulin availability (https://diabetesatlas.org/en/). The predicted number of diabetics in 2010 was estimated to reach 438 million in the world by 2025; however, this number has already been surpassed, and it is estimated that it will cross 578 million by 2030. Unless appropriate and relevant remedial measures are adopted, the problem may become pandemic. Besides, non-pharmacological methods of intervention including dietary restrictions and lifestyle modifications, availability of more effective pharmacological agents in the light of management guidelines for diabetics (Sattar 2019; Om et al. 2018), and the recent advances in the surgical management of diabetes by transplantation of the pancreatic islets have met with limited success, although it works similarly in terms of MACE in both the young and old patients who receive pancreas transplantation (Montagud-Marrahi et al. 2020). Gene therapy-based protocols have been developed for supporting the G0 β-cells transition to the G1 phase of proliferation to repopulate the pancreas with functionally competent β-cells (Chen et al. 2012). For example, manipulation of cMyc expression in β-cells alters their proliferation rate but with resultant functional immaturity (Puri et al. 2018). Similarly, concomitant delivery of Pdx1,

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Ngn3, and Mafa transgenes into the liver cells has been reported that increased insulin production and effectively reduced the glucose levels in the experimental animals (Cim et al. 2012). Moreover, an efficient reversal of hyperglycemia has been achieved by a lentiviral vector-based insulin gene delivery in the experimental settings (Elsner et al. 2012). These genetic strategies are mostly intended to reenter the existing β-cells into cell cycle or to transform non-pancreatic cells into β-cells or pancreatic non-β-cells into morphofunctionally competent β-cells (Zhong and Jiang 2019). With the emergence of regenerative medicine based on stem cell therapy approach, therapeutic intervention with stem cells or using their derivative β-cell is gaining popularity (Godfrey et al. 2012; Neshati et al. 2010; Alipio et al. 2010). A recently published systematic review and meta-analysis of 27 cell therapy-based clinical studies for the treatment of diabetes using six different types of stem cells have shown that cell therapy is safer and more effective option for the treatment of diabetes as compared to islet or pancreatic engraftment (Rahim et al. 2018).

This book chapter explicitly focuses on the effect of diabetes on the functionality of the intrinsic pool of stem/progenitor cells and critically appreciates the published data defining the possibility to exploit exogenous stem cells for replacement of nonfunctional pancreatic β-cells to normalize insulin production. The data published from various research groups are equivocal in anticipation that a therapeutic intervention at the cellular and/or molecular level will lead to a reversal of the disease process to restore glucose homeostasis.

12.2 Diabetes and Stem Cell Function

Similar to the other tissues in the human body, the existence of tissue-resident putative precursors and their ability to differentiate into functionally competent insulin-secreting β-cells have been reported in the mammalian pancreas (Seaberg et al. 2004). Smukler et al. have identified a rare population of pancreas-derived multipotent precursors (PMPs) in the adult mouse and human pancreas, capable of extensive self-renewal and differentiation to adopt pancreatic and neural phenotypes (Smukler et al. 2011). The authors also assessed the therapeutic potential of the derived β-cells post-engraftment in a diabetic mice model to show that the cells continued to secrete insulin in vivo despite lacking mature β-cell phenotype, and the recipient diabetic mice showed weight loss as well as improved hyperglycemic control. Similarly, Lee et al. have reported the presence of mesenchymal stem cells (MSCs) in the exocrine pancreatic tissue which could differentiate to form insulin-secreting β-cells. These cells expressed most of the classic biomarkers attributed to MSCs, i.e., CD73, CD90, and CD105, besides expressing pancreatic transcription factors including Pdx1, Ngn3, and Mafa (Lee et al. 2016). A recent study has reported the presence and isolation of pancreatic resident endocrine progenitors (PREPS) having an MSC-like phenotype from mice pancreas (Srivastava et al. 2019). Treatment of PREPS with activin-A and swertisin differentiated the cells into islet clusters, while intravenous transplantation studies in streptozotocin (STZ)-induced diabetes mice model alleviated the diabetic conditions in the animals receiving cell therapy. In vitro studies revealed that the cells were able to develop into functionally mature islet clusters within 4 days of induction. Interestingly, the authors showed that the PREPS specifically lodged in the pancreas with minimal sequestration in any other organ. Moreover, the differentiated cells had a rich expression of transcription factors needed for endocrine pancreatic homeostasis.

12.2.1 Hyperglycemia Alters Stem/Progenitor Cell Morphology and Surface Marker Expression

Although the existence of stem/progenitor cells offers hope for diabetics (Jebaraj and Bhuvaneswari 2020), their functional status in diabetic animals and humans needs to be ascertained because diabetes is one of the major risk factors which cause aberrant stem/progenitor cell functionality, i.e., clonogenicity, proliferation potential, mobilization, differentiation capacity, and reparability (Albiero et al. 2011; Poljak-Blazi et al. 1980; Neef et al. 2012; Kränkel et al. 2005; Fadini et al. 2006; Liu et al. 2013). The aberrant functionality of stem cells is being attributed to changes in the metabolomic profile of the cells, which may be significantly impacted by hyperglycemia (Surrati and Haider 2019). For example, similar to the other cells in the body (Rajab et al. 2018), innate kidney stem cells isolated from rat renal papilla show less proliferative potential and altered differentiation capacity upon persistent exposure to osmotic and glycemic stress in their microenvironment (Yang et al. 2015). The stem/progenitor cells become senescent, less tolerant of hypoxia, and their epithelial differentiation is significantly reduced due to prolonged exposure to hyperglycemia. Similar results have also been reported by other research groups that showed senescence and reduced differentiation potential attributed to the presence of advanced glycation products and their specific receptors thus leading to cellular exhaustion (Stolzing et al. 2010; Cramer et al. 2012). Many of the major diabetic complications have been ascribed to the impaired function of the stem/progenitor cells with the involvement of diverse signaling pathways (Rodrigues et al. 2015).

Persistent exposure to high glucose concentration promotes adipogenic differentiation of vascular stem cell-derived mesenchymal progenitor cells while sparing the derivative EPCs. These data show a differential effect of

hyperglycemia on cellular functions in different cell types (Keats and Khan 2012). Incidentally, this altered stemness of the cells in the hyperglycemic environment is considered as an important contributory factor toward impaired wound healing in diabetic patients. While elucidating the cellular basis of diabetes-induced osteoporosis, the widespread existence of insulin and proinsulin-positive cells has been observed in the liver, spleen, thymus, adipose tissue, and BM in the experimentally induced diabetic mice and rats (Kojima et al. 2004). The authors reported the appearance of GFP+ proinsulin and insulin-expressing cells within 3 days in response to experimentally induced hyperglycemia in transgenic mice having mouse insulin promoter-driven GFP. Interestingly, most of the extrapancreatic insulin-positive cells had BM origin. Similar results have also been published by Chen et al. who used the bioluminescence imaging approach to monitor extrapancreatic insulin gene expression (Chen et al. 2010). Parallel experiments investigating the transplantation of genetically marked BM cells in diabetic animals showed that the extrahepatic and extrathymic insulin and proinsulin positive cells from the BM origin in response to the persistent hyperglycemia and colonized in other tissues in the body. Similar observations have also been reported when the BM cells from rats and mice were cultured in high glucose conditions in vitro (Oh et al. 2004). More than 50% of the cultured cells differentiated into islet of Langerhan-like cells and secreted insulin, glucagon, somatostatin, and pancreatic peptide-C. Upon transplantation into an experimental mice model, the aggregates of the cells continued their hormonal secretions, which successfully maintained blood glucose homeostasis in experimentally induced diabetic mice. Moreover, removal of the donor cell graft resulted in a relapse of hyperglycemia and the ultimate death of the recipient mice. The culture of progenitor cells under high glucose conditions impacts their survival and proliferation capacity besides altered gene expression profile (Katagi et al. 2014). A 24–48 h culture of tendon-derived stem cells under high glucose conditions alters the expression of tendon-specific markers tenemodulin and collagen-I besides inhibiting their proliferation in vitro (Lin et al. 2017). These findings have been accredited to the underlying cause of diabetic tendon pathologies. A recent study has shown that resident cardiac stem cells (CSCs) cultured in high glucose medium have poor nuclear translocation of β-catenin/TCF-4 and expressed adipogenic transcription markers (PPARγ, ADD1, and C/EBPα) with a concomitant abrogation of CSC-specific markers including Ckit, Sca-1+, MDR-1, and Isl-1 (Zhang et al. 2018). Therefore, we must take into account the effect of hyperglycemia on stem cell functionality while designing cell therapy protocols, especially when the cells to be used are autologous (Grohová et al. 2019).

A case-control study using amniotic fluid-derived MSCs from pregnant women with gestational diabetes revealed that the inflammatory response genes including TNF-a, MCP-1, CD40, and CTSS were upregulated, while anti-inflammatory IL133 gene expression was downregulated (Algaba-Chueca et al. 2020). These gene-level expression changes were related to the metabolic status of the mother and her fetus affects the fetus. However, a recently published study has reported that MSCs cultured under high glucose conditions have similar in vitro properties such as trilineage differentiation and colony formation besides showing similarity in their therapeutic potential (José et al. 2017). These data warrant further mechanistic investigations to explain the divergence in the data. Moreover, it would be interesting to find out if there is a differential effect of pulsatile vs chronic constant hyperglycemic exposure on cellular functions (Frost et al. 2012).

12.2.2 Hyperglycemia Affects Stem/Progenitor Cell Mobilization

In addition to the altered surface marker expression and changes in observed in clonogenicity, hyperglycemia significantly impairs the mobilization potential of stem/progenitor cells. Endogenous stem/progenitor cell mobilization and their retention at the site of injury remain an integral part of the intrinsic repair process. Nevertheless, endogenous BMSC mobilization and their recruitment to the site of injury as an integral part of the intrinsic repair process also get diminished over time in the hyperglycemic environment (Shin and Peterson 2012). While elucidating the molecular mechanism of unresponsiveness to G-CSF treatment, the poor mobilization response of Sca+Ckit+CD34+ cells to G-CSF treatment was ascribed to the reduced dipeptidyl peptidase-4 (DPP-4) activity in the BM in diabetic animals as compared to their wild-type counterparts (Fadini et al. 2013). The same group of researchers has later reported that treatment with CXCR4 antagonist plerixafor alleviates the negative effects of hyperglycemia on hematopoietic stem cell (HSCs) mobility in response to G-CSF treatment in patients receiving chemotherapy (Fadini et al. 2015). Sustained exposure to hyperglycemia, besides insulin resistance and dyslipidemia, also causes decreased CXCR4 expression in EPCs. This negatively impacts the SDF-1α/CXCR4 axis and reduces EPCs responsiveness to mobilization and homing-in cues emanating from the site of injury. In vitro experiments with EPCs support these findings, and the data provide compelling evidence that hyperglycemia accelerates the onset of EPCs senescence thus leading to the impaired proliferative activity via phosphorylation of p38 MAPK (Kuki et al. 2006). Impaired mobilization of HSCs in diabetic experimental animal settings has been attributed to defective adhesion and chemotactic properties of HSCs as well due to diabetes-relevant changes in the physiology and microanatomy of the BM (Ferraro et al. 2011). Experimental animal studies have

revealed diabetes-related microvascular remodeling (significantly reduced microvascular density in the diabetic BM) negatively impacts the integrity as well as homeostasis in the BM (Oikawa et al. 2010). Therefore, protocols are being developed to optimize systemic therapy to overcome these changes in the BM and enhance mobilization and recruitment of BM-derived EPCs using a combination of FK506 (tacrolimus) and AMD3100 (Plerixafor/Mobizol; a chemokine receptor antagonist) (Qi et al. 2020).

Cultured endothelial cells from diabetic BM are characterized by higher oxidative stress, elevated β-galactosidase activity (a marker of senescence), reduced migratory and network forming potential, and increased adhesiveness with the BM mononuclear cells (Tepper et al. 2002; Caballero et al. 2007; Jarajapu et al. 2011). Microangiopathy in diabetic hearts has been ascribed to endothelial dysfunction, which can be corrected by the restoration of angiomirs-126 and angiomir-132. Restoration of angiomir-126 and angiomir-132 expression abrogates the deleterious effects of diabetes and high glucose culture conditions on endothelial cells for their survival, proliferation, and angiogenic potential (Rawal et al. 2017; Meng et al. 2012). On the same note, abrogation miR-15 and miR-16 restore the angiogenic functions of EPCs (Kane et al. 2014). In a prospective clinical study (NCT01102699) involving 24 diabetic patients, impaired mobilization of CD34+ cells into peripheral circulation was observed in response to treatment with 5 ug/kg human recombinant G-CSF as compared with the normoglycemic controls (n = 14). The number of circulating CD34+CD133+ was significantly lower in diabetic patients as compared to the normoglycemic controls. Moreover, in vitro Matrigel assay also showed a significantly impaired proangiogenic function of the mobilized CD34+CD133+ HSCs obtained from diabetic patients. The observed mobilopathy of BM-derived CD34+ cells was related to the structural alterations affecting the BMSC niche (microangiopathy) besides the functional defect, which impaired their mobilization from their BM niche [Fadini et al. 2013]. The problem of BM-derived HSCs mobilopathy has been linked to myelopoiesis with the mechanistic involvement OSM-p66Shc signaling (Albiero et al. 2019).

12.2.3 Hyperglycemia Modulates the Paracrine Activity of Stem/Progenitor Cells

Each stem cell type secretes a specific combination of trophic factors that constitute its specific paracrine secretion profile under a defined set of culture conditions (Haider and Aslam 2018). The paracrine activity of stem cells, like other cellular functions, gets altered due to chronic exposure to hyperglycemia. CSCs isolated from cardiac biopsies of diabetic patients not only yield a lower number of CSCs with cardiac-specific markers, but they are also deficient in reparability when they are transplanted in the infarcted mice heart (Molgat et al. 2014). The cells and their conditioned medium have poor angiogenic potential as compared to the nondiabetic donor-derived CSCs and their conditioned medium. A cytokine profiling the conditioned medium shows significantly reduced expression of pro-angiogenic factors in the conditioned medium of CSCs from diabetic patients. Similar observations have been reported when normoglycemic donor-derived CSCs were cultured under hyperglycemia conditions and profiled for angiogenic factors. Similar observations have also been reported in the experimental animal models. Kim et al. showed that MSCs derived from diabetic rats had suppressed the angiogenic growth factor profile as compared to normoglycemia (Kim et al. 2015). The authors observed that MSCs form the diabetic rats had lower expression of the major angiogenic growth factors including VEGF, Ang-1, Ang-II, PGF, FGF-2, and HGF-1 as compared to the normoglycemic MSCs used as control. The cells also had poor tubulogenic activity during in vitro matrigel assay and thus failed to alleviate ischemia post-engraftment in an experimental model of hind-limb ischemia.

Despite similarities in morphological characteristics and biological behavior in the experimental settings, MSCs derived from diabetic and wild-type animals significantly differed in their proliferative potential and differentiation capability besides their paracrine secretions from the differentiating pancreatic cells that recruit chromatin modulators to ensure maturation of the islet cells (Jin et al. 2010; Phadnis et al. 2011). Strategies are also being developed to revamp the stem cell functions including paracrine activity. Treatment of MSCs with salidroside reverses the hyperglycemia (cells cultured under high glucose conditions) and suppresses the expression of wound-healing factors hemoxygenase-1 (HO-1), HGF-1, and FGF2 besides significantly reducing the intracellular load of reactive oxygen species thus enhancing their survival (Ariyanti et al. 2019). Salidroside is a small molecule that is a known regulator of ROS activity, inflammation, and apoptosis. While elucidating the underlying mechanism, the same group of researchers has previously reported that salidroside abrogated the prolyl hydroxylase domain 3 (PHD3) which is upregulated in the hyperglycemia-exposed cells thus suppressing the release of proangiogenic factors, i.e., VEGF-A and PDGF-BB, from the muscle cells (Zhang et al. 2017). Put together, these studies validate the hypothesis that exposure to hyperglycemia is detrimental for the paracrine activity of stem cells thus significantly impacting their reparability. However, it would be of interest to see if hyperglycemia-induced impairment of paracrine activity may be reversed to restore the functional impairment of the cells.

12.3 Stem Cell Therapy and Diabetes

12.3.1 Cell-Based Therapy in Regenerative Medicine

More than two decades of experimental studies in animal models (Haider et al. 2004a, b, 2008; Takamiya et al. 2011; Ozdemir et al. 2012; Wu et al. 2013; Ionescu et al. 2012; Fang et al. 2012) and clinical trials in patients (Veronesi et al. 2013; Herreros et al. 2012; Yau et al. 2019) supported by the mechanistic studies in vitro (Liu et al. 2011; Sassoli et al. 2012; Vishnubalaji et al. 2012) have shown the prowess of stem cell-based interventions as a safe and an effective alternative to the conventional treatment modalities. Skeletal myoblasts (SkMs), BMSCs, and CSCs are some of the most extensively studied cell types with proven therapeutic efficacy both in experimental animal models and clinical studies. SkMs are skeletal muscle-derived unipotent cells with excellent myogenic differentiation characteristics (Durrani et al. 2010). Moreover, SkMs inherently express Oct4, one of the Yamanakas quartets of transcription factors for reprogramming, which render the cells more amenable to reprogramming to pluripotency.

The latest entrant to the long list of stem cells characterized by regenerative capacity is the induced pluripotent stem cells (iPSCs) (Ahmed et al. 2011a, b; Buccini et al. 2012; Okawa et al. 2013; Kawamura et al. 2012; Carpenter et al. 2012; Espejel et al. 2010; Sumi, 2011). The iPSCs are surrogate ESCs derived after the reprogramming of the somatic cells. Similar to ESCs, iPSCs differentiate into cells of all the three germ layers; however, their use is without ethical and moral issues regarding availability and use. Moreover, the possibility of disease specificity and patient-specific autologous availability places iPSCs as choice cells for the treatment of various pathologies including diabetes. Since the publication of the first report that somatic cells can be reprogrammed by ectopic induction of pluripotency determinant transcription factors (Oct4, Sox2, cMyc, and Klf4) (Takahashi et al. 2007), reprogramming protocols have been optimized to enhance the efficiency and safety of iPSCs for human use (Si-Tayeb et al. 2010; Hiratsuka et al. 2011; Li and Rana 2012; McGrath, et al. 2018). However, the tumorigenicity of iPSCs is one of the main impediments for their progress to clinical application (Ahmed et al. 2011a, b). Current research is primarily centered on optimization of the classical reprogramming protocol with special focus to curtail tumorigenicity of iPSCs (Park et al. 2012; Martinez-Fernandez et al. 2010).

Efforts are also underway to combine stem cell transplantation with other strategies to enhance their therapeutic efficacy. Physical, genetic, and/or chemical manipulation of stem cells before transplantation augments their survival, paracrine behavior, and stemness (Suzuki et al. 2010; Afzal et al. 2010; Noiseux et al. 2012; Yan et al. 2012; Jeong et al. 2017). We have already pioneered a novel method based on ischemic preconditioning of stem cells (Kim et al. 2009). Our novel subcellular preconditioning approach is the latest addition to the in vitro manipulation strategies to alter stemness-related characteristics of the donor cells (Lu et al. 2010). With an emerging mechanistic role of miRs as critical regulators of diverse signaling pathways, various research groups including ours have shown that genetic manipulation of stem cells for altered expression of miRs is an effective alternative approach to enhance their stem cell functionality (Kim et al. 2012a, b; Lai et al. 2012).

12.3.2 Stem Cell-Based Therapy and Diabetes

Unlike the acinar and ductal cells in the pancreas which retain their capacity of self-renewal and growth, postnatal pancreatic β-cells are terminally differentiated and mitotically inactive, and hence, their replacement as a part of endogenous repair process occurs via a neogenetic process. This encompasses the differentiation of duct-like epithelial cells to become morphofunctionally competent hormone-secreting β-cells. Alternatively, strategies are being developed to stimulate the quiescent β-cells to reenter into the cell cycle and replace the nonfunctional β-cells. To this end, targeted inhibition of DYRK1A and GSK3b signaling successfully promotes β-cell proliferation (Shen et al. 2015). Efforts are underway to find out novel targets and genomic modulators to promote β-cell proliferation and self-renewal (Robitaille et al. 2016; Shirakawa et al. 2016; Shuen-ing et al. 2017).

Given that stem cells can cross lineage restriction to adopt various phenotypes, the possible application of stem cells for the treatment of diabetes remains an area of intense research (Calafiore et al. 2014). The prime advantage of stem cell therapy will be the restoration of an endogenous mass of insulin-producing cells (IPCs) that will be responsive to glucose changes in their microenvironment. The strategy is considered as an alternative to the conventional therapeutic interventions to replenish the loss of functionally competent β-cells, thus restoring insulin secretion to achieve the reversal of hyperglycemia and maintenance of glucose homeostasis (Li et al. 2012; Prabakar et al. 2012; Kim et al. 2012a, b). The target patients expectedly benefitting from this novel approach will be those who required exogenous insulin as a support therapy to replenish their functionally deficient insulin

source due to progressively declining β-cell failure (Chhabra and Brayman 2013).

12.3.3 Using Stem Cells as Magic Bullets to Cure Diabetes

Beyond the management of diabetes by lifestyle changes and pharmacological intervention, stem cell transplantation provides an opportunity to cure diabetic patients by their differentiation to insulin-secreting β-cells (Wehbe et al. 2016). Stem cells from various tissue sources and differentiation potential have been successfully used for trans-differentiation into insulin-secreting β-cells and for treatment in experimental animal models with encouraging data (Amer et al. 2018; Xu et al. 2019a, b). Transplantation of stem cells in these studies repopulated the pancreatic tissue in the recipient animals with insulin-producing β-cells in glucose concentration-responsive manner which gave improved glycemic control. Moreover, the newly differentiated cells expressed pancreatic β-cell-specific markers including Pdxq and Glut-2 expression.

SkMs have been used as magic bullets to reduce hyperglycemia in experimental animal models by an as yet undefined mechanism (Lei et al. 2009). Using a KK Cg-Ay/J mice model of type 2 diabetes, we showed that intramuscular transplantation of human SkMs significantly attenuated hyperglycemia and improved glucose tolerance. Supported by immunosuppression, these studies evidenced extensive survival of the transplanted human SkMs until 12 weeks of observation (Ma et al. 2013). Although transplantation of non-autologous stem cells ensures logistic advantages in terms of off-the-shelf availability, the survival of the cell graft and immunosuppressive therapy of the recipients to support non-autologous donor cell survival are the major drawbacks that necessitate further experimentation. We have reported that SkMs enjoy conditionally immune-privileged status, and hence, their long-term engraftment may be supported by transient immunosuppression (Haider et al. 2003). Starting immunosuppression 3−4 days before and until a short time after SkMs transplantation improved the survival of the xenografted SkMs in a porcine heart model (Haider et al. 2004a, b). As an alternative strategy, encapsulation of the islets confers immune isolation of the non-autologous donor cells (allogenic or xenogenic) and enhances the cell graft survival after transplantation besides alleviating the need for immunosuppression (Ngoc et al. 2011; Tomei et al. 2015). Strategy based on multiple repeated injections has also been adopted to ensure long-term antihyperglycemic effects of donor stem cells (Ho et al. 2012). More than 51% of the transplanted cells survived and were observed until 6 months of follow-up after fortnightly repeated intravenous injections of MSCs in STZ-induced diabetic mice. Multiple cell injections reversed glucose homeostasis and significantly reduced the systemic oxidative stress with concomitant engraftment of the donor cells in the liver and their differentiation into IPCs. These data implied the importance of repeated administration of stem cells to sustain the therapeutic benefits over a long time as compared to single-dose administration. These data have been substantiated by other research groups; nevertheless, the time of stem cell infusion after experimental induction of diabetes was critical for the outcome in terms of therapeutic benefits. Stem cell transplantation is also intended to gain metabolic control in the cells to overcome the loss of glucose homeostasis (Mabed and Shahin 2012). Intravenous infusion of MSCs can increase GLUT4 expression besides the phosphorylation of insulin receptor substrate-1 (IRS-1) and Akt in the insulin target tissues (Si et al. 2012).

Although neogenesis of IPCs and support of islet remodeling are the two main mechanisms by which transplanted stem cells contribute to the repair of injured islets cells (Iskovich et al. 2015), failure of the transplanted cells to differentiate into IPCs has also been reported that suggests their differentiation-independent contribution to diabetes reversal (Choi et al. 2003; Lechner et al. 2004; Dor et al. 2004; Taneera et al. 2006). The donor cells remain undifferentiated in the host pancreas, and none of the donor BMSCs expresses insulin but still manage to lower hyperglycemia. In-depth mechanistic studies have been carried out to explain the differentiation-independent mechanism of stem cell therapy by focusing on their anti-inflammatory and immunomodulatory properties to support islet graft survival (Yagi et al. 2010). In vitro studies have shown that antiapoptotic activity of the trophic factors released by MSCs protects the islets cells upon their subsequent exposure to proinflammatory cytokines in a co-culture system (Yeung et al. 2012). The antiapoptotic, anti-inflammatory, and pro-survival activity of stem cells via the release of bioactive molecules has been demonstrated by many research groups (Karaöz et al. 2010; James et al. 2010; Ohnishi et al. 2007). Extrapolation of these data in experimental animal models of diabetes has shown that co-transplantation of islet cells with MSCs gave better survival and functionality of islet graft due to improved regional revascularization as compared to the control without MSCs engraftment (Ito et al. 2010). Wang et al. have reported that of human umbilical cord, MSCs cause reversal of dedifferentiated β-cells to alleviate their dysfunction by secreting ILI-Ra that interferes with the diabetes-related inflammatory process by blocking the access of IL-1 to its receptors (Wang et al. 2020).

Encouraged by these experimental animal data, stem cell therapy for β-cell regeneration has progressed to the clinical studies in diabetic patients as a plausible therapeutic intervention to achieve glucose homeostasis. A recently published meta-analysis of 206 participants from 6 studies has reported a significant reduction in HA1cA at 12-month follow-up

after autologous BM-derived HSCs treatment that reduced their daily insulin dose requirement (Guo et al. 2019). These data reveal a multifactorial mechanism by which donor stem cells may contribute to resolving diabetes.

12.4 Stem Cells Reprogramming to Insulin-Secreting β-Cells

Most of the reported protocols for reprogramming of somatic stem cells to differentiate into IPCs are based on the manipulation of culture conditions or by genetic modulation of the cells for the expression of key regulatory genes responsible for the development of pancreas during embryogenesis (Zanini et al. 2011; Santamaria et al. 2011; Tsai et al. 2012). Successful programming of BMSCs has been reported by single or combined ectopic expression of repressor element-1 silencing transcription factor (Rest), sonic hedgehog (Shh), neurod1, Mafa, and Pdx1 with/without treatment with growth factors (McKimpson and Accili 2019). The genetically reprogrammed IPCs secrete insulin in response to glucose changes in their microenvironment (Paz et al. 2011). Alternatively, abrogation of intrinsically expressed ARX (Aristaless-related homeobox; with a significant role in pancreatic endocrine development), and induction of exogenous Pax4 transcription factor (Paired box4; one of the members of transcription factors Pax1-Pax9 with a significant role in embryonic organogenesis), results in monohormonal, glucose-responsive IPCs that secreted insulin at 15−30% of the human islets (Lima et al. 2016). The expression of Isl1β gene expression further enhances the production of insulin from the differentiated IPCs in a glucose-responsive manner (Jung et al. 2018). Induction of the cells by betacellulin treatment, a ligand for an epidermal growth factor (EGF), besides induction of EGF receptors-1, 2, and 3 also causes the cells to express insulin receptor substrate-2 (IRS-2) and proliferation of cells (Oh et al. 2011). Following an identical approach, the transduction of cDNA encoding for betacellulin transformed the cells into insulin secretors which expressed copious amounts of insulin both in vitro (0.4 ng/mL per 104 cells) and in an STZ-induced experimental mice model of diabetes. In a similar study, delivery of betacellulin was combined with the transgenic overexpression of Ngn3 (Neurogenin3) (Yechoor et al. 2009). The neo-islets thus obtained displayed ultrastructural characteristics, glucose-responsiveness to secrete insulin, and transcriptional profile similar to the native β-cells. Moreover, transplantation of the neo-islets ameliorated hyperglycemia in the STZ-induced diabetes model. Nevertheless, the protocols for gene modification of stem cells are under scrutiny for patient safety issues. Hence, the transplantation of stem cells engineered with regulatable vectors for controlled insulin expression may be a safer option (Unniappan et al. 2009). Likewise, methods without genetic modification, such as the ones based on optimization of culture conditions, have been reported to make β-cells safer for clinical application (Sun and Ji 2009; Kadam et al. 2012).

Stem cells modified for ectopic overexpression of therapeutic gene/s serve as a continuous source of the transgene expression product via altered paracrine behavior to impart their therapeutic benefits (Haider et al. 2008; Lei et al. 2008; Ahmed et al. 2010; Noiseux et al. 2012; Konoplyannikov et al. 2013). The strategy is also used for the reprogramming of stem cells to insulin-secreting β-cells and enhances their lineage commitment before transplantation (Barcala et al. 2013). For example, BMSCs were successfully reprogrammed in vitro to become IPCs and were subsequently transplanted into experimentally induced diabetic animals (Zhang et al. 2009a, b). A follow-up until 8 weeks showed the persistence of the transplanted cells with concomitant normoglycemia in diabetic animals. BMSCs have also been genetically modified for overexpression of human pancreatic duodenal homoeobox-1 (Pdx1) enhanced the rate of their differentiation to become functionally competent β-cells (Karnieli et al. 2007). Similar results were also obtained by ectopic transgene expression of Ngn3 either alone or in combination with Pdx1 transgene in BMSCs which were already immortalized by human telomerase reverse transcriptase (hTERT) induction (Limbert et al. 2011; Cao et al. 2011). On the same note, a reliable protocol for high-efficiency differentiation of ESCs into β-cells by ectopic transgenic expression of Pdx1 has been reported (Raikwar and Zavazava 2012). Assessment of the rate of efficiency of β-cell differentiation using an insulin-II-GFP reporter revealed that less than 3% of ESCs were differentiated into IPCs (Ben-Yehudah et al. 2009). This poor differentiation rate besides the teratogenicity of genetically modified ESCs also raised safety concerns about their clinical application.

A comparison of the transcriptome profile of the differentiated IPCs with the islets and undifferentiated BM-derived MSCs revealed that activation of Ins1, Ins2, Glut2, and glucagon genes was low in the differentiated IPCs as compared to islets cultured in the same basal glucose medium (Hyder 2019). These issues have been addressed by Xin et al. who developed a three-stage protocol to enhance the differentiation efficiency of human BM-derived MSCs (Xin et al. 2016). The differentiated IPCs expressed β-cell-specific markers, and 43% of the cells expressed L-type calcium channel activity in response to glucose changes in their microenvironment. Treatment of STZ-induced diabetic nude mice successfully lowered their blood glucose. Future in-depth studies to identify the genes and growth factor cues responsible for the differentiation of cells to adopt the β-cell phenotype will be helpful for the treatment of diabetic patients. For example, a recent study shows that fate determination of the differentiating IPCs is a dynamic process that is

influenced by the presence of laminin rather than fibronectin in the microenvironment that compels the differentiating cells to adopt duct cell phenotype (Spagnoli 2018).

Another major impediment to the success of reprogrammed β-cell transplantation is their massive death after transplantation. Chronic exposure to hyperglycemia further increases β-cell apoptosis which is accentuated by impaired vascularization and poor regional perfusion, thus reducing the effectiveness of the procedure (Biarnés et al. 2002). To this end, β-cells have been genetically modified to abrogate the expression of proapoptotic caspase-3 and X1AP to interrupt the apoptotic cascade (Cheng et al. 2008; Emamaullee et al. 2005). Moreover, delivery of the genes encoding for angiopoietic growth factors, having regulated or unregulated gene expression, is used to restore regional blood via activation of survival signaling as well as angiogenesis to enhance islet graft survival (Dai et al. 2004; Lee et al. 2011; Lopez-Talavera et al. 2004). Effectiveness of this remedial approach may be supported by multimodal approach wherein stem cell therapy may be combined with the delivery of angiopoiesis-encoding factors either alone or in combination with molecules involved in the pro-survival signaling pathway (Bone et al. 2012; Fiaschi-Taesch et al. 2007; Wu et al. 2011). Besides alleviating the technical challenges of gene transfer to the β-cells and maintenance of a continuous source of transgene expression product to improve islet survival, angiogenesis may effectively restore diabetic wound healing (Castilla et al. 2012).

Even though different cell types have been used as parent cells for IPCs differentiation, there is little evidence whether their derivative IPCs also differ in their functional characteristics. Novel multipotent precursor cells in the islets have been identified having MSC-specific surface marker expression (Carlotti et al. 2010). These results have been substantiated by the studies, which showed that the precursors also expressed ESCs-specific markers of primitiveness, i.e., Oct4, Sox2, and Rex1, and their comparison with MSCs revealed higher telomerase activity (Karaoz et al. 2010). Interestingly, proteomic studies revealed that the derivative β-cell populations differed in their respective parent cell-specific protein expression profile. Similarly, a comparison of diverse populations of MSC isolated from BM, Wharton jelly, adipose tissue, and peritoneum showed that all four types of MSCs can differentiate into IPCs. However, the peritoneal MSC-derived β-cells were most efficient in responding to glucose changes in their microenvironment. Some other stem cell types used as starting material for IPCs generation include human urine-derived stem cells, gallbladder stem cells, etc. (Hwang et al. 2019; Chen et al. 2019). Despite encouraging data, the rationale for preference and criteria of parent stem cell selection in terms of quality of the respective derivative β-cells remains less well-understood and, therefore, warrants future in-depth studies to address this important issue.

12.5 Pluripotent Stem Cells for β-Cell Regeneration

12.5.1 ESCs as a Renewable Source of β-Cells

Distinct from the adult stem cells due to their pluripotent status, ESCs have been studied for more than a decade as a promising renewable source for β-cell generation (Rezania et al. 2012; Schulz et al. 2012; Russ et al. 2015). The two main properties that make ESCs a promising choice for stem cell therapy are their unlimited undifferentiated self-renewal potential in vitro and pluripotency that enables them to differentiate into cell types of the three germ layers. The earlier studies have shown that undifferentiated ESCs can inherently secrete insulin under appropriate culture conditions (Soria et al. 2000; Soria 2001). The novel insulin-secreting ESCs population was successfully isolated by the cell-trapping technique and characterized in vitro. Besides the expression of insulin, the cells were positive for β-cell-specific markers including Pdx-1, Nkx6.1, Glut2, and Sur-1. Although the purified clone of the insulin-secreting ESC successfully corrected STZ-induced hyperglycemia in the experimental animals, 40% of animals in the treatment group reverted to hyperglycemia within 12 weeks of observation. These results necessitate manipulation of the donor cells to achieve a more stable expression of insulin. Various research groups have optimized the ESCs differentiation protocols to enhance the rate of β-cell differentiation (Soria 2001; Leon-Quinto et al. 2004; Bruin et al. 2014). Upon differentiation, ESCs self-assemble to form 3D clusters similar in topology to the islets in the pancreas and display glucose-responsiveness to release insulin (Lumelsky et al. 2001). A new protocol for IPCs generation from human ESCs has been reported, which is based on 3D culturing of the cells under hypoxic conditions (Rattananinsruang et al. 2018). The differentiation protocol based on hypoxia treatment successfully inducted the expression of pancreas-related genes, including Pdx1, Ngn3, Nkx6.1, and Glut2. The IPCs thus generated were encapsulated and transplanted in an STZ-induced diabetic mouse wherein the cells successfully reduced the level of inflammatory cytokines as well as treated hyperglycemia.

Many other research groups have also reported successful transplantation of IPCs to treat experimentally induced hyperglycemia in experimental animal models (Hua et al. 2014). As described earlier, for optimal functioning of the transplanted cells, it is important to immune-isolate the cells to enhance their acceptance by the host after engraftment

(van der Torren et al. 2017). In this regard, strategies such as microencapsulation have given markedly superior results (Tuch et al. 2014; Kirk et al. 2014). However, technical limitations to achieve fully differentiated mature β-cells from ESCs, ethical issues surrounding their availability and the use of immunosuppression due to lack of autologous availability have hampered their way for routine patient use. Moreover, the undifferentiated remnants of ESCs may be an important contributory factor toward the teratogenic nature of ESCs. These are serious issues that need to be addressed to ensure their safety and efficacious use in clinical settings.

12.5.2 iPSCs as a Renewable Source of β-Cells

Somatic cell programming to pluripotent status has reinvigorated the interest of researchers in stem cell research (Ibrahim et al. 2016). Deviating from the classical reprogramming protocol of Takahashi et al. based on transduction of a quartet of transcription factors, protocols are being optimized to achieve somatic cell reprogramming with lesser number of stemness factors without genome-integrating viral vectors to make the derivative iPSCs safer for human use (Nakagawa et al. 2008; Stadtfeld et al. 2008; Okita et al. 2008; Carey et al. 2009; Kim et al. 2016; McGrath et al. 2018). A summary of the advancements in the protocols has been provided by Shahjalal et al. (2018). Multiple iPSC lines using different somatic cell types of mouse and human origins have been produced and characterized (Kunisato et al. 2010; Ahmed et al. 2011a, b; Buccini et al. 2012). There is a special focus on the epigenetic characterization of the derivative iPSCs in relation to differentiation potential as the reprogramming process shares several features with the early human embryos such as extensive DNA hypomethylation (Watanabe et al. 2013; Perrera and Martello 2019). In-depth transcriptional profiling of ESCs and iPSCs shows two distinct groups of reprogramming-induced and reprogramming-resistant genes with preferential methylation marks (Polouliakh 2013). It is generally concluded that both these groups of genes in general, but the reprogramming-resistant genes in particular, are important determinants of iPSC functionality.

As an alternative to ESCs, iPSCs are currently being studied for the generation of hyperglycemia-responsive β-cells capable of insulin secretion. One of the main advantages of iPSCs is that they allow a continuous source of patient-specific β-cells thus raising the possibility of long-term functional survival of the transplanted cells without immunosuppression (Ohmine et al. 2012). The success of differentiation of any protocol depends upon the appropriate cues that stimulate specific signaling pathways in iPSCs that support the cellular machinery to adapt to the needs of new cell phenotype. In the absence of appropriate cues, immature/fetal-like β-cell will be developed due to incomplete differentiation. Therefore, various protocols are being reported that involves treatment with agents such as glucagon-like peptide-1/exedin-4, genetic manipulation of iPSC for overexpression of single or multiple embryonic transcription factors and miRNA manipulation to achieve complete differentiation of iPSCs before transplantation (Raikwar et al. 2015; Xu et al. 2019a, b). Mostly, these protocols have been designed in line with the sequential role of various growth factors and transcription factors during embryogenesis of the pancreas, development of β-cells, and insulin secretion in the vertebrates. In one such multistep protocol, human iPSCs were treated with Activin-A/Wnt3 for endoderm fate followed by priming with FGF10 and KAAD/cyclopamine and subsequent treatment with pancreatogenic cocktail containing retinoic acid boosted by the inclusion of indolactum-V (Thatava et al. 2011). The pancreatic progenitors thus obtained expressed PDX1, Ngn3, and NeuroD1 markers. Further guidance with IGF-1 and HG-1 treatment, enhanced by the inclusion of glucagon-like peptide-1, gave glucose-responsive mature β-cells in vitro. Simultaneous overexpression of Pdx1, MafA, and NeuroD or Ngn3 with concomitant culture on laminin-5 extracellular matrix facilitated the transformation of mouse-derived iPSC into insulin-secreting cells (Kaitsuka et al. 2014). An interesting feature of the protocol was the use of feeder-free culture conditions. The transformed cells released C-peptide in response to glucose challenge in vitro and successfully reverted hyperglycemia in diabetic mice. Similar multistep protocols with modifications have been reported by other research groups (Zhang et al. 2009a, b; Kredo-Russo et al. 2012; Shahjalal et al. 2014; Kuise et al. 2014). A careful analysis of these protocols, irrespective of the steps involved therein, shows a common modus operandi of the systematically and sequential entrance of the pluripotent stem cells into endoderm fate specification and pancreatogenic fate followed by priming for glucose-responsive insulin secretion. The latest addition to the list of these protocols is the use of small molecules to prime the iPSCs to IPCs (Thakur et al. 2020).

12.6 Development of Direct Reprogramming Protocol Using miRNA Approach

Efforts are underway to simplify the protocols discussed above while maintaining an enhanced rate of mature β-cells differentiation without tumorigenic potential. With the recent progress and understanding of the crucial regulatory involvement of miRNAs in the genesis of the pancreas, β-cell function, and insulin signaling (both synthesis and exocytosis) (Martinez-Sanchez et al. 2017), researchers are attempting to harness the significance of miRNAs to optimize β-cell differentiation protocols. They are manipulating the miRNA

expression profile of iPSCs for directed differentiation to the β-cell phenotype without achieving pluripotent status. MiRNAs are functionally important regulators of multiple cellular functions including their differentiation as well as maintenance of the differentiation status to their functional standing (Haider et al. 2015). Circulating miRNA profile during a study involving 295 patients with metabolic syndrome has revealed the involvement of miR-23a, miR-509-5p, and mIR-197 as the major contributors in metabolic syndrome (Karolina et al. 2012). Similarly, the miRNA-17-92 knockout mouse shows significantly impaired glucose tolerance after STZ treatment which is associated with reduced β-cell mass and decreased proliferation, thus making the animals susceptible to experimental induction of diabetes (Wan et al. 2020). These data show a plausible implication of miRNAs in pancreatic β-cell function in health and disease. Lentiviral vector-based delivery of miRNA-375 is sufficient for directed differentiation of iPSCs into functionally mature β-cell phenotype, which is capable of secreting insulin in a glucose-responsive manner (Lahmy et al. 2014). Unlike the current protocols which mostly rely on treatment of iPSCs with multiple growth factors and transcription factors, overexpression of miRNA-375 was simple in differentiating iPSCs into fully functional β-cells. Similar results have also been reported by the transfection of human iPSC with combined transgenic induction of miR-375 and miR-186 (Shaer et al. 2014). This protocol provides the opportunity of patient-specific iPSCs genetically manipulated for miRNAs, albeit without viral vectors, thus enhancing its safety for human application. The latest development in this regard is the finding that miRNA-181c-5p delivery into human iPSCs induced endodermal markers SOX17, FOXA2, CXCR4, and GATA4 besides induction of endocrine-specific gene expression of Pdx1, Nkx6.1, Mafa, and insulin. Transplantation studies revealed that recipient animals were protected from chemically induced diabetes (Li et al. 2020).

Despite these encouraging data from ESC- and iPSC-derived β-cells, there are several lacunae including tumorigenicity, which need to be addressed to ensure their progress to clinical settings (Kroon et al. 2008). Hence, intensive future studies are warranted to optimize differentiation protocols to eliminate tumorigenicity of the contaminating undifferentiated iPSCs, which remains an integral part of our proposal.

12.6.1 Advances in Insulin-Producing Cells for iPSCs and Future Perspective

Efforts are underway to develop protocols that ensure better acceptance of the iPSCs-derived IPCs from the clinical perspective. One of such strategies is 3D culturing of iPSCs on synthetic scaffolds. Endrami et al. have reported successful differentiation of iPSCs to IPCs using the 3D culture of the cells on poly-L-lactic acid and polyvinyl alcohol (PLLA/PVA) nanofiber scaffolds (Enderami et al. 2018). It was observed that the differentiating iPSCs formed a homogeneous population of spherical cells that expressed pancreatic beta cell-specific transcription factors including Pdx1, insulin, Glut-2, and Ngn3. The same group of researchers has reported polyethersulfone nanofibers coated with collagen and polycaprolactone and polyvinyl alcohol (PCL/PVA) to support IPCs differentiation of human iPSCs (Reyhaneh et al. 2018; Abazari et al. 2018). Such is the popularity of this approach that a recently published systematic analysis has reported more than 60 different scaffold materials in 197 research papers to show that scaffold-based differentiation approach significantly enhances the rate of cell survival and differentiation of IPCs in vitro as well as after transplantation (Salg et al. 2019). These data are in line with the recently published results from real-time observation of 3D-cultured human iPSCs into pancreatic β-cells which showed that 3D-culture conditions prompted the cells to become mature, glucose-responsive IPCs unlike the 2D-culture conditions wherein the derivative cells were functionally immature (Wang et al. 2019). The improved viability and successful differentiation of iPSCs on scaffolds have been attributed to the optimal structural support and mechanical stability provided by the scaffold material to ensure microenvironment that is conducive for cellular activities required during the process of differentiation.

Another approach to overcome functional immaturity of iPSCs-derived beta cells is their engineering using CRISPER-Cas-9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein-9) to develop specific genetic variants of the cells; however, such an approach would require an in-depth understanding of the underlying molecular mechanism (Balboa et al. 2019). The combination of CRISPR-Cas-9 technology and iPSCs has been successfully used for gene therapy of beta-thalassemia in the experimental mice model (Ou et al. 2016). Maxwell and colleagues have used this approach to genetically correct iPSCs derived from a Wolfram syndrome (WS) patient and differentiate the genetically corrected iPSCs to become functionally competent β-cells. Transplantation studies in a mice model of revealed successful reversal of STZ-induced hyperglycemia (Maxwell et al. 2020)

Future studies are warranted to make a direct comparison of various stem cells in terms of their β-cell differentiation potential and functionality in terms of glucose-responsive insulin release besides studying their transplantation-relevant characteristics such as tumorigenesis and immune acceptance. Besides enhancing their immunological acceptance, the encapsulation strategy reshapes the proteomic profile and its landscape thus supporting their higher rate of differentiation thus necessitating further refinement and optimization

of encapsulation protocols (Legøy et al. 2020). Protocols are required to support direct differentiation of somatic cells into IPCs without completely reprogramming the cells into iPSCs, which will help to curtail their tumorigenesis.

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Induced Pluripotent Stem Cells in Pediatric Research and Clinical Translation

13

Duygu Uçkan-Çetinkaya and Khawaja Husnain Haider

Abbreviations

ALS	Amyotrophic lateral sclerosis
ATMP	Advanced therapy medicinal products
BM	Bone marrow
CMML	Chronic myelomonocytic leukemia
3D	Three-dimensional
EBMT	European Blood and Marrow Transplantation
ESCs	Embryonic stem cells
GMP	Good manufacturing practice
HSCs	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
ISCBI	International Stem Cell Banking Initiative
IRB	Institutional Review Boards
iPSCs	Induced pluripotent stem cells
Klf4	Kruppel-like factor-4
MSCs	Mesenchymal stem cells
Oct 3/4	Octamer-binding transcription factor-3/4
PDXs	Patient-derived xenografts
SNP	Single nucleotide polymorphism
SOx2	Sex-determining region Y-box 2
QC	Quality control
UCSCs	Umbilical cord stem cells

13.1 Introduction

Through their adhesive, migratory, secretory, and immunomodulatory properties, all of which confer functional plasticity, these cells play critical roles in tissue repair (Bruder et al. 1994; Caplan, 2015; Caplan and Hariri, 2015). Stem cells are becoming an indispensable cell type in both research and clinical practice. The therapeutic role of hematopoietic stem cells (HSCs) in transplantation practice has been well-documented since the 1960s till now, current hematopoietic stem cell transplantation (HSCT) numbers exceeding 40.000 patients annually in Europe alone (and additional reporting countries to European Blood and Marrow Transplantation/EBMT Registry) (Passweg et al. 2020). This experience, besides demonstrating the lifelong achievement of donor-derived hematopoiesis, also suggested the possible role of stem cells in the regeneration of non-hematopoietic tissues. Studies have shown migration of donor-derived HSCs to damaged tissues of HSCT recipients and contribution to the repair process (Kollet et al. 2003; Idilman et al. 2006). Similarly, the presence of microchimerism (feto-maternal or vice versa) years after pregnancy or detection of hematopoietic microchimerism following organ transplantation also suggested the contributory role of chimeric stem cells in repair of injured tissues or, sometimes, in development of diseases (Castela et al. 2017; Jeanty et al. 2014; Tan et al. 2005; Gilliam 2006). In the field of regenerative medicine, mesenchymal stem cells (MSCs) have attracted the most interest. Being of connective tissue origin, these cells are the main components of the stem cell microenvironment in several tissues, including the bone marrow "niche." Through their adhesive, migratory, secretory, and immunomodulatory properties, all of which confer functional plasticity, these cells play critical roles in tissue repair (Bruder et al. 1994; Caplan, 2015; Caplan and Hariri, 2015). These properties have led to an exploding interest in the clinical use of MSCs in many pathological conditions with acceptable safety but fair/unsatisfactory efficacy profile.

Induced pluripotent stem cells (iPSCs) have emerged as a highly potent stem cell type which can differentiate into all cell types of the three germ layers in the body, thus carrying a high potential in regenerative medicine (Ibrahim et al. 2016). These cells are obtained in vitro by reprogramming of any somatic cell to gain pluripotency by

D. Uçkan-Çetinkaya (✉)
Department of Pediatrics, Division of Hematology/Bone Marrow Transplantation Unit, Center for Stem Cell Research and Development (PEDI-STEM) and Department of Stem Cell Sciences, Institute of Health Sciences, Hacettepe University, Ankara, Turkey

K. H. Haider
Department of Basic Sciences, Sulaiman AlRajhi University, Al Bukayriyah, Saudi Arabia

K. H. Haider (ed.), *Stem Cells*, https://doi.org/10.1007/978-3-030-77052-5_13

methods defined by Japanese scientists Takahashi and Yamanaka in 2006 (Takahashi and Yamanaka in 2006; Takahashi et al. 2007). Stem cell research expanded dramatically since then with the understanding that these cells are a unique source for disease modeling and drug research (Shi et al. 2017; Chhabra 2017; Cagavi et al. 2018; Omole and Fakoya 2018; Hou et al. 2013; Raab et al. 2014). Generation of iPSCs in the lab is time-intensive and requires excessive manipulations, including the use of gene insertion and epigenetic modification techniques from various sources of somatic cells and successfully used for myocardial repair in experimental animal studies (Ahmed et al. 2011a; Buccini et al. 2012). Considering the extent of in vitro procedures, the use of high-throughput, advanced techniques are needed to monitor, improve, and ensure the quality and safety profile of iPSCs for use in the clinic (Muller et al. 2011; Bock et al. 2011; Tsankov et al. 2015). Clinical translation of this state-of-the-art technology involves the use of progenitors or differentiated cells derived through in vitro differentiation of iPSCs toward the tissues of interest (e.g., cardioprogenitor cells, cardiomyocytes, neuroprogenitors, neural cells, hepatocytes). Several groups are conducting clinical trials for selected diseases in a small number of patients. Some of the ongoing or approved clinical study topics include age-related macular degeneration, corneal transplantation, Parkinson's disease, heart failure, spinal cord injury, cancer, and the use of iPSC-derived platelets in blood transfusion practice (Karagiannis et al. 2017; Attuale et al. 2018; McNeish et al. 2015; Trounson and DeWitt 2016; Norbnop et al. 2020; Mandai et al. 2017). Given the broad range of quality control tests combined with extended monitoring to prove the safety of iPSC-derived cell products, the progress in clinical trials is expected to be time-intensive. In the meantime, enormous research is being performed in the field of iPSCs in disease modeling, tissue engineering, drug discovery, toxicity testing, and organoid studies.

Currently, many centers are focusing on drug screening for the repurposing of existing drugs/small molecules on iPSC-derived cells or systems generated from patients with incurable severe/progressive disorders. The encouraging results of in vitro screening studies have led to the initiation of clinical trials with the use of known drugs for new indications in patients with unmet medical needs (Lee et al. 2009; Cai et al. 2015). Till the safety issue for the application of iPSC-derived cells in the clinics is solved, the use of iPSCs for drug repurposing appears to be a reasonably fast track strategy to timely reach patients in need by providing therapy alternatives or disease-modifying treatment options. At the same time, application and approval of interventional clinical trials involving clinical use of iPSC-derived cellular products are progressing faster than expected, with great enthusiasm, and cautiously.

13.2 Stem Cell Research and Clinical Use in Pediatrics

13.2.1 Stem Cell Experience in Pediatrics Toward iPSCs

A huge amount of experience has been gained in the clinical use of stem cells in pediatrics in HSCT practice dating back to the 1960s. First successful bone marrow transplantation was performed by Good and his team in 1968 in a child with severe combined immunodeficiency, followed by many hematological, immunological, and other diseases (Gatti et al. 1968; Buckley et al. 1999; Wagner et al. 2007; Simpson and Dazzi 2019). First cord blood transplantation was performed in 1989 by E. Gluckman and her team in a child with Fanconi anemia (Gluckman et al. 1989). In the 1980s, in addition to hemato-immunological diseases and hematological cancers, HSCT practice involved inborn errors of metabolism/selected lysosomal diseases (e.g., Hurler's) as well (Hobbs et al. 1981; Galieva et al. 2017; Tan et al. 2019). Donor-derived healthy hematopoietic cells, by providing the missing enzyme and by differentiation toward tissue macrophages (in the central nervous system and other tissues), are suggested to contribute to the healing process and recovery in these metabolic diseases of childhood. These data have paved the way to elucidate the underlying of the mechanisms of the regenerative role of stem cells in-depth.

Extensive experience gained by HSCT research and clinical use contributed to a better understanding of stem cell biology, trafficking, niche, and cellular interactions in determining cell fate under basal and stressed/disease conditions (Magnon and Frenette 2007; Mendez-Ferrer et al. 2009, 2010; Kotha et al. 2018). Disease modeling by the use of biological samples of humans is a critically important task for the advancement of modern medicine. In that regard, iPSCs generated from patients with inherited diseases are invaluable sources for the invention of state-of-the-art therapy alternatives.

13.3 Increased Incidence of Inherited Diseases in Childhood

13.3.1 Disease Modeling and Drug R&D with iPSCs

Considering the increased ratio of inherited diseases in childhood compared to adult ages, the discovery of iPSC technology has brought the field of pediatrics to the most interesting point in the stem cell area. In childhood, especially in societies where the incidence of consanguineous

marriages is frequent, there is a wide variety of diseases, especially autosomal recessive and rare diseases (Hamamy 2012; Lal et al. 2016). According to European Union definitions, a disease is considered as rare when it affects less than 1 in 2000 citizens. Approximately 7000 different rare diseases have been identified to date, >70% of rare diseases are genetic and 70% of those start in childhood (Sun et al. 2017; Wakap et al. 2020). Rare diseases when together bring an enormous burden to the health system. Treatment alternatives are limited; response to therapy is variable among patients due to heterogeneity of diseases (Melnikova 2012). As the number of patients with serious and progressive disease is smaller, it raises the financial cost per patient besides making the procedure more labor-intensive. Despite the accelerated orphan drug approvals, shorter development timeline, and marketing of drugs (small molecules, biologics, enzymes, recombinant proteins, antibody, cell, and gene therapy products) in the recent years, there are still a variety of rare diseases in which the medical needs of many patients cannot be met. There is a need for the development of new therapeutic agents, strategies, and biomarkers for targeted therapies. A much better understanding of biology and the pathophysiology of rare diseases is needed for the discovery of efficient new treatments. Disease modeling and screening platforms by the use of iPSCs, organoids, and advanced reprogramming and engineering techniques are breakthrough steps toward the establishment of efficient, safe therapeutic measures, hopefully at reasonable prices.

13.4 Limited Amount of Biological Samples in Pediatrics

Pediatric research is often hampered by the inadequate sample availability in general and from the infants in particular. This is a major obstacle for disease modeling and functional studies since there is a need for a high number of cells. This disparity between the amount of original sample from the donors, especially those with a rare/very rare disease and in whom the majority of the studies until recent years were performed on genetic materials, is of more concern. Discovery of iPSC technology has enabled the expansion of iPSCs generated from a limited number of patient cells almost indefinitely and, by differentiation toward cells of interest contributed to organ-specific, disease-specific, or patient-specific research (Merkle and Eggan 2013; Durbin et al. 2018). This is a major step toward personalized medicine. By the current technologies, studies have shown iPSCs' derivation even from murine samples, which is a major advantage in pediatrics (Shi et al. 2016; Qi et al. 2018; Mulder et al. 2020).

13.5 Increased Regenerative Potential of Children and Future Use of iPSCs in Clinics

High regeneration ability in children gives them an advantage in stem cell applications (Traister et al. 2018; Tanaka and Ferretti 2009). The efficacy of stem cell therapy in children is expected to be better as compared to the adult patients. The results of HSCT in diseases with similar diagnoses are better in children when compared to adults who experience higher toxicity due to the transplant procedure (Tomizawa et al. 2017; Warren and Rossi, 2008). Moreover, donors of pediatric transplant patients are usually of younger age; thus, the quality of stem cell product is better when compared to older-age donors (Friedrich et al. 2001). This is in part due to the presence of more potent stem cells having increased engraftment potential and better regeneration potential to repair the injured tissues with more active repair mechanisms. Another contributing factor to the regenerative potential of the cells during in vivo applications is the stem cell dose. Cells are administered to patients at defined doses/kg of recipient weight in cell therapy applications. Generally, in humans >3×10^6/kg CD34+ hematopoietic stem/progenitor cells are infused for successful engraftment in HSCT, whereas >1–3×10^6/kg MSCs are used in clinical practice (Pulsipher et al. 2009; Parekkadan and Milwid 2010). Therefore, cell preparation takes less time in children, a higher number of cells/kg is available, the cost may be less, and a sufficient number of cells may be available for repeated applications. These factors have led to more favorable results in pediatric stem cell applications. However, the issue of limited cell number is surmounted by the fact that iPSCs can be cultured for extended time period in vitro and therefore serve as a renewable source for the cells of interest and that too in high number (Takahashi et al. **2006**). The feasibility of the derivation of many mature cell types with iPSC technology makes iPSCs very promising cell type for future regenerative applications in children with inborn errors of multisystem problems, birth defects, birth complications, and other childhood problems such as intoxication/trauma-related severe pathologies.

13.6 Congenital Anomalies, Birth Complications, and Availability of Umbilical Cord Stem Cells

13.6.1 Future Use of Cord Blood for iPSC Generation

The newborn period is suitable for regenerative applications due to the increased regenerative potential and decreased risk

of rejection of cellular products due to their immunologic immaturity. The special pathologies of the neonatal period, such as birth complications especially in preterm and congenital anomalies, may be life-threatening and, therefore, may necessitate stem cell use, which may even be more efficacious in the prenatal period in the developing fetus (Yun 2015; Seifert and Voss 2013; Touraine et al. 1999). In that regard, umbilical cord or amnion fluid-derived stem cells, usually MSCs, hold great promise in organ/tissue repair. MSCs derived from cord tissues, Wharton jelly, and placenta possess significant immunomodulatory/immunosuppressive activity and, thus, are being increasingly being used in clinical trials not only for neonates but in older children and adults as well.

On the other hand, umbilical cord blood is an easily accessible and commonly used stem cell type in HSCT. Cord blood carries great potential in regenerative medicine as well and has the advantage to be collected in premature births with expected complications. Both autologous and allogeneic cord blood cells have been used in conditions such as hypoxic-ischemic encephalopathy, bronchopulmonary dysplasia, and cerebral palsy (Kurtzberg 2017; Huang et al. 2019a; Yang et al. 2018; Cotten et al. 2014; Sutsko et al. 2013).

Stem cells from different sources, including BM-derived MSCs, cord-derived, or cord blood-derived cells, have been studied in congenital anomalies, mainly in cardiac defects (Tsilimigras et al. 2017). More recently, the potential role of iPSC-derived cardiomyocytes or directly differentiated cardiomyocytes from cardiac fibroblasts is being emphasized (Sadahiro et al. 2015; Taguchi and Yamada 2017; Srivastava and DeWitt 2016). Although the current time is premature for the clinical application of iPSC-derived or reprogrammed cells in pediatric practice, this area holds great promise for the future. The feasibility of banked cord blood and cord-derived cells as starting cells for iPSC generation is an exciting topic (Abberton et al. 2018). Recently, clinical-grade iPSC lines were generated under good manufacturing practice (GMP) conditions from cryopreserved cord blood units selected from those with homozygous HLA haplotypes to be made available for a larger population of patients in need (Rim et al. 2018; Morishima et al. 2018, 2020). However, the authors emphasize the ethical and regulatory issues to be solved to obtain consent to make iPSCs from cord blood donors.

13.7 Gene Therapy Applications: iPSCs and CRISPR-Cas9 Gene Editing

A major advantage of pediatrics for stem cell research and clinical use is the suitability of inherited diseases to gene therapy. HSCT is a curative option in only selected diseases and is associated with high morbidity and mortality. Besides, the need to find an HLA-matched donor is a limiting factor. Gene therapy is a suitable and promising treatment alternative in inherited disorders, especially in monogenic diseases, mostly caused by loss-of-function mutations. Phase I/II clinical studies involving gene transfer/modification to stem cells, e.g., autologous HSCs, are ongoing and are being ready to be presented as commercial products/drugs (Pai 2019; Ferrari et al. 2020; Shahryari et al. 2019; Gidaro and Servais 2019; Rao et al. 2018). Still, the lack of adequate vector systems remains as a major limitation in the gene therapy field. The CRISPR-Cas9 genome-editing tool is a significant step forward in overcoming some limitations, but still awaiting several obstacles to be overcome including the off-target effects, immunogenicity, and less optimal efficacy, for in vivo gene therapy applications (Lu et al. 2015; Wei-Jing et al. 2016; Ashmore-Harris and Fruhwirth 2020).

13.8 Adverse Drug Reactions in Children: Personalized Therapy with iPSCs

Human iPSCs (hiPSCs) are rewarding tools to assess drug toxicity particularly in cardiology (Magdy et al. 2018). The response of pediatric patients to medications, particularly in the newborn period, is different than adults partly due to different metabolic, pharmacokinetic, and pharmacodynamics profiles. This may lead to the occurrence of different kinds of adverse drug reactions, in some cases causing severe organ dysfunction. A recent review focuses on the use of iPSC and organoid technologies in the prediction of drug toxicity in intestinal, hepatic, pancreatic, renal, cardiac, and neuronal tissues (Genova et al. 2019). The toxicity of medications during the process of drug development can be predicted by studying drug effects on iPSC-derived cells (e.g., hepatocytes) derived from the patients with different polymorphisms in cytochrome p450 enzymes (Anson et al. 2011). Considering the ethical issues and moral limitations in performing clinical trials in children, the authors state the value of iPSC technology in the prevention/management of drug reactions in pediatric patients. Studies with iPSCs for the assessment/prediction of drug effects and adverse reactions are particularly valuable in rare diseases since there is limited knowledge in many cases, due to lack of clinical trials in a sufficient number of patients (Easley 2019). Drug repurposing of previously approved drugs for new indications is a very promising area in the field of rare diseases where iPSC-derived cells are used for assessment of response in the relevant cell types for that disease (Zhu et al. 2020; Tamer et al. 2020; Luz and Tokar 2018; Kim et al. 2019a; Tiscornia et al. 2013).

13.9 Regulations in Pediatric Clinical Research

There are strict regulations and ethical restrictions in performing basic and clinical research or clinical trials in pediatrics. Following the publication of the national regulations and ethical principles/guidelines regarding pediatric research in the USA in the 1970s, up-to-date, stricter regulations have become a requisite for protection of the rights of children in an era when the number of clinical trials involving children has shown a rapid increase due to the need for the invention of better treatment protocols in pediatric medicine (Jonsen, 1978; Rose 2017; Stroustrup et al. 2008). Subsequent to the Orphan Drug Act in the USA in 1983, many orphan drugs have been approved to treat rare ailments. Moreover, the establishment of critical review of research protocols, informed consent, child assent, and determination of upper limits on the acceptable degree of research-related risks by Institutional Review Boards (IRBs) has led to the further protection in pediatric research. In this regard, reprogramming technologies, by enabling research using a limited amount of biological materials, have brought major advantages to pediatric research particularly for in vitro drug screening studies. However, strict regulations and ethical limitations are to be implemented for clinical use of cell and gene products, evaluated according to advanced therapy medicinal products (ATMP) guidelines. The clinical use of iPSCs is a very recent topic requiring specific regulations to prove safety and ensure efficacy. Such interventional clinical trials will necessitate much stricter regulations for children. Clinical, scientific, and regulatory authorities from several parts of the world are extensively working on development or revision of quality assessment and quality control criteria for iPSCs' banking and clinical use.

13.10 Technical Issues and Need for iPSCs Banking: For Research and Clinic

13.10.1 Generation and Characterization of iPSCs

iPSCs are generated in vitro through reprogramming of almost each somatic cell type, including skin fibroblasts, skeletal myoblasts, bone marrow-derived MSCs, blood, and other cells obtained from any biological material, including urine, synovial fluid, dental tissues, hair, and other tissues. The pioneering research involving the transfer of the classical OSKM quartet of Yamanaka's embryonic transcription factors including Octamer-binding transcription factor-3/4 (Oct 3/4/), sex-determining region Y-box 2 (Sox2), Kruppel-like factor-4 (Klf4), and c-Myc using retroviral vectors to mouse and human fibroblasts led to generation and expansion of iPSC in culture (Takahashi and Yamanaka in 2006; Takahashi et al. 2007). This discovery was rewarded by 2012 Nobel Prize for both Gurdon and Yamanaka "for the discovery that mature cells can be reprogrammed to become pluripotent" (Colman 2013; Campbell et al. 1996). Reprogramming of somatic cells is a challenging and technically demanding process with low efficiency. The dramatic advancements in the reprogramming protocols were achieved by the discovery of alternative transcription factors and their combinations and by using more efficient and/or non-integrating safer viral vectors (e.g., non-integrating Sendai RNA virus), nonviral transfection methods, the use of small molecules, and their combinations to increase the reprogramming efficacy or to eliminate the need for genetic manipulations (Takahashi and Yamanaka 2016; Fusaki et al. 2009; Grob et al. 2013). Also, rising interest and experience in directed differentiation/trans-differentiation techniques also helped a better understanding of reprogramming mechanisms and the establishment of new protocols.

Undifferentiated self-renewal potential of iPSCs can be exploited for their in vitro expansion akin to the embryonic stem cells (ESCs). The reprogrammed cells in culture form iPSC colonies similar to the ESCs and show pluripotent characteristics. This is confirmed by the expression of intrinsic pluripotency markers of the reprogrammed cells (i.e., Oct4, Sox2, SSEA-1, Nanog) that can be detected by molecular and/or flow cytometric techniques, immunofluorescence, RT-PCR, and Western blotting. Additionally, differentiation potential of iPSCs into cells from the three germ cell layers, i.e., endoderm, mesoderm, and ectoderm, is confirmed either by the demonstration of teratoma formation upon injection to immunocompromised mice, embryoid body formation, or in vitro/in vivo differentiation assays. Alternatively, global gene expression profile assays can be used for verification of the molecular signature of pluripotency (PluriTest-Compatible Prime View Assay, Thermo Fisher Scientific, USA). Another TaqMan hPSC ScoreCard assay (Thermo Fisher, USA) has been developed to evaluate the pluripotency and expression signatures that define the differentiation potential of iPSCs (Bock et al. 2011; Tsankov et al. 2015).

13.10.2 Banking of iPSCs

The use of standardized assays (e.g., Pluritest, ScoreCard) for characterization and quality testing is particularly important in the banking of iPSCs. In the last decade, several banks or initiatives for research-grade iPSC banking have been established and started collecting biological samples to generate a large number of human iPSCs from different diseases. The opportunities provided by iPSC technology in disease

modeling, drug research, regenerative medicine, and personalized cell and gene therapies have led to an understanding of the requirement of iPSC banking for both scientific research purposes and for clinical use. The collection of biological materials from patients with different diseases, isolation, and purification of the cells from the biological samples, their reprogramming and storage in biobanks are important for the pharmaceutical industry to ensure the supply of well-characterized cells for R&D activities (Stacey 2017; Kim et al. 2019a, b; Ching-Ying et al. 2019).

Generation and characterization of iPSCs are a very expensive, labor-intensive, and technically challenging procedure that necessitates daily manipulation of cultures for medium change and assessment, characterization, authentication, and/or quality testing. However, the use of iPSCs is not without risk of chromosomal instability and tumorigenesis incurred by the possible insertional mutagenesis due to the viral vector delivery-based protocols or epigenetic alterations (Yu et al. 2009; Sadelain et al. 2011; Ahmed et al. 2011b; Yoshihara et al. 2017; Kanchan et al. 2020). Therefore, the real significance of iPSC banks is to serve as a source of high-quality cells to researchers rather than indulging in time-intensive and financially burdening activity of iPSCs generation in their laboratory facilities. This will allow the researchers to perform more efficient and productive research.

In parallel with the rapidly expanding iPSC research activities and promising results, clinical scientists have become interested in the therapeutic use of iPSC-derived cell products for the regeneration of severely damaged organ functions. Patient-derived fibroblasts were used in vitro to derive iPSCs, which were then differentiated toward the cells of interest (e.g., retinal pigment epithelial cells for age-related macular degeneration) (Mandai et al. 2017). Although autologous iPSC generation and their subsequent differentiation toward cells of interest are advantageous for the prevention of immune reaction, it is a very expensive and lengthy procedure that may consume more than 3–6 months for each patient. These factors have paved the way for the establishment of clinical-grade iPSC banks for their allogeneic use. To prevent immune rejection of the allogeneic cells, preparation of iPSCs from HLA homozygous donors or the development of HLA manipulation techniques to achieve universal donor cells are being emphasized for allobanking practices. Several centers and organizations in developed countries have established iPSC banks operating under current GMP conditions and taken initiatives to establish international groups (International Stem Cell Banking Initiative, ISCBI) to ensure safety and efficacy of the final cell therapy products. Furthermore, this arrangement has also helped to quality assure of the source materials of the primary donor cells (Barry et al. 2015; Kim et al. 2019a, b, 2017; Shariatzadeh et al. 2020). Quality tests for clinical applications are extensive and therefore need high-throughput assays. In addition to the standard sterility and mycoplasma testing, mRNA expression analyses to identify stemness/pluripotency markers, chromosomal integrity testing with G-banding, or an high-throughput single nucleotide polymorphism (SNP) microarray to identify variations from the donor genome, genotyping of iPSC lines to compare donor as quality control (QC) testing and PCR analysis to detect residual plasmids (> passage 5) to confirm the removal of reprogramming transgenes are performed in active centers (Stacey et al. 2019).

13.11 Further Developments in the iPSCs Field

13.11.1 Disease Modeling and the Next-Generation Technologies with iPSCs

Disease modeling enables the establishment of in vitro systems mimicking the in vivo microenvironment for investigation of disease pathophysiology, the discovery of biomolecules and drug targets, evaluation of efficacy and toxicity of drugs, therapeutic agents/strategies toward a cure, particularly in human genetic diseases and in patients with unmet medical needs. Specific cell types derived by directed differentiation of iPSCs toward tissue(s) of interest provides the opportunity to establish organ-like 3D functional structures, organ-on-a-chip microfluidic systems by the use of advanced engineering strategies, organoids, and eventually sophisticated organs suitable for transplantation purposes (Ramme et al. 2019; Clevers et al. 2017; Lancaster et al. 2013; Kim et al. 2020; Bredenoord et al. 2017). Organ-on-a-chip technology combines microfluidics, iPSCs, and tissue engineering in a micro-device that enables 3D self-organizing tissue production. This technology is suitable for the prediction of drug effects on different organs/tissues, including the liver, lung, heart, and bone marrow. By iPSC technology, all cell types of the desired tissue/organ may be generated in vitro from a limited number of cells; the influence of genetic modifications by gene-editing tools such as CRISPR-Cas9 can be investigated, and in vivo stress conditions may be mimicked by exposing the cells carrying the patient's genetic material to different stressors to estimate/predict the in vivo response to the environmental factors (Ran et al. 2013; Kumar et al. 2012).Therefore, iPSC technology has been a major step in the advancement of personalized therapies.

With these major advantages, iPSC technology appears to diminish the need for animal studies for disease modeling. However, data derived from in vitro models are not sufficient to obtain approval for clinical trials. Therefore, preclinical animal studies to determine the pharmacokinetics, dosage,

and toxicity of drug candidates are still invaluable when considering the limited number of patients in clinical trials, particularly in case of rare diseases. The generation of valuable data in optimized in vitro systems will be useful in decreasing the number of animals used in preclinical studies.

iPSC technology is also used in some human cancer models in experimental animals for the establishment of patient-derived xenografts (PDXs) using immunodeficient or humanized mouse (Murayama and Gotoh 2019). PDXs that are established by the direct transfer of human tumors are more efficient than using cell lines. These PDX models provide opportunities for better management of cancer by simulating human tumor biology and evaluating their efficacy in animals. By preserving the original features of patient tumors, these models study the sensitivity of chemotherapeutic agents in animals. Cancer cells have been suggested as more refractory to reprogramming than the normal cells due to their genetic alterations. Therefore derivation of iPSCs from malignant cells is challenging and depends upon the type of cancer (Kumano et al. 2012; Kotini et al. 2015; Papapetrou 2016; Zhu et al. 2018). Myeloid malignancies such as myeloproliferative neoplasms, chronic myeloid leukemia, polycythemia vera, primary myelofibrosis, myelodysplastic syndromes, and juvenile myelomonocytic leukemia appear to be more suitable for iPSC generation for use in PDX models.

13.11.2 Organoid Research with iPSCs: A Step Toward Organoid Medicine

iPSCs can be used to mimic organ structure and function in a 3D culture system by providing multiple cell types of the organ through recapitulation of developmental organogenesis program and leading to a self-organized organ/tissue, called as organoids or the in vitro miniature organs. Organoids can be also be derived by direct culture of tissues obtained from biopsy/surgery specimens (e.g., mini-gut organoid) embedded into an extracellular matrix (commonly used Matrigel) (Clevers et al. 2017; Lancaster et al. 2013; Kim et al. 2020; Bredenood et al. 2017). However, in some organs or a tissue such as the brain, accessibility remains problematic, and hence, iPSCs are used for in vitro generation of organoids. These "mini-brains" provide a 3D functional platform representing several different brain regions (e.g., midbrain) to study neurological development and disease processes of the human nervous system. During the initial studies, cerebral organoids were established from microcephaly patient-derived iPSCs, and small-size organoids were generated mimicking patient's condition (Lancaster et al. 2013). This system provided a platform to study the effects of the Zika virus on brain size (Mesci et al. 2018). In recent studies, autism spectrum disorders have been modeled by organoid techniques, and by functional studies, candidate gene FOXG1 was identified (Mariani et al. 2015).

Tumor organoids are increasingly being used to study tumorigenesis and tumor biology. Comparison of organoid cultures has been shown to possess similar characteristics as the tissue of origin as documented by mass cytometry in single-cell studies (Drost and Clevers 2018). The current developments in organoid research are heading toward organoid medicine in the near future, perhaps in less than years. The use of organoids in the clinic may be suggested as a bridging therapy before transplantation. At present, the direct clinical application of organoids is a very demanding issue. Vascularization problem, particularly as the organoid size becomes bigger, remains an unresolved issue (Grebenyuk and Ranga 2019). The risk of malignant transformation is a major concern. The presence of animal-derived matrix (e.g., Matrigel) or growth factors may carry infection risk as well as may cause immunological reactions in the recipient. However, organoids are promising structures for use in personalized medicine by drug/small-molecule screening high-throughput systems (Yin et al. 2016).

13.11.3 Drug Repurposing/Repositioning with iPSCs for Fast-Track Clinical Trials

iPSCs generated from patient biological samples, carrying the patient's genetic background, are a perfect tool for personalized medicine. Drug/small-molecule screen on iPSC-derived differentiated cell types by high-throughput assays using large chemical libraries have become a rewarding area leading to potential new medical indications for the FDA or other regulatory agency-approved drugs or novel discovery. These studies are particularly useful to screen candidate molecules on cell and tissues with difficult accessibility, i.e., cardiomyocytes, neurons, and hepatocytes. In vitro disease models using patient-derived iPSCs provide information about the relationship between genotype, phenotype, and drug response and study the bioactivity and toxicity of the drugs in early clinical stages of drug development. Combining the data from the patients with the experimental data from in vitro cell-based studies may provide in-depth information about the drug effect and its feasibility in treating a disease condition. The preclinical safety assessment and tests for efficacy tests using cell-based assays for novel lead compounds and drug candidates can be established in patient-derived iPSCs, control iPSCs, and genetically corrected iPSCs from the patients (isogenic lines) (Hinz et al. 2019).

Recent drug repurposing studies on iPSCs-derived cell types have revealed many exciting achievements that have led to initiation of clinical trials for new indications. One of

the first studies was performed on familial dysautonomia (Lee et al. 2009). More than 6000 candidate compounds were screened on iPSC-derived neural crest progenitors to ascertain the transcription of IKBKAP gene expression. Initially eight and then one small molecule were identified as the lead molecule. In another study on fibrodysplasia ossificans progressiva, rapamycin has been found as a promising therapeutic compound to prevent the development of ossification in the patients (Taoka et al. 2018). Being a progressive neurodegenerative disorder, amyotrophic lateral sclerosis (ALS) is a disease necessitating drug screening studies. The antiepileptic drug ezogabine was effective in an iPSCs-based drug repurposing model on motor neurons from ALS patients, and a clinical trial has been initiated. There are several other diseases and conditions that produced promising results using this technology (Shi et al. 2017; Kim et al. 2019a, b).

As an example of drug repositioning, iPSCs generated from a myeloproliferative disorder, CMML, were used in a humanized mouse model, and small molecules and a bisphosphonate drug, clodronate, have been identified as potential drugs for treatment. In this study, the authors have shown that liposomalization of clodronate enhanced its effectiveness and suggests this variation of clodronate to be a candidate repositioned drug (Taoka et al. 2018).

13.11.4 Clinical Use of iPSCs in Regenerative Medicine

At present, due to their extensive self-renewal and pluripotency, iPSCs are being regarded as the most promising cell source in regenerative medicine for cell-based therapy and in tissue engineering (Ibrahim et al. 2016). Despite the significant risk of tumorigenesis and long-term cell culture period, the iPSC-based cell therapies have progressed rapidly, however, with a meticulous selection of candidate diseases and application of stringent quality control measures.

Similarly, despite the autologous availability of iPSCs and their derivative cells for cell replacement therapy that give them immunological advantage of nonrecognition, majority of clinical trials are using allogeneic cells due to their off-the-shelf availability and logistic advantage. In the allogeneic settings, there is a need for long-term immunosuppression to obviate the risk of immunological rejection (Ching-Ying et al. 2019). This issue has been addressed by the establishment of iPSC banks for storage of HLA homozygous cell lines to cover a reasonable percentage of the population, which is more feasible in a population like Japan where common haplotypes are frequently observed. CiRA Centre in Kyoto has established a clinical-grade iPSC bank from the healthy HLA homozygous donors. This center is dedicated for "iPSCs stock development projects for regenerative medicine" (Shi et al. 2017; Umekage et al. 2019; Huang et al. 2019b). iPSC lines generated from bone marrow and cord blood HLA homozygous donors (n=7) of the most frequent four haplotypes provided by the Japanese Red Cross Society are suggested to cover 40% of the Japanese population. Since 2015, these homozygous iPS cell lines referred to as the super donor iPSCs stock are being used in the clinical trials. To cover very high populations' genome-edited second generation, iPSC stocks (at HLA locus) are being prepared for the near future use (Xu et al. 2019). There are also some ongoing studies to optimize iPSCs and differentiated cell derivation methods to significantly cut the cost and time for autologous use for personalized therapies which is a great advantage enabling gene correction, especially in the genetic diseases, by using state-of-the art genome-editing technologies (Papasavva et al. 2019).

Here are some examples of interventional clinical trials that define the administration of iPSC-derived cells to patients. The majority of the ongoing approved or in-preparation clinical studies are originating from Japan Keio University, CiRA, RIKEN, and Osaka University play roles as clinical research centers to promote iPSC-based cell therapy. Age-related macular degeneration from Japan RIKEN Centre initially started with the use of autologous cells and then followed with allogeneic retinal pigment epithelial cells derived from iPSCs from HLA-matched healthy donors. Studies from Osaka University consist of the use of allogeneic corneal stem cells for limbal stem cell deficiency, and cardiomyocytes derived from allogeneic donors for heart failure. Sawa and colleagues from Osaka University are conducting clinical trials to test allogeneic sheets of tissue derived from iPSCs for the treatment of three patients with heart disease. Similarly, a phase I/II study is underway using allogeneic iPSCs-derived dopamine precursor cells for Parkinson's disease patients, while another one study is using autologous iPSC-derived platelets for patients with aplastic anemia. Retinitis pigmentosa, spinal cord injury, arthritic disorder, and iPSC-derived platelets for transfusion are other diseases/conditions that have been approved in Japan, and clinical trials have just started or are about to start. Type I diabetes mellitus and immunotherapy for cancer are the other conditions that are in the process of application for approval. Recently the use of iPSC-derived natural killer cells in different types of cancers was approved in the USA, at Minnesota University, in collaboration with Fate Therapeutics. Another approval for a clinical trial is from Cynata Therapeutics, Australia, which involves the use of iPSC-derived MSCs for graft-versus-host disease, and the study has proceeded to phase II trials. At present, it is premature to conclude about the results of these studies (Mandai et al. 2017; Shi et al. 2017, Karagiannis et al. 2017; McNeish et al. 2015; Attuale et al. 2018; Trounson and DeWitt 2016; Norbnop et al. 2020; Nagoshi et al. 2019; Shin et al. 2020; Bloor et al. 2018).

13.12 Future Perspective and Conclusions

IPSC technology brought many opportunities and expectations to medicine and biotechnology. But, there are drawbacks. The main challenge, not yet been tackled, is in generation of high-quality reprogrammed cells. Reprogramming and culturing period is long, technically demanding, time consuming, involves heavy workload necessitating daily care/intervention, and has low efficiency. Additionally, the requirement for extensive characterization and quality control tests makes this technology a high-cost one. Establishment of research grade iPSC banks is an important step toward more efficient drug screening and disease modeling. Through this opportunity, good quality and standardized iPSCs generated from a wide range of diseases, including very rare ones, are made available to researchers, saving their time and budget. On the other hand, it may be necessary to establish autologous iPSC lines for personalized research. In genetic diseases, this provides an opportunity to perform functional studies and evaluate phenotypic, genotypic variations. The use of heterozygote carriers and isogenic (gene edited/gene corrected) cell lines as controls is helpful in assessment of mutation-specific effects. Gene editing has become a revolutionized and almost standard application in the iPSC field by CRISPR-Cas9 technique but has limitations as well, including off-target effects, immunogenicity, and suboptimal efficacy. Modeling of complex diseases poses difficulties due to their multifactorial nature and involvement of multiple genes and environmental factors.

The clinical use of iPSCs (derived cells) in humans, although very promising in regenerative medicine, is a very tough issue. The main challenge is the tumorigenicity risk of iPSCs (and derived cells). There are technical difficulties as well. Preparation of the cells under GMP conditions for clinic use in humans requires a featured infrastructure and operation. The processing takes long time and is very costly, particularly for generation of autologous iPSCs. HLA homozygous donor banks and HLA-gene-edited clinically grade allogeneic cell lines are being generated to supply cells to cover large populations. Important issues waiting to be solved in iPSC banking are informed consent issue, requirement of extended consent forms, covering, e.g., commercial use, reconsenting issues after the age of 18 years, ownership issues of processed cell lines.

In spite of these challenging and demanding factors, iPSC basic and translational research offers many opportunities for advancement of medicine, science, and biotechnology and drug industry. Directed differentiation of somatic cells, in vivo reprogramming, activation of endogenous stem/progenitor cells, and in vivo gene editing are additional reprogramming topics of interest. A flow diagram of iPSCs in R&D and highlights toward clinical translation are summarized in Fig. 13.1. The iPSCs field is one that involves many

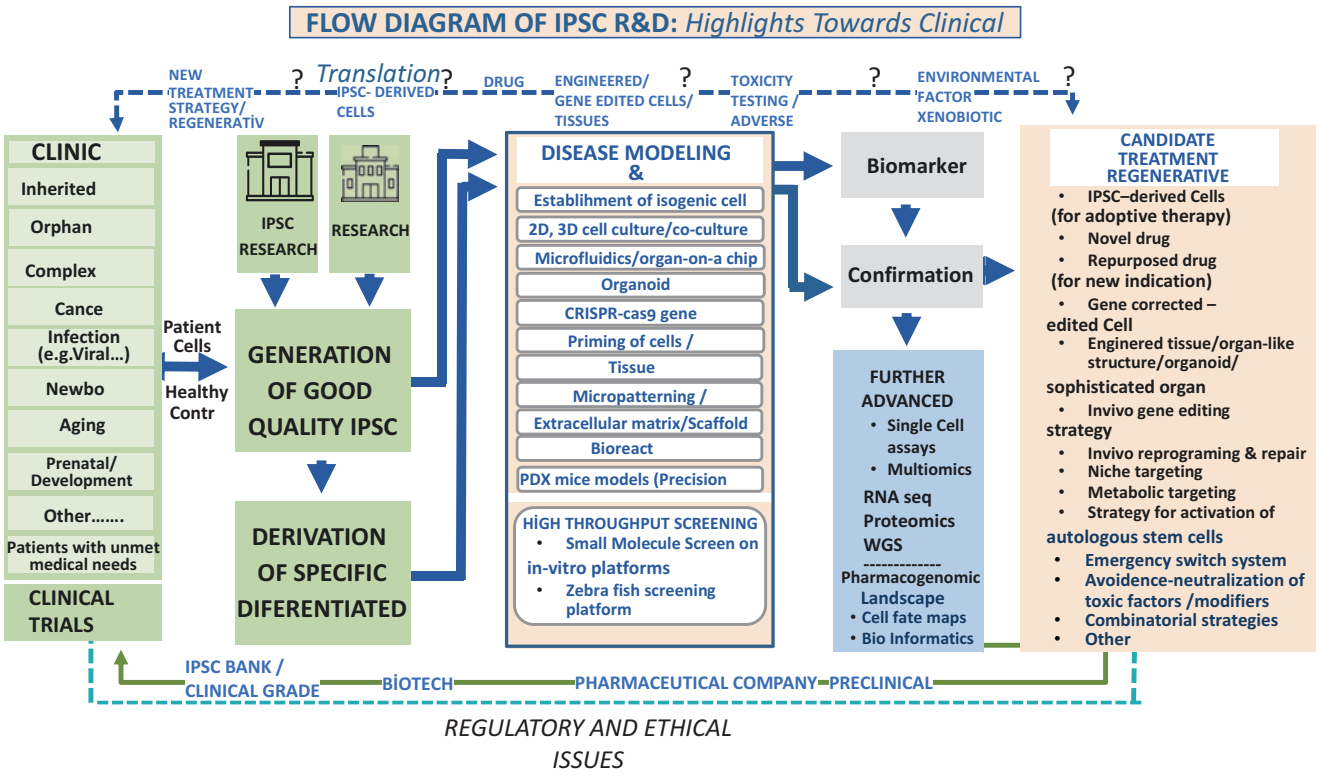

Fig. 13.1 Flow diagram of IPSC R&D: highlights towards clinical translation

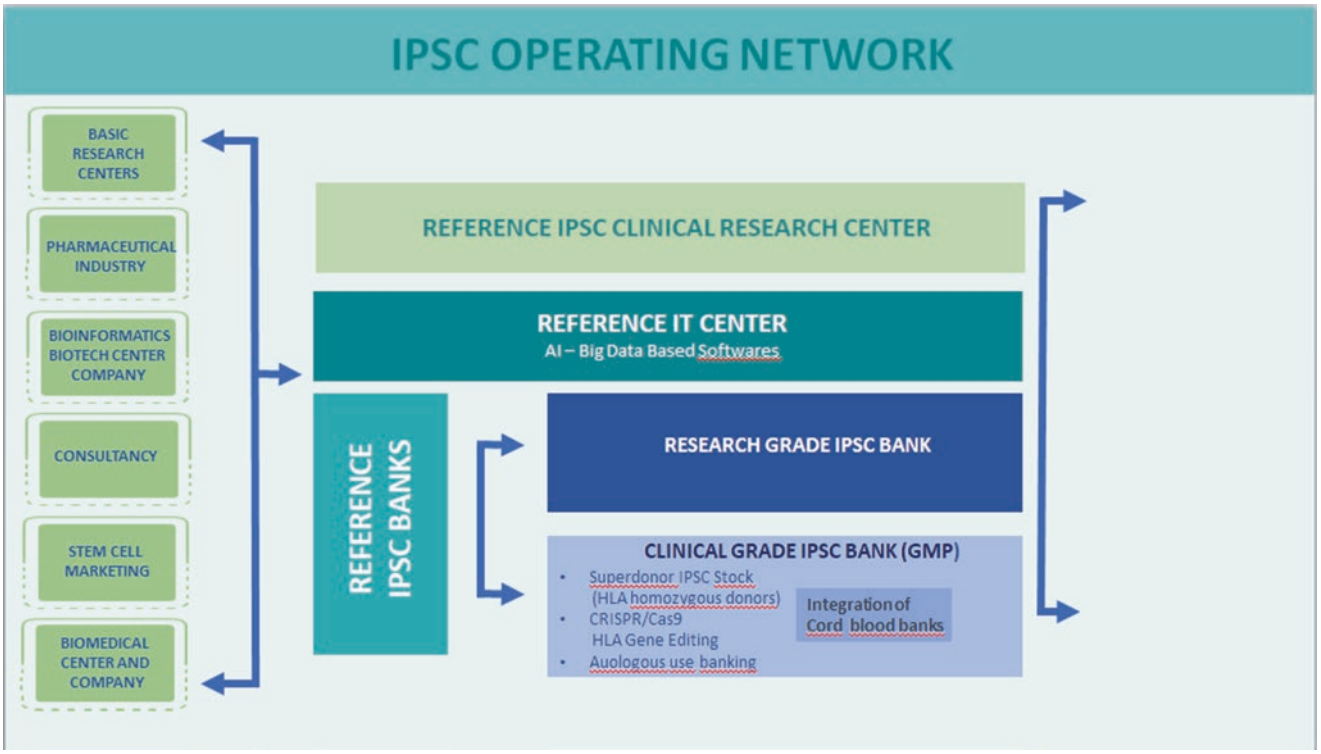

Fig. 13.2 Schematic representation of IPSCs operating network

sectors, disciplines, and institutions, all getting organized for next-generation management of patients. The iPSC operating network/the macroenvironment is summarized in Fig. 13.2.

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14 Maturity of Pluripotent Stem Cell-Derived Cardiomyocytes and Future Perspectives for Regenerative Medicine

Nawin Chanthra and Hideki Uosaki

Abbreviations

AP	Action potential
ARVD/C	Arrhythmogenic right ventricular dysplasia/cardiomyopathy
BMP	Bone morphogenetic protein
Cx43	Connexin 43
CDK	Cyclin-dependent kinase
DCM	Dilated cardiomyopathy
ES cells	Embryonic stem cells
ECMs	Extracellular matrices
FAO	Fatty acid β-oxidation
hCMPs	Human cardiac muscle patches
hERG	Human ether-a-go-go-related gene
HCN4	Hyperpolarization-activated cyclic nucleotide-gated channel 4
iPS cells	Induced pluripotent stem cells
LTCCs	L-type calcium channels
MI	Myocardial infraction
MHC	Myosin heavy chain
NRVM	Neonatal rat ventricular myocytes
PKP2	Plakophilin-2
PSCs	Pluripotent stem cells
PSC-CMs	Pluripotent stem cell-derived cardiomyocytes
RBM20	RNA-binding motif protein 20
RYRs	Ryanodine receptors
SR	Sarcoplasmic reticulum
SERCA2a	Sarcoplasmic/endoplasmic reticulum calcium ATPase 2a
NCX	Sodium-calcium exchanger
T-tubules	Transverse tubules

N. Chanthra · H. Uosaki (✉)
Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan
e-mail: uosaki.hideki@jichi.ac.jp

14.1 Introduction

Pluripotent stem cells (PSCs) can be classified into two types—embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. ES cells are derived from the inner cell mass of a blastocyst (Evans and Kaufman 1981; Thomson et al. 1998). iPS cells can be obtained by inducing transcription factors (e.g., Oct3/4, Sox2, Klf4, and c-Myc) into somatic cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007). Both types of PSCs can be maintained in a pluripotent state and expanded unlimitedly in a dish. Thus, PSCs provide an unlimited cell source for fundamental researches and patient-specific cell therapies.

A heart is the first functional organ during vertebrate development (Miquerol and Kelly 2013). Initially, gastrulation is driven by gradients of signaling proteins. For example, bone morphogenetic protein (BMP) is required for extraembryonic ectoderm, and primitive streak formation is driven via Wnt signaling. With the ingression through the primitive streak, epiblast cells fate to mesoderm and endoderm along the anterior-posterior axis (Tam and Loebel 2007). A part of newly formed mesoderm cells migrates from the primitive streak to the lateral side of an embryo. Then, the cells further migrate toward the anterior side of the embryo to commit to the cardiac lineages and form heart fields (Abu-Issa and Kirby 2007).

With the understandings of dynamic changes of the vital signaling factors during heart development, presently, cardiomyocytes can be successfully differentiated from ES and iPS cells, called pluripotent stem cell-derived cardiomyocytes (PSC-CMs), in adherent (Lian et al. 2012, 2015; Minami et al. 2012; Burridge et al. 2014) and suspension culture (Yang et al. 2008; Chen et al. 2015; Kempf et al. 2015). Recapitulating heart development, treatments of Wnt modulators, activin A, and BMP4 at the specific concentrations and the particular time windows (Kattman et al. 2011; Friedman et al. 2018) are critical to inducing efficient

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differentiation of PSC-CMs (Galdos et al. 2017). Spontaneously beating cardiomyocytes can be observed right after cardiac differentiation for 7 days (Uosaki et al. 2011; Chanthra et al. 2020). However, these conventional methods lack physical and environmental cues, resulting in heterogeneous cell populations (Karbassi et al. 2020). Diverse purification methods have been established, such as sorting the cells with specific antibodies for cardiac surface proteins, to obtain a highly purified population of cardiomyocytes (Dubois et al. 2011; Uosaki et al. 2011; Skelton et al. 2017). Other methods are culturing the PSC-CMs with glucose/glutamine depletion with lactate supplement (Tohyama et al. 2013, 2016) and using cardiac promotor-driven antibiotic resistance gene cassette (Yamanaka et al. 2008). In this chapter, we discuss PSC-CMs in the context of their maturity, current methods to enhance cardiomyocyte maturation, and their future perspectives for regenerative medicine.

14.2 Difference Between the Characteristics of Immature and Mature Cardiomyocytes

Although PSC-CMs hold a great promise for a wide range of medical applications, it is widely known that PSC-CMs resemble fetal cardiomyocytes in morphological, physiological, and functional features rather than adult ones. Different phenotypes between immature (fetal-type) and mature (adult-type) cardiomyocytes are showed in Fig. 14.1a.

14.2.1 Morphology

During heart development, cardiac muscle cells undergo a sophisticated series of structural changes and eventually turn to adult phenotypes (Fig. 14.1b). Adult cardiomyocytes have a rod-like shape of approximately 20μm in width, 150μm in length, and 15μm in height (Gerdes et al. 1992). The morphology of adult cardiomyocytes provides a structural framework of the cells that facilitates the different cell functions. For instance, an adult cardiomyocyte assembles complex ultrastructures of transverse tubules (T-tubules) and sarcoplasmic reticulum (SR) along the Z-lines of sarcomeres that is crucial for calcium handling and efficient cardiac contractility (Bers 2002). Adult cardiomyocytes also have higher membrane capacitance than smaller cells because of their larger surface area (Spach et al. 2000, 2004). The elongated adult cardiomyocytes accompany by long myofibrils mediating cell contractility (McCain and Parker 2011). In contrast, culturing PSC-CMs in a dish is insufficient to support physiological hypertrophy, which is observed in vivo during the maturation process, because physical and environmental cues are absent (Lundy et al. 2013; McCain et al. 2014; Dai et al. 2017). As such, PSC-CMs tend to be round in shape and small in size (approximately 10μm in width, 30μm in length, and 5μm in height) even after prolonged culture (Snir et al. 2003). These dissimilarities between immature and adult cardiomyocytes are the critical roadblocks that limit the potential of using PSC-CMs for disease modeling and drug discovery.

14.2.2 Contractile Apparatus

A sarcomere is an integral unit for cardiac contraction. Adult cardiomyocytes do not beat by spontaneously; however, upon receiving a stimulus, they respond by providing greater contraction force, upstroke, and conduction velocities (Karakikes et al. 2015; Denning et al. 2016). Indeed, PSC-CMs generate lower conduction and upstroke velocities while beat spontaneously (Karakikes et al. 2015; Denning et al. 2016). Sarcomeres of adult cardiomyocytes are very organized, well-aligned, and consist of A-band, I-band, H-zone, Z-line, and M-line, while those of immature cardiomyocytes lack these structural features (Fig. 14.1c) (Denning et al. 2016). In adult human cardiomyocytes, sarcomere length is ~2.2μm long, whereas that of human PSC-CMs is shorter (~1.7μm) and poorly organized (Bird et al. 2003; Mollova et al. 2013). The organization and formation of sarcomere structure depend on the expressions of various sarcomere proteins, i.e., troponin complex, myosin, and titin, of which different isoforms express between the immature and adult cardiomyocytes (Bedada et al. 2014; Iorga et al. 2017; Zuppinger et al. 2017). For troponin complex, cardiomyocytes express slow skeletal muscle isoform (ssTnI or Tnni1) during fetal development and switch to express cardiac isoform (cTnI or Tnni3) after birth (Sabry and Dhoot 1989; Siedner et al. 2003). Two different isoforms of myosin heavy chain (MHC) are also alternately expressed during heart development. In mice, β-MHC (encoded by *Myh7*) is the predominant isoform in fetal cardiomyocytes, while α-MHC (encoded by *Myh6*) is the adult isoform (Lompre et al. 1979). Conversely, α-MHC is the fetal isoform, and β-MHC is the adult isoform in human hearts (Yang et al. 2014a; Jiang et al. 2018). Moreover, fetal cardiomyocytes primarily express the N2BA isoform of titin, and after birth, N2B becomes the major isoform (Hinson et al. 2015).

14.2.3 Calcium Handling

Calcium ions play an essential role in cardiomyocyte contraction. During action potential (AP) propagation along the sarcolemma and T-tubules of adult cardiomyocytes, L-type calcium channels (LTCCs) are activated thus facilitating an influx of calcium ions into the myocyte cytosol (Bers 2002).

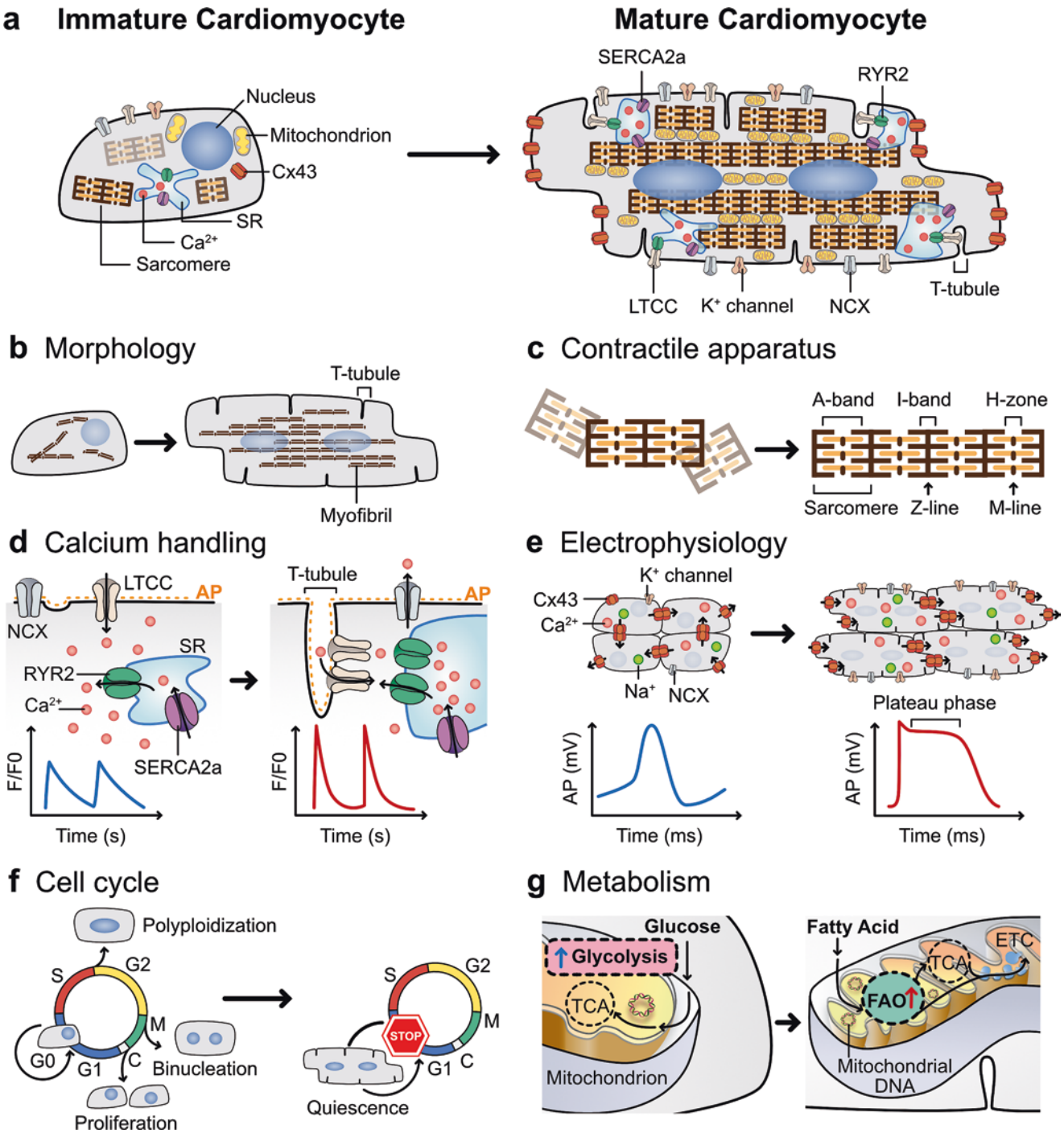

Fig. 14.1 Different characteristics between immature and mature cardiomyocytes (**a**) During heart development, cardiomyocytes undergo sophisticated changes in both structure and function. SR; sarcomere, Cx43; connexin 43, SERCA2a; sarcoplasmic/endoplasmic reticulum calcium ATPase 2a, RYR2; ryanodine receptor 2, LTCC; L-type calcium channel, NCX; sodium-calcium exchanger. The major hallmarks for cardiomyocyte maturation are summarized in parts (**b–g**). (**b**) Mature cardiomyocytes are increased cell size and become anisotropic, whereas immature cells are round or polygonal. (**c**) Mature cardiomyocytes show organized sarcomere patterns and increase expressions of mature myofibril protein isoforms, resulting in efficient contraction. (**d**) Mature cardiomyocytes have effective calcium handling than immature cells due to well development of T-tubules, SR which is calcium storage, and also the increased expressions of calcium handling proteins. AP; action potential. (**e**) Adult heart show polarization of the Cx43, gap junction protein, in the intercalated discs, but Cx43 protein are circumferentially distributed in immature cells, resulting in different patterns of action potentials. (**f**) Immature cardiomyocytes have highly proliferative potential, whereas mature cells are quiescent. M; mitosis, C; cytokinesis. (**g**) Metabolic substrates are switched from glucose to fatty acid during cardiomyocytes become more mature. FAO; fatty acid β-oxidation, TCA; tricarboxylic acid cycle, ETC; electron transport chain

The calcium ions initiate calcium-induced calcium release from SR through ryanodine receptors (RYRs) (Bers 2002). Calcium ions bind to troponin C to induce conformational changes of the troponin complex, which allows myosin binding to actin and leads to cardiac muscle contraction. Once SR uptakes calcium ions back via sarcoplasmic/endoplasmic reticulum calcium ATPase 2a (SERCA2a), cardiac muscle turns to the relaxation phase. In adult cardiomyocytes, calcium handling occurs efficiently because of the proximity of LTCCs to RYRs on the SR. By contrast, the lack of T-tubules results in the spatial uncoupling of the LTCCs and RYRs, leading to low calcium dynamics in PSC-CMs (Fig. 14.1d) (Bers 2002; Kane et al. 2015). Furthermore, the function of SR for calcium storage is also critical for calcium dynamics. Previous studies have demonstrated that PSC-CMs displayed dissimilarity in the function of SR compared to adult cardiomyocytes in several animal models such as mice (Liu et al. 2002; Janowski et al. 2006), rat (Tanaka and Shigenobu 1989; Escobar et al. 2004), and rabbit (Huang et al. 2008). An efficient calcium handling property is one of the most common parameters that is characterized in PSC-CMs. However, their expression of calcium handling proteins is much lower than adult cardiomyocytes (Liu et al. 2007; Satin et al. 2008; Zhu et al. 2009; Germanguz et al. 2011; Itzhaki et al. 2011). For example, their LTCC β-subunit Ca_v β2 and RYR expression levels were 20-fold and 1000-fold lower (Satin et al. 2008; Tanaka and Shigenobu 1989; Liu et al. 2002; Escobar et al. 2004; Janowski et al. 2006; Huang et al. 2008; Satin et al. 2008). In contrast, transduction of calsequestrin (encoded by Casq2) to PSC-CMs increases peak amplitude, upstroke velocity, and time to decay, implying the role of calsequestrin in the functional maturation of calcium handling in PSC-CMs (Liu et al. 2009). Moreover, calsequestrin expression supports the presence of functional SR-depending calcium handling (Satin et al. 2008; Germanguz et al. 2011; Itzhaki et al. 2011).

14.2.4 Electrophysiology

AP propagation along the sarcolemma mediates dynamic changes inward and outward movement of ions into the cardiomyocytes. These ionic changes are responsible for the contractility of the heart. While an isolated single adult ventricular cardiomyocyte is mostly quiescent in electrophysiology, depolarization of a neighboring cell in vivo or their electrical stimulation ex vivo efficiently induces beating of the cells. Conversely, PSC-CMs have a higher automaticity than mature cardiomyocytes that is partly attributed to the high levels of hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4) (Carmeliet 2019). The resting membrane potential of adult cardiomyocytes is approximately −85 mV (Liu et al. 2010), but immature PSC-CMs is less negative (~−50 to 60 mV) due to the low level of inward rectifier potassium current (I_{K1}) mediated by inward rectifying potassium channels (Kir) 2.1 and Kir2.2 (Goversen et al. 2018). The upstroke velocity of the AP is initiated by the rapid opening of the voltage-gated sodium channels ($Na_v1.5$), which allows sodium influx (I_{Na}) and membrane depolarization (Guo and Pu 2020). PSC-CMs predominantly express fetal isoform of α-subunit of the sodium channels, resulting in slower upstroke velocity (Veerman et al. 2017). Following depolarization, transient outward potassium current (I_{to}) occurs, leading to the transmembrane voltage decrease and presenting a notch shape in the AP of adult cardiomyocytes (Guo and Pu 2020). Membrane depolarization triggers the opening of L-type calcium channels ($Ca_v1.2$) that provides the calcium current ($I_{Ca,L}$) responsible for the plateau phase in mature cardiomyocytes (Fig. 14.1e) (Guo and Pu 2020). Then, the repolarization phase is controlled by the effects of $I_{Ca,L}$ and repolarizing potassium currents including slow delayed-rectifier potassium current (I_{Ks}), rapid delayed-rectifier potassium current (I_{Kr}), and I_{K1} (Guo and Pu 2020). I_{Kr} is mediated through voltage-gated potassium channel Kv11.1, known as KCNH2 or human ether-a-go-go-related gene (hERG), one of the responsible channels for long QT syndrome. In PSC-CMs, the repolarization phase is mainly control by I_{Kr} (Hoekstra et al. 2012; Zhao et al. 2018). Consequently, the membrane potential is rapidly reduced. After $Ca_v1.2$ inactivation, resting membrane potential is reestablished by I_{K1} (Guo and Pu 2020). Moreover, the cell-cell coupling is also necessary for AP propagation. One of the key components in the intercalated disc is a gap-junction protein (connexin 43, Cx43). In immature cardiomyocytes, Cx43 is circumferentially distributed and gradually polarized to the intercalated disc during the process of maturation (Fig. 14.1e) (Scuderi and Butcher 2017).

14.2.5 Cell Cycle

Studies in both mouse and human have shown that embryonic, fetal, and early postnatal cardiomyocytes have high proliferative activity, whereas the adult cardiomyocytes are mostly dormant (Fig. 14.1f) (Soonpaa et al. 1996). In mice, cardiomyocytes' cell-cycle exit occurs within a week during the post-natal life, but the proliferative activity gradually decreases to a low level by the second decade of life in humans (Porrello et al. 2011; Mollova et al. 2013). During cardiomyocyte maturation, the cardiomyocytes become polyploid in most mammals (Hirose et al. 2019). In mouse hearts, cardiomyocytes undergo cell cycle without cytokinesis (C), resulting in binucleation of cardiomyocytes (~90% of the cells contain two diploid nuclei, Fig. 14.1f) (Soonpaa

et al. 1996). By contrast, human cardiomyocytes are predominantly mononuclear (~75%) but achieve polyploidy due to DNA replication without karyokinesis (Brodsky et al. 1994; Olivetti et al. 1996). Cardiomyocyte polyploidization negatively correlates with cell cycle arrest. Overexpression of the positive regulators for cell cycle such as cyclin Ds (Zhu et al. 2018) and combined the overexpression of cyclin-dependent kinase 1 (CDK1), CDK4, cyclin B, and cyclin D efficiently stimulated adult cardiomyocyte proliferation (Mohamed et al. 2018), both of which resulted in smaller cardiomyocytes and more mononuclear diploid cells. Moreover, some of the adult cardiomyocytes which still retain proliferative potential are mononuclear diploid cells (Mollova et al. 2013; Bergmann et al. 2015; Patterson et al. 2017). On the other hand, cells' ploidy is positively correlated with the cell size (Frawley and Orr-Weaver 2015). Forced polyploidization has been shown to increase physiological hypertrophy of cardiomyocytes (González-Rosa et al. 2018; Liu et al. 2019). At present, though, little is known about the role of polyploidization and its effect on cellular physiology.

14.2.6 Metabolism

After birth, cardiomyocytes produce a high-energy level (ATP) to meet the workload demand and efficient contraction. The primary energy consumers are myosin ATPase and SERCA2a, responsible for cardiac muscle contraction and relaxation, respectively (Hirose et al. 2019). Energy production occurs in mitochondria via fatty acid β-oxidation (FAO, Fig. 14.1g) (Lopaschuk and Jaswal 2010). During cardiomyocyte maturation, mitochondria are increased to 20–30% of cell volume (Schaper et al. 1985). In line with the high number of mitochondria in cardiomyocytes, oxidative capacity is increased, representing to switch in metabolic substrates from glucose to fatty acid (Lopaschuk and Jaswal 2010). To efficiently transport ATP to ATPase in sarcomere and SERCA2a in SR, mitochondria are potentially attached to SR through endoplasmic reticulum-mitochondria contact sites, suggesting the link between mitochondrial location and cardiac function (Seppet et al. 2001). Adult cardiomyocytes also have dense cristae that provide enough space for efficient mitochondrial respiration (Porter et al. 2011; Feric and Radisic 2016). By contrast, fetal cardiomyocytes share similarities of mitochondrial phenotypes to those in PSC-CMs, including the lower number and small size (~5% of cell volume), few and poorly aligned cristae, and exiting in the perinuclear cell (Karbassi et al. 2020). In terms of metabolic activity, both fetal cardiomyocytes and PSC-CMs rely on glycolysis rather than FAO (Kim et al. 2013) (Fig. 14.1g).

14.3 Current Maturation Strategies

Maturation of PSC-CMs arrested at late-embryonic cardiomyocytes after long-term culture in vitro (Uosaki et al. 2015). In contrast, PSC-CMs underwent structural and functional maturation within 2 months when transplanted into a neonatal rat heart (Cho et al. 2017). However, human cardiomyocytes require more than 5 years of maturation in human heart. These results indicated that PSC-CMs possessed the potential undergo maturation in the presence of a conducive and appropriate environment. Subsequently, there are numerous efforts mimicking microenvironments during heart development to enhance the maturation of PSC-CMs as described below.

14.3.1 Prolonged Culture

Fundamentally, cardiomyocyte maturation is a gradual process that requires a long time. Indeed, human neonatal cardiomyocytes develop their adult phenotype about 6−10 years in vivo (Peters et al. 1994), while beating human and mouse PSC-CMs emerge within just 7 days of differentiation (Laflamme and Murry 2011; Uosaki et al. 2015). To imitate the maturation process, PSC-CMs were cultured longer in a dish (Fig. 14.2a). The in vitro culturing of human PSC-CMs for up to 120 days resulted in morphological changes toward adult-like mature cardiomyocytes, increased sarcomere length, cell size, an elongated shape, as well as binucleation (Lundy et al. 2013). Moreover, a detailed sarcomere organization analysis demonstrated that Z and I bands have appeared after culture the PSC-CMs for 30 days. Then, extended culture for 30−90 days resulted in A-bands development, followed by induction of M-bands formation after culture up to 360 days (Kamakura et al. 2013). Transient outward and inward rectifier potassium currents (I_{to1} and I_{k1}) also increased through a prolonged culture (Sartiani et al. 2007). Although the long-term culture of PSC-CMs enhances several aspects of cardiomyocyte maturation, as mentioned above, it is still low-throughput and time-consuming. In order to accelerate cardiomyocyte maturation, diverse maturation strategies have emerged.

14.3.2 Extracellular Matrices (ECMs)

ECMs provide structural support during heart development and contain signaling molecules for transmitting signals between cardiomyocytes and neighboring tissues (Herron et al. 2016). A previous study has demonstrated that culturing neonatal cardiomyocytes on cardiogel, containing cardiac fibroblast, laminin, fibronectin, collagen

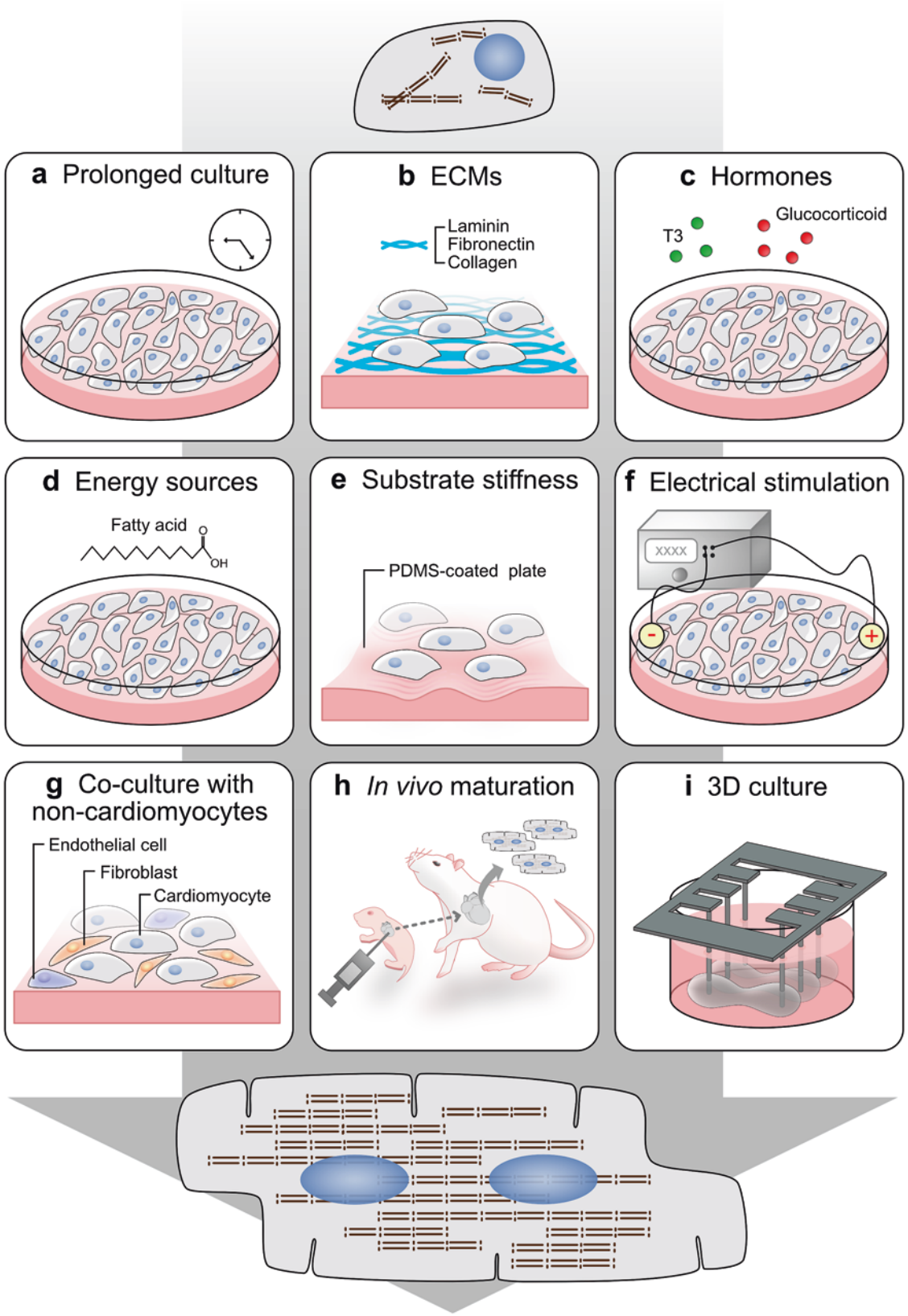

Fig. 14.2 Summary of current methods for promoting cardiomyocyte maturation To enhance cardiomyocyte maturation, several strategies have been developed including (**a**) prolonged culture, (**b**) culturing on ECMs, (**c**) postnatal hormone treatments, (**d**) alterations in metabolic substrates, (**e**) adjusting substrate stiffness, (**f**) electrical stimulation, (**g**) co-culture with non-cardiomyocytes, (**h**) *in vivo* maturation, and (**i**) applying 3D culture system

type I and III, and proteoglycans, exhibited more matured phenotypes such as spontaneous contractility, hypertrophy, and cytoskeleton development faster than two-dimensional (2D) culture system (VanWinkle et al. 1996). Consistently, culturing PSC-CMs on overlaid matrigel consisted of laminin, collagen-type IV, and proteoglycan also improved electrophysiological properties of the treated PSC-CMs (Zhang et al. 2012). In addition to these studies, laminin 511 and 521 were identified from a screening of ECMs to enhance cardiomyocyte maturity through improving several aspects such as increasing sarcomere length and cell size, calcium transient, and also mitochondrial function toward adult phenotypes (Chanthra et al. 2020). These results raise the important roles of ECMs on cardiomyocyte maturation (Fig. 14.2b).

14.3.3 Hormonal Treatments

Postnatal hormones are considered as one of the enhancers for cardiomyocyte maturation (Fig. 14.2c). Specifically, treating PSC-CMs with triiodothyronine (T3) has been shown to increase cell size and elongation, contractility, and sarcomere length compared to nontreatment (Yang et al. 2014b). Furthermore, T3 treatment is effective for several cardiac gene expressions such as α-MHC, titin, and SERCA2a (Krüger et al. 2008; Yang et al. 2014b). T3 even showed a significant increase in mitochondrial activity both maximal respiratory capacity and respiratory reserve capacity (Yang et al. 2014b). In addition to T3 treatment, PSC-CMs treated with dexamethasone, a synthetic glucocorticoid, showed significantly faster calcium decay, increased forces of contraction, and sarcomere length (Kosmidis et al. 2015). A combination of T3 and dexamethasone applied to PSC-CMs also induced T-tubule formation and increased excitation-contraction coupling (Parikh et al. 2017). These results provide important evidence that hormones are essential for cardiomyocyte maturation.

14.3.4 Alternations of Energy Source

Adult cardiomyocytes primarily generate energy by using fatty acids as substrates through FAO, whereas glucose is the main energy source for immature cardiomyocytes (Guo and Pu 2020; Karbassi et al. 2020) (Fig. 14.2d). The metabolic substrate switch from glucose to fatty acids occurs after birth and is considered as a hallmark for cardiomyocyte maturation (Guo and Pu 2020; Karbassi et al. 2020). Cardiac differentiation protocols rely on basal RPMI 1640 media supplemented with B27 that was originally designed for hippocampal neuronal culture (Lian et al. 2012). This media provides excellent support for cardiac differentiation and maintenance of PSC-CMs (Uosaki et al. 2011; Burridge et al. 2014). However, the low level of lipid and high glucose media can promote de novo lipogenesis and suppress FAO (Saggerson 2008; van Weeghel et al. 2018). Glucose-free and lactate-containing media were increasingly used to remove non-cardiomyocyte and enrichment PSC-CMs (Tohyama et al. 2013; Burridge et al. 2014). In addition to the enrichment, supplementation of fatty acids, such as palmitate, oleic acid, linoleic acid, and carnitine, to low-glucose media promotes cardiomyocyte maturation (Correia et al. 2017; Nakano et al. 2017; Horikoshi et al. 2019; Yang et al. 2019). In contrast, culturing PSC-CMs with high glucose media leaves them in an immature state (Nakano et al. 2017). Moreover, a recent study has shown that using metabolic maturation media, which comprises of albumin-bound fatty acid, L-carnitine, taurine, and creatine, improved physiological and electrophysiological properties of PSC-CMs toward adult-like phenotypes (Feyen et al. 2020). Interestingly, PSC-CM models of dilated cardiomyopathy and long-QT syndrome displayed their phenotypes after the in vitro culture in the media, indicating the importance of fatty acid and fidelity of using PSC-CMs for cardiac diseases (Feyen et al. 2020).

14.3.5 Substrate Stiffness

Spatial change of microenvironment generally occurs during heart development, for instance, a collagen accumulation in mice heart. This change, combined with other dynamic changes, results in a threefold increase of myocardium elasticity from embryo to neonatal stage (Jacot et al. 2010), and a twofold increase from neonatal to adult heart (Prakash et al. 1999). Coincidentally, this process appears postnatally with the elevation of blood pressure and the capability of pumping blood by a heart. Therefore, substrate stiffness is one of the microenvironmental factors that have been widely investigated for its effect on cardiomyocyte maturation (Fig. 14.2e). A study demonstrated that cardiomyocytes were well-developed on the optimal substrate of comparable stiffness to native tissue (Bajaj et al. 2010). Consistent with this study, neonatal rat ventricular myocytes (NRVM) plated on collagen-coated polyacrylamide with substrate stiffness similar to native myocardium, 10 kPa, and appeared aligned sarcomere better than stiffer or softer substrates. The treated cells generated greater mechanical force and showed the highest calcium transients with increased SERCA2a expression, on 10 kPa gel (Jacot et al. 2008). Altogether, substrate stiffness affects to physical and functional maturation of cardiomyocytes.

14.3.6 Electrical Stimulation

Cardiomyocytes are subjected continuously to electrical impulses conferring spontaneous rhythmic contraction. Thus, electrical stimulation is expected to promote cardiomyocyte maturation (Fig. 14.2f). Interestingly, a transcriptome analysis of rat cardiomyocytes found that known cardiac-specific genes such as *Myh6*, *Cx43*, and L-type calcium channel (such as *Cacna1c*) were highly upregulated following electrical stimulation. Moreover, NRVM in collagen sponges increased contraction amplitude and improved their alignment after applying electrical stimulation (Radisic et al. 2004). Stimulation of NRVM monolayer improves several maturation aspects of cardiomyocytes, including the expression of a sodium-calcium exchanger (NCX), AP duration, conduction velocity, and even mitochondrial activity (Xia et al. 1997; Sathaye et al. 2006). Although electrical stimulation has impacted cardiomyocyte maturation, little is known how it impacts, and it is often combined with other maturation strategies (Nunes et al. 2013). Thus, several confounding factors, i.e., ECM interaction, may interfere with the effects of electrical stimulation (You et al. 2011).

14.3.7 Co-culture with Non-cardiomyocytes

A rat heart consists of 30% cardiomyocytes, 6% endothelial cells (ECs), and 64% fibroblasts (FBs) (Zhou and Pu 2016). During heart development, cardiomyocytes interact closely with other cell types. Thus, non-cardiomyocytes can contribute to cardiomyocyte maturation in vivo either through cell-to-cell contact or paracrine effects or both (Yang et al. 2014a) (Fig. 14.2g). The in vitro simulation of the cardiac microenvironment is essential for cardiac differentiation and also maturation. Non-cardiomyocytes might be necessary for the electrophysiological growth and maturation of human PSC-CMs and the expression of intracellular calcium handling proteins and ion channels (Kim et al. 2010). Gao et al., who generated human cardiac muscle patches (hCMPs) consisting of cardiomyocytes, smooth muscles, and ECs differentiated from human iPSCs, showed that hCMPs displayed improved electromechanical coupling, calcium handling, and forced generation after 7 days of dynamic culture in vitro (Gao et al. 2018). Co-culture with other cell types affected cardiomyocyte's functional improvement in vitro and also showed clinical relevance when engrafted to animal myocardial infraction (MI) models (Gao et al. 2018).

14.3.8 In Vivo Maturation

Presently, PSC-CMs can be generated routinely with high yield and purity (Karbassi et al. 2020). However, the generation of mature cardiomyocyte-like cells in a dish has remained challenging. The transcriptional analysis demonstrated that the generated PSC-CMs became more mature after extended culture, but the maturation was arrested at the late embryonic/neonatal stage (Uosaki et al. 2015). This suggests the complexity and unknown factors for cardiomyocyte maturation, which occurs within the second decade of human life and 2 weeks after birth for rodents (Guo and Pu 2020). Mouse PSC-CMs mature into adult cardiomyocytes when transplanted into rat neonatal myocardium (Cho et al. 2017) (Fig. 14.2h). For instance, the transplanted cells had developed T-tubules with a regular pattern, as observed in adult cardiomyocytes with indistinguishable transcriptome to adult ones. Moreover, human PSC-CMs resembled adult cardiomyocytes and displayed the disease phenotypes that observe only in adulthood as well when transplanted to rat neonatal hearts (Cho et al. 2017). Curiously, PSC-CMs failed to reach the adult stage when directly transplanted to adult hearts (Shiba et al. 2012), indicating that the critical time point exists for cardiomyocyte maturation. Altogether, in vivo maturation system is considered a maturation strategy that can be used to generate late-onset cardiac diseases.

14.3.9 3D Culture System

Individual maturation strategies improve many aspects of cardiomyocyte maturation. But it is insufficient to generate fully matured cardiomyocytes. Recently, combinations of several available techniques have been incrementally used to generate more mature PSC-CMs. For example, the combination of electrical and mechanical stimulations showed a two-fold increase in the contractile force (Ruan et al. 2016). Moreover, 3D engineering of heart tissues with other cell types combined with ECMs enhanced the morphological and functional maturation of the cells (Fig. 14.2i). 3D cardiac patches using modified substrates and co-culture with non-cardiomyocytes improved the morphology and function of PSC-CMs and were used to treat animal MI models (Shadrin et al. 2017; Gao et al. 2018). Although combinations of maturation methods are documented, the maturity of PSC-CMs with those manners compared to in vivo cardiomyocytes remains unknown.

14.4 Future Perspectives of PSC-CMs

14.4.1 Cardiac Disease Modeling

Although experimental animal models have been enormous for our overall understanding of diseases, experimental animals still show differences from humans such as size, heart rate, ion channel contributions, and even developmental pro-

cesses. With the promise of iPS cells, functional cardiomyocytes can be efficiently obtained from an individual patient via the direct cardiac differentiation of patient-specific iPS cells. Human PSC-CMs have been generated from patients including long QT syndrome (Moretti et al. 2010), dilated cardiomyopathy (DCM) (Sun et al. 2012; Hinson et al. 2015), Leopard syndrome (Carvajal-Vergara et al. 2010), Timothy syndrome (Yazawa et al. 2011), and familial hypertrophic (Lan et al. 2013), have shown great promise for investigating the pathogenesis and determining new therapeutic targets (Fig. 14.3).

A previous study has highlighted that mature PSC-CMs are required to model adult-onset diseases (Kim et al. 2013). This study has developed an arrhythmogenic right ventricular dysplasia/cardiomyopathy (ARVD/C) model from patients containing mutation in the plakophilin-2 (PKP2) gene. The PSC-CMs derived from the PKP2 mutant patient could reproduce pathological phenotypes such as exaggerated lipogenesis and ARVD/C apoptosis when introducing the cells to adult-like metabolic energetics (from glycolysis to FAO) (Kim et al. 2013). More examples of the requirement of maturation have been increasing lately. For instance, isogenic iPS cells carrying RNA-binding motif protein 20 (RBM20) mutation, R634Q, recapitulated the contractile deficits, a hallmark of DCM, only when PSC-CMs were cultured in metabolic maturation media (containing albumin-bound fatty acid, L-carnitine, taurine, and creatine) (Feyen et al. 2020). These researches provide pieces of evidence that induction of adult-like metabolism has a pivotal role in establishing adult-onset disease model both patient-specific iPSCs and isogenic mutated iPSCs.

14.4.2 Pharmacological Studies

Although new drugs are extensively tested in animal models before getting approval to test in humans and spreading out to the market, unexpected results like cardiovascular toxicity are still observed (Braam et al. 2009). This indicates the significant difference between humans and animals. With limited access to human cardiac tissues, PSC-CMs are attractive models and are widely used (Fig. 14.3). PSC-CMs response to tested drugs in terms of beating frequency and contractility was similar to what had been observed clinically (Yokoo et al. 2009). Consistently, PSC-CMs treatment with 28 different compounds with known cardiac effects led to changes in the amplitude of the impedance measurement, compatible with empirical results for clinical application (Guo et al. 2011). These findings suggest that PSC-CMs are excellent tool for novel therapeutic drug screening. Not only at a single-cell level but as a tissue (i.e., single-layered or multilayered sheets), PSC-CMs modeled reentrant arrhythmia and torsade de point (Kadota et al. 2013; Kawatou et al. 2017). Applying high-frequency stimulation resulted in the generation of reentrant spiral-wave propagation, which can be terminated by antiarrhythmic drugs (Kadota et al. 2013). With more sophisticated sheets with PSC-CMs and non-myocytes, QT elongation and torsade de point can be recaptured with a known I_{Kr} channel blocker (Kawatou et al. 2017). These studies highlight the utility of PSC-CMs for drug testing, specifically for drugs with antiarrhythmic potential and proarrhythmic cardiotoxicity.

As mentioned earlier, generated PSC-CMs remain immature compared to those of adult heart. This point hinders the

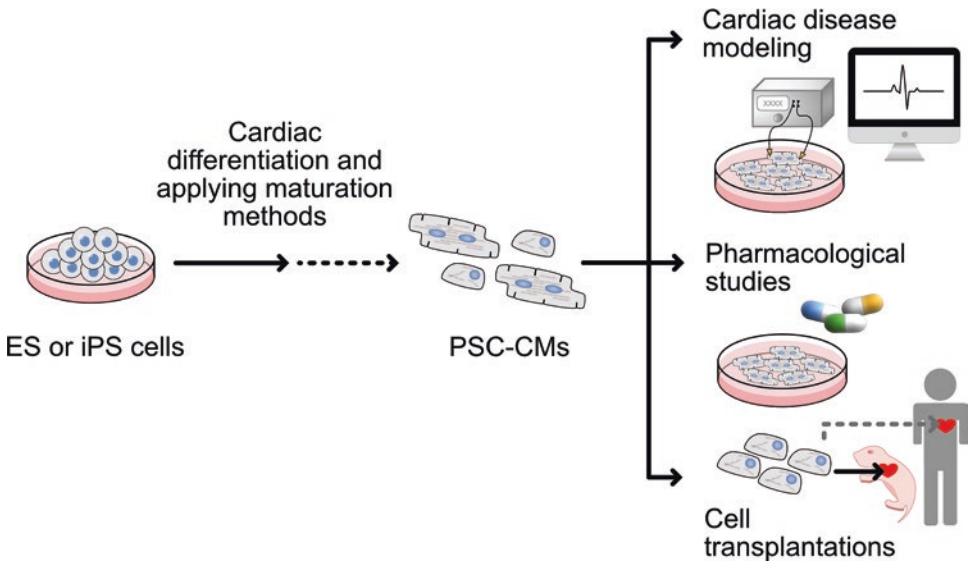

Fig. 14.3 Applications of PSC-CMs in regenerative medicine PSC-CMs can be efficiently and reproducibly obtained by direct cardiac differentiation of ES or iPS cells and used for regenerative medicine such as cardiac disease modeling, pharmacological studies, and even cell transplant therapy

utility of PSC-CMs in pharmacological studies. For example, PSC-CMs lack functional potassium channels and shifted to sodium channel activation, resulting in immature electrophysiological phenotype, thus giving incorrect responses to proarrhythmic triggers beyond hERG block (Jonsson et al. 2012). Therefore, the development of mature cardiomyocytes is necessary for safety pharmacology.

14.4.3 Cell Transplantations

The use of fully matured cardiomyocytes in cell transplantations is restricted due to their inability to survive in the host myocardium. Isolated adult rat cardiomyocytes fail to thrive when transplanted into acutely cryoinjured myocardium and even in the normal heart (Reinecke et al. 1999). Conversely, fetal and neonatal cardiomyocytes survive under all conditions posttransplantation. Grafted neonatal cardiomyocytes initially show polarization of N-cadherin, an adherent junction protein, into the intercalated discs on day 6 post-engraftment, which is followed by gradual localization of Cx43 in a similar fashion to N-cadherin. Importantly, grafted cells can form adherent and gap junctions with host cardiomyocytes, indicating the electromechanical coupling between the host cardiomyocytes and the donor cells. It is highly likely that the collapse of the contractile skeleton through enzymatic dispersion of the cells affects to electrical and mechanical function. It is also noted that there might be a critical time window for the transplantation of PSC-CMs as well in terms of in vitro maturity and posttransplantation maturation (Funakoshi et al. 2016; Cho et al. 2017; Kadota et al. 2017). For instance, PSC-CMs successfully recapitulated adult cardiomyocyte phenotype post-engraftment in the neonatal rat hearts (Cho et al. 2017) but not in adult hearts (Shiba et al. 2012), thus indicating the critical time window for cardiomyocyte maturation. As immature cardiomyocytes maintain the electrophysiological properties, such as automaticity, after transplantation, they may cause arrhythmias (Kadota and Shiba 2019). Graft-associated arrhythmias are transient and can be controlled when the transplanted PSC-CMs mature in vivo (Nakamura and Murry 2019).

14.5 Conclusion

Development of iPSCs and ESCs has shown substantial progress during the last two decades. Achievement of generating PSC-CMs by cardiac differentiation from iPS cells and ES cells provides a great promise in regenerative medicine. Hope is high that soon we can discover novel therapeutic drugs by using PSC-CMs and, perhaps, use these cells in cell transplantation someday. Building on these successes, generating either fully matures PSC-CMs or controlling the cells to certain maturity is still an essential step, and a current challenge is to recapture mature cardiomyocyte phenotypes in vitro and produce safer cells for transplantation. To this end, understanding of cardiomyocyte maturation process is crucial.

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Availability of Pluripotent Stem Cells from Normal Cells in Cancer Science

15

Ghmkin Hassan, Said M. Afify, Juan Du, Akimasa Seno, and Masaharu Seno

Abbreviations

ADM	Acinar to ductal metaplasia
AFP	Alpha-fetoprotein
AML	Acute myeloid leukemia
CAFs	Cancer-associated fibroblasts (CAFs)
CEA	Carcinoembryonic antigen
CFU	Colony-forming unit
ciPSCs	Converted iPSCs
CK19	Cytokeratin-19
CM	Conditioned medium
Colla1	Collagen type I alpha 1 (Col1α1)
CSCs	Cancer stem cells
Dox	Doxycycline
ESCs	Embryonic stem cells
FSP1	Fibroblast-specific protein 1
GPC3	Glypican-3
HSCs	Hematopoietic stem cells
iCSCs	Induced cancer stem cells
iPSCs	Induced pluripotent stem cells
LIF	Leukemia inhibitory factor
miPSCs	Mouse iPSCs
NACs	Non-adherent cells
NPCs	Neural progenitor cells
PDGFα	Platelet-derived growth factor-α
PTEN	Phosphatase and tensin homolog
α-SMA	α-smooth muscle actin
PRCC	Papillary renal cell carcinoma
PDAC	Pancreatic ductal adenocarcinoma
RNA-seq	RNA sequencing
SDF-1α	Stromal-derived factor-1α
TGFβ1	Transforming growth factor β1
TOFT	Tissue organization field theory
VPA	Valproic acid

G. Hassan
Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama, Japan

Department of Microbiology and Biochemistry, Faculty of Pharmacy, Damascus University, Damascus, Syria

Department of Genomic Oncology and Oral Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University, Japan

S. M. Afify
Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama, Japan

Division of Biochemistry, Chemistry Department, Faculty of Science, Menoufia University, Shebin El Koum-Menoufia, Egypt

J. Du
Department of cancer institute, Shanxi provincial cancer hospital, Taiyuan, P.R. China

A. Seno
Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama, Japan

R&D Division, The Laboratory of Natural Food & Medicine Co., Ltd., Okayama, Japan

M. Seno (✉)
Graduate School of Interdisciplinary Science and Engineering in Health Systems, Okayama University, Okayama, Japan
e-mail: mseno@okayama-u.ac.jp

15.1 Introduction and a Short History of Cancer Research

Attempts to understand the origin and cause of cancer are back into the earliest period of life when human began to observe diseases. Throughout history, a gradual understanding of tumors by the researchers and the different treatments, such as simple herbal, salt mixtures, and primitive surgery techniques, was applied. The first available documented description of cancer as a disease is back to 3000 BC, founded in the ancient medical text Edwin Smith Papyrus, which contained a description of breast cancer as a deadly disease. The ancient Egyptian also tried to treat this disease with arsenic paste. A notable progress, after that, was introduced by Greeks around 400 BC, where Hippocrates began to give more details about cancer; he gave the name of "cancer" to the disease as he believed the similarities between the disease and moving crab and described the disease as a natu-

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ral cause. More comparison between crab and cancer, especially the crab's ability to adhere by its claws to a different direction, was done. At the same time, there were attempts to treat cancer by using a mixture of egg and honey (Hajdu 2011).

Much data have also been documented by different cultures, including ancient Chinese, Indian, Persian, and Muslims in this regard. When Baghdad City was the center of the scientific world, remarkable information about cancer and its treatments were documented by different scholars such as Avenzoar and Avicenna, who described cancer in his famous book *The Canon of Medicine*. Some of his special notes were that some cancers are more common in women and internal tumors could be removed by specific surgery techniques such as polypectomy. He also suggested that external tumors are possible to treat than internal ones that grew continuously and became difficult to treat (Golzari et al. 2013). During the period between 1500 and 1700, the progress in the cancer field was due to surgery and pathology specialties describing the difference between benign and malignant tumors and distinguishing sarcoma from carcinoma. At that time, lung tumors were also diagnosed either as primary or secondary tumors coming from other parts, and their treatment was more difficult. The tumor's origin was also introduced by Deshaies Gendron, who proposed that cancer was arising from the transformation and continuous growth of different solid structures of the body. At the same time, chronic inflammation and tobacco were also suggested as causes of cancer. More types of cancer have been documented in this period through notes of Theophilus Bonetti, who recorded 43 case reports on colon, pancreatic, liver, lung, stomach tumors, and so on (Manchester 1997).

The first attempt to transplant human cancer sample into animals was performed by Bernard Peyrilhe. He injected human breast cancer extracts into a dog in 1775 as the first experiment in this direction even before developing the concept of cells (Androutsos and Karamanou 2009). From the eighteenth century up to the beginning of the nineteenth, cancer was also presumed to rise from chronic exposure to environmental agents such as soot, tobacco, coal tar, and hot paraffin or flow obstruction of body fluids. In this period, cancer terms, such as "soft cancer" referring to lymph glands and soft tissue cancers and "metastasis" referring to invasion and spreading of tumor far from original places, were introduced (Anttila and Boffetta 2014).

One of the most distinguished findings that impacted cancer research was the cell theory established by Theodor Schwann, who proposed cells as blocks of human and animal tissues. After that, Muller described cancer as groups of abnormal cells and stroma. Cell theory introduced the routine use of microscopes for medical research giving more details and descriptions about tumor tissues and classifying cancers depending on microscale features illustrating cancer cells (Ribatti 2018). In the second half of the nineteenth century, much of the groundwork done by the researchers enormously contributed to the progress and advancements in the cancer field. Coley injected toxins to support the patient immune system against tumors. Novinsky successfully transplanted dog and rat tumors into healthy animals. Controlled exposure to X-rays was being used to treat cancers. Ehrlich introduced the term "chemotherapy," that worked to develop chemical compounds as drugs for cancers and suggested that the tumor consisted of chemically resistant and sensitive cells (Hajdu 2012).

At the beginning of the twentieth century, a remarkable experiment by Peyton Rous showed the ability of cell-free filtrates from hen sarcoma to induce cancer in another hen, and the cancer-causing agent was later identified as a virus named the Rous sarcoma virus. At the same period, Theodor Boveri proposed the basics of somatic mutation theory. In this theory, Boveri assumed that cancer occurred due to mutations, "abnormal chromosomal rearrangement," which lead to cell proliferation and cancer initiation (Di Lonardo et al. 2015). During the twentieth century, cancer research grew significantly wherein many groundbreaking discoveries were made. Yamagiwa was the first to succeed in inducing an invasive skin cancer in animals by applying crude coal tar, which was considered as a mixture of pro-inflammatory chemicals (Yamagiwa and Ichikawa 1918). X-rays exposure was found to induce skin cancer in radiation workers (Shore 1990).

These early findings allowed observing differences between malignant and normal cells and establishing cancer cell lines. Since Yamagiwa's results promoted the investigation of carcinogenesis, the effects of hundreds of chemicals were assessed during the decade after the discovery in various research laboratories. Simultaneously, Warburg found that cancer cells consumed glucose at a rate higher than normal cells, a phenomenon called "the Warburg effect," which formed the basis of cancer metabolism. Watson and Crick's discovery of DNA structure had enormous influence on molecular oncology (Liberti and Locasale 2016). In 1953, the experiments by Helene Toolan were among the first successful attempt of human cancer sample transplantation into animals. In these experiments, Toolan reported that human tumors could successfully grow and proliferate in cortisone-treated laboratory animals (Xu et al. 2020). Breast cancers were linked to the familial breast carcinoma-related gene mutations such as BRCA1 and BRCA2, besides patients' family history. Viruses were suggested to be responsible for transforming normal cells to cancer cells by viral-derived genes, so-called oncogenes.

On the contrary, tumor suppressor genes, such as retinoblastoma Rb1 and P53, inhibited cell proliferation and affect cell cycle. The loss of the p53 gene and its mutations were linked to the malignant transformation of normal cells. Many oncogenes and suppressor genes have already been identified since then (Buchholz et al. 1999; Miller and Stebbing 2018).

The accumulated data focusing on the mutations and chromosome abnormalities guided the researchers to the somatic mutation theory. In light of this theory, the normal cells required multiple mutations (3 to 7) for cancer initiation, and its subsequent progression. Therefore, tumor formation was proposed to depend on a series of mutations through multiple intermediary stages (Fisher 1958). Cancer growth was shown to rely on blood vessels when Folkman and his colleagues provided an evidence that neovascularization was vital for solid tumor growth. The term "tumor angiogenesis" began to be used widely in cancer research (Ribatti 2008). The discovery of nude mice, which were hairless, immunocompromised mice lacking thymus glands, accelerated cancer research by enabling to simply reestablish tumor models in animals without drug or radiation since nude mice were mutant defecting the immune system (Neff 2016; Szadvari et al. 2016).

On the same note, isolated from a wide range of cancer types, various cancer cell lines have been developed and used for in vitro or in vivo experimental models, which were extensively used to investigate the cell characteristics, such as tumorigenesis, drug resistance, and metastasis in different cancer types. However, these cells often fail to provide insights for tumor development and progression due to the alteration of characters after years of careless maintenance in vitro since their genomic and/or morphological characteristics have changed over time. If the preclinical experiments are carelessly performed under this situation, the following clinical phase testing will encounter frequent failures. This lack of translational success is often ascribed to multiple parameters, including tumor heterogeneity, diverse and complex cellular interactions, and limited availability and access to the in vitro the 3D tumor microenvironment models to mimic in vivo microenvironment (Ben-David et al. 2019).

For decades, somatic mutation theory remained the predominant theory of cancer origin, thus providing a considerable number of studies based on this notion. This bias decreased the opportunity to investigate other hypotheses of the origin of cancer in a more sophisticated way or neglected sometimes in favor of somatic mutation theory (Brucher and Jamall 2016; Soto and Sonnenschein 2014). At the end of the twentieth century, the epigenetic field has begun to emerge, and cancer tissues were subjected to epigenetic analysis. Epigenetic changes refer to the changes in genetic information rather than the DNA sequence, such as mutations (Brucher and Jamall 2014b). Epigenesis includes DNA methylation and/or histone methylation/acetylation, etc. In early 1980, the epigenetic changes were reported to involve oncogenes and tumor suppressor genes to regulate their expression, thus altering the resultant phenotypes. Therefore, epigenesis was assigned a predictive role in cancer theranostic applications (Baylin and Jones 2016). Feinberg et al. found that specific genes in human tumor tissues were hypomethylated compared to those in normal adjacent tissues.

A significant difference in DNA methylation was found between different human malignant tumors, benign tumors, and normal tissues (Feinberg and Vogelstein 1983). Cancer epigenetics is primarily focused on activating oncogenes and inactivating cancer suppressor genes. Epigenetic alterations due to environment or aging have also been linked to carcinogenesis and possible role to initiate cancer. Recently, new hypotheses have been put forward regarding the mechanism underlying cancer initiation. A large number of scientists began to think that cancer is a tissue-based disease instead of being a cell-based disease. Carlos Sonnenschein suggested the tissue organization field theory (TOFT) as one of the cancer initiation mechanisms where abnormal interaction between tissue microenvironment and different types of cells, i.e., stromal or epithelial cells, could result in cellular transformation and cancer initiation. This is not necessarily dependent on mutations or clonal dominance of mutant cells (Soto and Sonnenschein 2011). In this context, biophysical forces and interactions between cells and tissues are pivotal factors in the carcinogenesis process. This insight is also supported by a long history of studies and the accumulation of evidence supporting the crucial roles of chronic inflammation in initiating cancer.

Different stimuli and pathogens could induce chronic inflammation, which is a long-term disruption of homeostasis in tissues. Abnormal secretome profile and cell-to-cell interactions could eventually lead to cellular transformation. Experimental exposure of chemicals to different animal tissues for an extended period was among the first cancer induction experiments. Some viruses such as hepatitis B and C and bacteria such as helicobacter pylori were linked to different types of cancers such as the liver and gastric cancers. The link between these factors is the disruption of chronic inflammation-inducing signaling, which seems to be crucial in the early stages in cancer development (Brucher and Jamall 2014a, 2019). Figure 15.1 summarizes major discoveries and events in cancer research.

Different tools exist for cancer studies, such as cell lines, patient-derived xenografts, and different experimental animal models. However, it is insufficient to encompass all the cancer initiation mechanisms. Therefore, additional disease modeling strategies are needed to complement existing techniques in cancer research. In this regard, induced pluripotent stem cells (iPSCs) could go a long way provide a powerful tool in this area.

15.2 Cancer Stem Cells in Cancer Science

The concept of cancer stem cells (CSCs) is long-standing and dates back to the nineteenth century when Julius Cohnheim mentioned the similarity between cancer cells and embryonal cells. Cohnheim suggested that the origin of

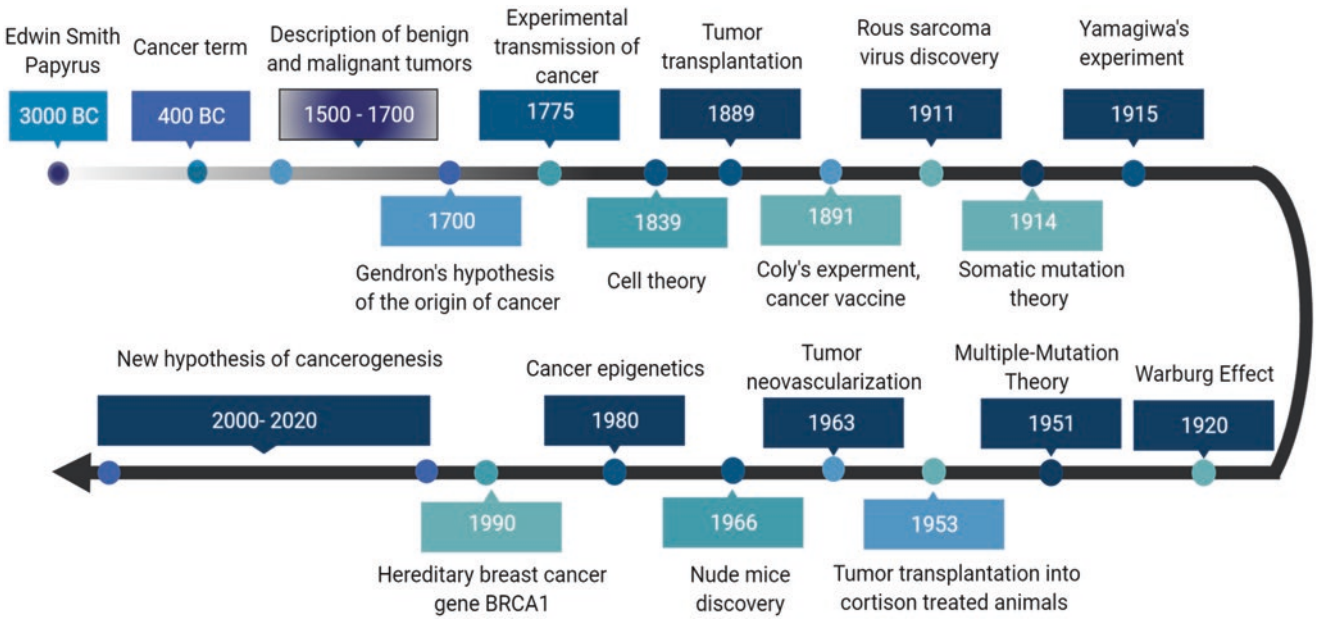

Fig. 15.1 Timeline of some major events and discoveries in cancer research history

cancer is from cells misplaced during embryonal development and/or retaining the embryonal characteristics. In 1977, Hamburger and Salmon developed a cell culture protocol for the tumor stem cells by culturing tumor cells in semisolid conditions where some of tumor cells selectively formed colonies, while cells from healthy volunteers failed to colonize under the same set of culture conditions (Capp 2019). CSCs were isolated for the first time by John E. Dick from acute myeloid leukemia (AML) specimens. In this study, a rare subpopulation, identified using $CD34^+/CD38^-$ expression, showed the ability to initiate cancer upon injection into immunodeficient mice. On the other hand, $CD34^+/CD38^+$ and $CD34^-$ cells, the majority of myeloid leukemia cells, failed to give the same results as $CD34^+/CD38^-$. Therefore, $CD34^+/CD38^-$ cells were proposed as CSCs with their self-renewal and colony-forming ability. In this study, Dick also suggested the hierarchy of leukemia stem cells, which gave more mature cells in AML colonies (colony-forming units, CFU), which had less proliferative potential when cultured for a long time. Dick observed that both normal hematopoietic stem cells and leukemia stem cells were sharing the same phenotype, $CD34^+/CD38^-$. Therefore, he suggested comparing the gene expression between them to find significant genes and markers for leukemia stem cells. Dick focused on hematological malignancy because many hematological malignancies did not have an appropriate in vitro assay besides limited proliferation capacity of the responsible cells, unlike solid tumor cells, which could be easily cultured and maintained in vitro using the available optimized protocols (Bonnet and Dick 1997).

At the end of the twentieth century, the concepts of tumor heterogeneity and CSCs were prevalent, and CSCs were successfully isolated from hematological malignancies. In 2000, the first report of isolation and identification of CSCs in solid tumors came out. In this study, Al-Hajj et al. found that a minority of breast cancer cells, characterized as $CD44^+/CD24^{low}/lineage^-$, could form tumors at a very low number of 100 cells when injected in immunodeficient mice (al-Hajj et al. 2003). The authors reported that breast cancer cells contained phenotypically diverse populations where $CD44^+/CD24^{low}/lineage^-$ population could be highly tumorigenic and form tumors containing various populations of cancer cells. On the other hand, cells with phenotypes diverging from $CD44^+/CD24^{low}/lineage^-$ failed to form tumors even when tens of thousands were injected. Shortly after that, CSCs were also isolated from brain tumors and identified as $CD133^+$ cells, which could produce tumors when injected as 100 cells in the brain of immunodeficient mice. However, injection of 10000-fold more of $CD133^-$ cells failed to grow as tumors (Singh et al. 2003).

Colon CSCs were also identified as $CD133^+$ cells, which were highly tumorigenic and represent only 2.5% of cancer cells (Al-Hajj et al. 2003). CSCs were isolated almost from all types of cancers, and different surface and intercellular markers were reported as CSC markers. Recent advance in this field shows that a panel of markers is better than only one to distinguish CSCs and isolate themselves. Characterization of CSCs by more than one marker gives different populations of cells with different tumorigenicity. For example, pancreatic cancer cells expressing $CD44^+/CD24^+/ESA^+$ showed higher tumorigenicity when compared with

$CD44^+/CD24^+$ or $CD44^+/ESA^+$ cells, while c-$Met^{high}/CD44^+$ cells were recently identified as high tumorigenic pancreatic CSCs (Li et al. 2011). In the published literature, the presence of CSCs have been reported in almost all types of cancers where their existence is being considered as vital for tumor initiation, chemotherapeutic resistance, metastasis, and cancer relapse (Fig. 15.2).

Since the isolation of CSCs mainly depends on their specific surface markers, identification of these markers is a crucial task for CSC research. However, considering the requirements for antibody-based targeting, the lack of CSC-specific surface markers and the low rate of CSC existence in tumor specimens, identification and isolation of CSCs remain as unsurmountable challenge. The complexity and dynamic nature of cancer further render its theranostics a significant challenge. Moreover, the maintenance conditions of CSCs in vitro also need to be optimized where their stemness and differentiation status could be assessed.

Therefore, CSC-relevant research requires novel technology that could handle CSC-based experiments in optimum fashion and mimic their in vivo microenvironment to the best for their optimal maintenance in vitro. The novel in vitro models applying innovative technologies to identify, isolate, or alter normal cells into CSCs are anticipated.

15.3 Utilizing iPSC in Cancer Science

In 2006, the first iPSCs were introduced by Shinya Yamanaka, who used four pluripotency-related transcription factors (Oct4, Sox2, Klf4, and c-Myc) to reprogram mouse fibroblasts into ESCs-like cells (Takahashi and Yamanaka 2006). The publication of Yamanaka's reprogramming protocol paved the way for reversing the terminally differentiated somatic cells by reprogramming, such as fibroblasts, bone marrow cells, skeletal myoblasts, or peripheral blood monocytes, into ESCs-like cells (Ahmed et al. 2011; Buccini et al. 2012). The iPSCs are now considered as surrogate ESCs that have the ability to differentiate to all cell phenotypes of the three germ layers. One of the advantages of iPSCs generation technology is patient-specific and disease-specific iPSCs with wide range of applications in regenerative medicine, drug development, and disease modeling in vitro.

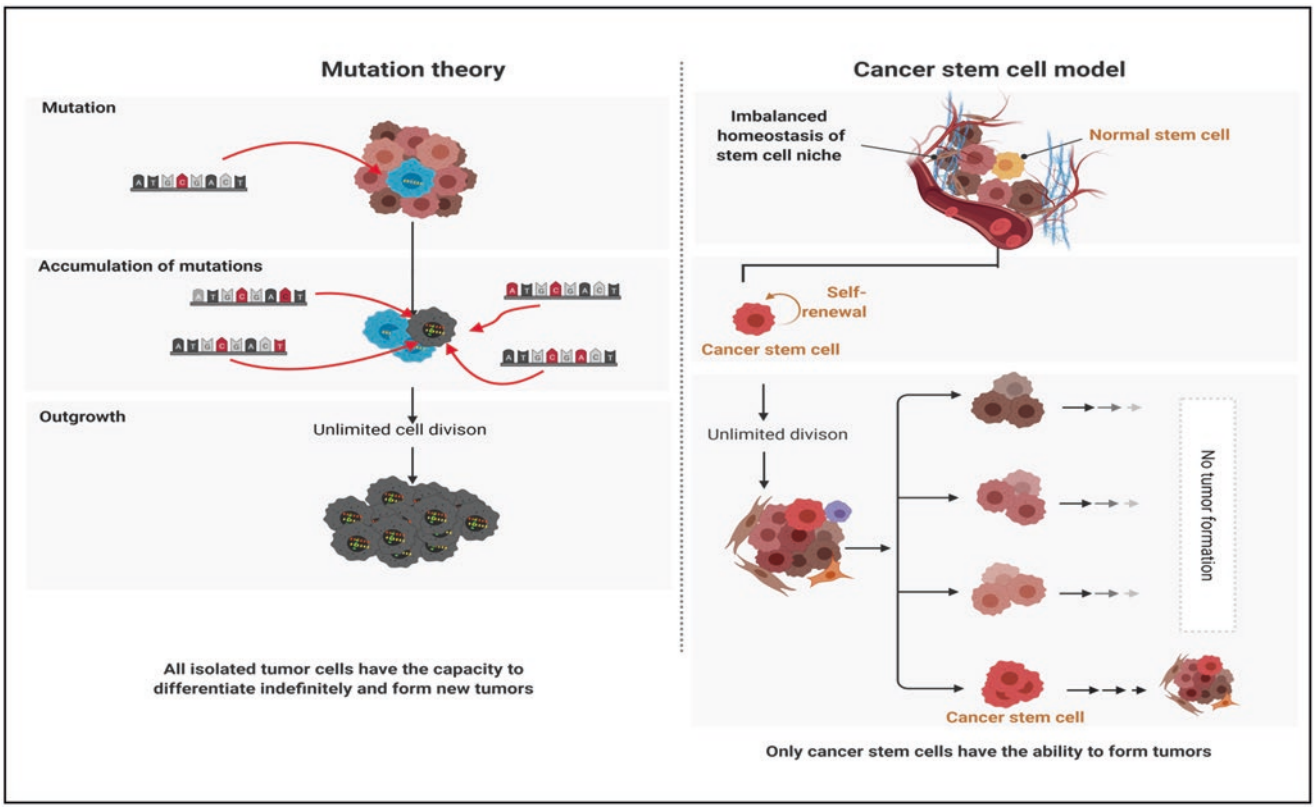

Fig. 15.2 Schematic illustration for the somatic mutation theory and cancer stem cell (CSC) models of tumorigenesis. The somatic mutation theory suggests that the accumulation of multiple mutations or genetic defects transforms cells into cancer cells and acquires unlimited divisions. The cancer stem cell model through epigenetics suggests that the disturbance of homeostasis in stem cell niches leads to their transformation into CSCs. CSCs, with self-renewal and differentiation abilities, form heterogeneous tumors where only CSC subpopulations can initiate tumor formation

iPSCs are similar to ESCs in terms of morphology, pluripotency-related gene expression profiles, epigenetic status, proliferation potential, teratogenicity, and differentiation. However, they are superior to ESCs in terms of availability autologous source and that too without ethical and moral issues. In iPSCs, each cell contains a full set of genomes, and its identity and function depend upon the activation status of genes contained therein. For example, skin cells have activated genes for skin function, while other cell types' specific genes are turned off. Given that iPSCs have pluripotent differentiation potential and infinite proliferation capacity, they usually form teratomas containing cells from the three germ layers but without the metastatic capability. The c-Myc is one of the exogenous reprogramming factors included in the classical quartet of Yamanaka's reprogramming protocol (Omole and Fakoya 2018). However, c-Myc is an oncogene that plays a vital role during embryonic development. c-Myc gets reactivated again after iPSCs generation causing the development of malignant tumors in rare cases. Therefore, it has been dispensed away during the subsequently reported protocols for iPSCs generation. Recently, Liu P et al. selected only two transcription factors (Oct4 and Sox2) of the four classical transcription factors by using CRISPR/Cas gene regulation technology to create iPSCs (Liu et al. 2018).

In succession, the accelerated development of iPSC technology by employing nonintegrating viral vectors, nonviral vectors, or removing the introduced foreign genes via gene knockout has ensured the yields of much safer iPSC (Ibrahim et al. 2016). Meanwhile, some researchers discovered that several chemical compounds were potent in accelerating cellular reprogramming. The process of reprogramming is complex and regulated delicately. Some compounds can significantly improve the reprogramming process's efficiency through activation or inhibition of multiple signaling pathways involved therein. Valproic acid (VPA) and sodium butyrate are histone deacetylase inhibitors found to increase the reprogramming efficiency by more than 100 times and 15−50 times, respectively (Huangfu et al. 2008; Mali et al. 2010). Shi et al. found that the combination of small molecules BIX-01294 and BayK8644 may be combined with only Oct4 and Klf4 for successful reprogramming of mouse embryonic fibroblasts thus indicating that these two small molecules can increase the efficiency and rate of cellular reprogramming or to successfully replace Sox2 in the production of iPSCs (Shi et al. 2008). The combination of SB43142 and PD0325901 could also significantly improve the efficiency of reprogramming. The combination of thiazovivin, an inhibitor of the ROCK pathway, SB43142, an inhibitor of TGF-β receptor, and PD0325901, an inhibitor of the MEK signaling pathway, could increase the efficiency by more than 200 times and shortened the time of reprogramming (Lin et al. 2009). The iPSC technology has become one of the most sought-after topics in stem cell research and helped significant progress in this field.

The iPSC reprogramming technology has also provided new opportunities and insights for cancer research, especially the concept of the existence of CSCs (Fig. 15.3).

Since the discovery of iPSCs technology, scientists have been trying to invest in this technology to create CSCs and study their characteristics. For example, Wong et al. transformed keratinocytes with c-Myc, Ras, and IκB, resulting in the acquisition of CSC phenotypes with high tumorigenicity and similarity with ESCs. They proposed the term "induced cancer stem cells" (iCSCs) as the benefit of reprogramming technology. Exploiting the wide range of differentiation capacity of iPSCs, some scientists could also create patient-derived cancer models to study sequential stages and molecular events of cancer initiation and progression. To this end, either iPSCs may be reprogrammed from normal somatic cells followed by the induction of mutation(s) or disease-specific or patient-derived cells may be reprogrammed to study the role of specific genes in cancer initiation in the context of pluripotency (Wong et al. 2008). Recently, genetically engineered mice-derived cells, in which expression of exogenous reprogramming factors (Oct3/4, Sox2, Klf4, and c-Myc) are controlled by doxycycline (Dox), have been used to study the effects of reprogramming event in vivo. This study showed that transient expression of the reprogramming factors induced by Dox administration resulted in the tumor development in different organs where tumor cells were distinct from teratoma cells and gained the gene expression signature akin to ESCs. The same group also reported that KRAS and TP53 mutations are not sufficient for pancreatic cell transformation, while mutant pancreatic cells transiently reprogrammed by iPSC transcription factors showed characters of early stages of pancreatic cancer development represented by acinar to ductal metaplasia (ADM). Moreover, TP53 cooperates with KRAS and accelerates the induction of pancreatic ductal adenocarcinoma (PDAC) (Shibata et al. 2018).

In the case of prostate tumor development, Zhao et al. found that the deletion of Tgfbr2, and phosphatase and tensin homolog (Pten) genes, increased the reprogramming efficiency of somatic cells by more than fourfold. When mice models were engineered as $Pten^{-}/Tgfbr2^{-}$, the deletion of these two genes promoted cancer growth and its invasiveness besides the induction of pluripotency markers, i.e., Nanog, Sox2, Oct4, and Cripto genes. Moreover, the expression levels of Nanog, Sox2, and Oct4 increased when iPSCs were reprogramed from Pten and TGFβr2 knockout cells (Zhao et al. 2018). In modeling neural cancers, the neural progenitor cells (NPCs) were differentiated from iPSCs, which had P53, Src, and EGFR mutations. These cells exhibited glioma CSC characteristics, which were highly tumorigenic and led to aggressive tumor growth. Different anticancer agents were

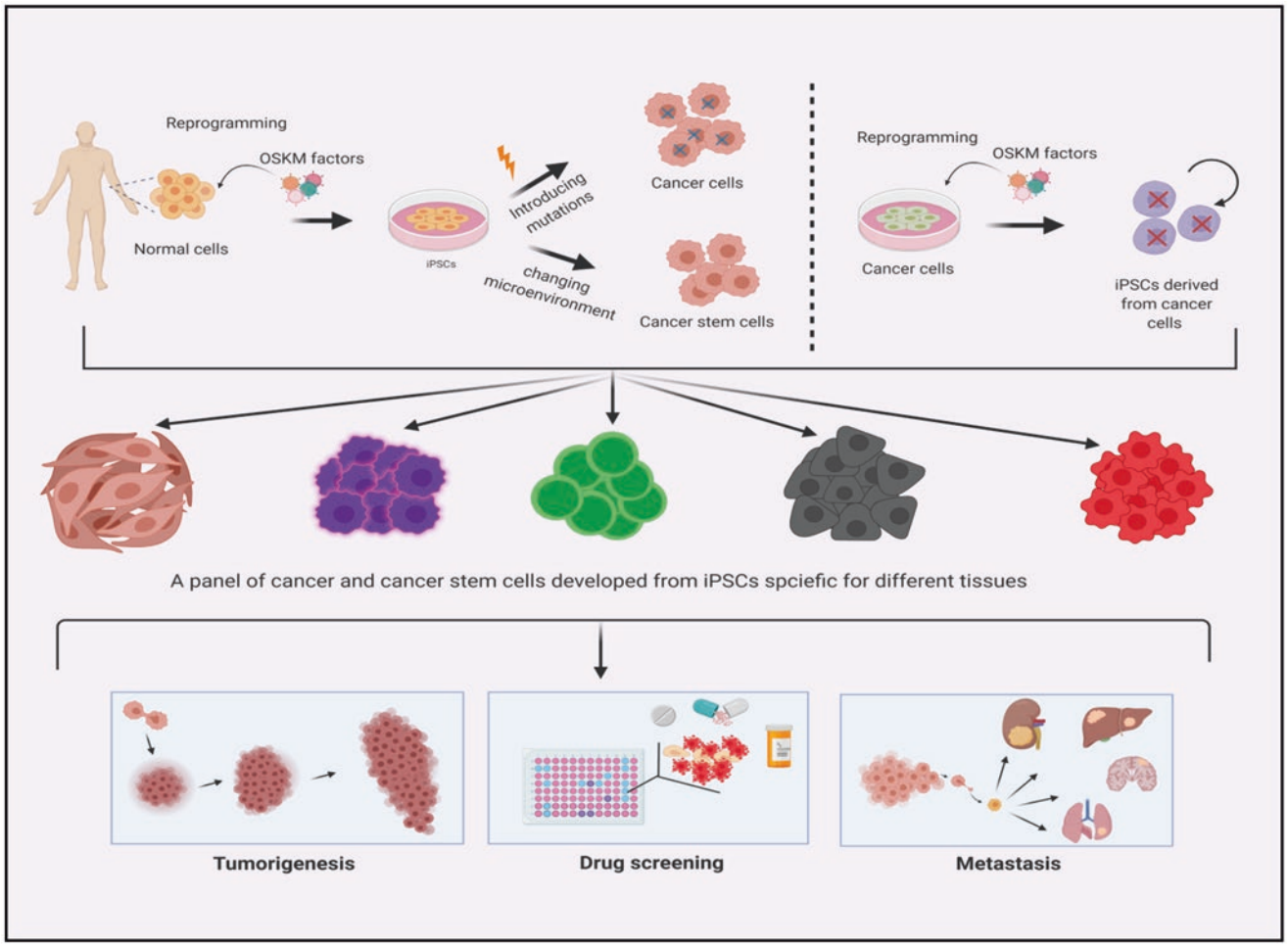

Fig. 15.3 Different approaches to using iPSCs technology in cancer science. Normal cells reprogrammed into iPSCs are being used to create cancer models by introducing mutations or being converted into CSCs by changing its microenvironment. At the same time, cancer cells could be reprogrammed into iPSCs giving iPSCs derived from cancer cells. Collectively, these cells can provide novel models for cancer stem cells or cancer cells deriving from iPSCs. These cells will be available to study tumorigenesis mechanisms, metastasis process, and drug screening

screened on this model to identify drugs targeting glioma CSCs efficiently (Sancho-Martinez et al. 2016) (Fig. 15.3).

In this approach, iPSCs are being used as tools to study tumor progress by introducing mutations in iPSCs or establishing iPSCs from genetically deficient cells. Thus, the effect and role of specific genes and their abnormalities and epigenetic changes in cancer induction and CSCs' maintenance could be assessed. Another more frequently adopted and systematic approach in cancer research is reprogramming of cancer cells into iPSCs to establish iPSCs-derived cell lines from cancer cells. This method helps us understand the nature and identity of cancer cells. In general, reprogramming of cancer cells changes their epigenetics and results in identity changes. If iPSCs derived from reprogramming of cancer cells are injected into immunodeficient mice, they will form teratomas. Melanocytes, pancreatic, and colorectal cancer cells were successfully reprogrammed with different factors to generate iPSCs from the respective cell type. It was interesting to observe that the derivative cells lost their tumorigenicity and chemoresistance characteristics in some cases (Marin Navarro et al. 2018; Czerwinska et al. 2018; Gong et al. 2019). On the contrary, reprogramming of breast cancer cell line MCF-7 cells into iPSCs did not reduce tumorigenicity, rather their tumorigenic properties were increased, resulting in a more aggressive undifferentiated invasive cancer phenotype. The reprogrammed cells were named cancer stem-like cells. Notably, iPSC markers, Oct3/4, Nanog, and SSEA-1 were not upregulated at this time after iPSC induction, unlike usual, but Sox2 was upregulated.

One of the most challenging issues in this method is the low efficiency of reprogramming. This may indicate that only a meager population of cancer cells, less than 1% of cancer cells, is reprogrammed, and the reprogramming cells

does not reflect the nature of cancer cell heterogeneity at the cellular or molecular levels (Chao and Chern 2018). Undeniably, iPSCs share many characters with CSCs, such as self-renewal and differentiation, thus making their investment in cancer science very attractive. At present, the research of iPSCs for cancer is in its infancy and is limited to experimental research. iPSCs reprogrammed from normal cells offer novel methods to generate CSCs without introducing any mutations and foreign genes exploiting the iPSC pluripotency.

During the development of a novel method in our laboratory, we used iPSCs reprogrammed from normal cells to generate CSCs for different cancer types. The conversion of iPSCs into CSCs was based on epigenetic changes and signaling pathway alterations under chronic inflammatory or cancerous microenvironment, exposing iPSCs to a cocktail of growth factors, cytokines, chemokines, and tissue-derived specific factors. The conditioned media (CM) were prepared from cancer cell lines creating such microenvironment, in which iPSCs were cultured for their conversion to CSCs. CM from different cell lines exhibited diverging potentials for conversion (Chen et al. 2012). In our method, we treated the iPSCs with CM prepared from different cancer cell lines. The iPSCs after treatment were named "converted iPSCs" (ciPSCs). This method's novelty was the usage of CM from cancer cell lines to direct the differentiation of iPSCs toward CSCs without any genetic modifications (Fig. 15.4).

To date, we successfully generate CSCs models for lung, pancreatic, breast, and liver cancers. Our first successful report was published in 2012 wherein the conversion of mouse iPSCs (miPSCs) into CSCs was performed using CM from Lewis lung carcinoma (LLC) cell line cells. The miPSCs used in this study were harboring GFP gene under the control of the Nanog promoter. The resulting cells stably expressed GFP in an undifferentiated state corresponding with the Nanog expression but lost its expression once differentiated. miPSCs required leukemia inhibitory factor (LIF) to maintain their stemness in vitro. The cells cultured without LIF underwent differentiation and did not survive. Interestingly, in our experiments, the miPSCs survived in CM without LIF. Cells treated with the CM from LLC cells for 4 weeks were named miPS-LLCcm cells, which kept expressing key markers of stemness and self-renewal such as Nanog, Eras, Rex1, and Cripto. Furthermore, these cells fulfilled the primary criteria to define CSCs by exhibiting sphere-forming ability in low adherent culture conditions and tumorigenicity in Balb/c nude mice (Chen et al. 2012). The pancreatic CSCs were generated from miPSCs following their treatment with CM derived from human pancreatic cancer cell lines, PK8, and KLM-1. In this study, CSCs converted in vitro were enriched via subcutaneous transplantation in nude mice, just as described by the previous studies, followed by transplantation into the pancreas. This orthotropic transplantation led to the enrichment of pancreatic CSCs, which in turn generated tumors imitating pancreatic ductal adenocarcinoma phenotype (PDAC) with liver metastasis. The analysis of RNA sequencing (RNA-seq) data for established CSCs indicated an elevation in the expression of transcription factors specific to pancreatic progenitor cells such as Pdx1, Hes1, Foxa2, Hnf1a, Hnf4a, Pax6, Nr5a2, Rbpj, Rbpgl, MafA, and MafB. PDAC-related hallmarks such as Kras, Krt19, Col8a1, Col1a1, Cxcr4, Muc1, Muc5aC, Mmp2, and Malat1 were also upregulated as well as the most representative pancreatic CSCs-specific markers including CD133, CD24, EpCAM, and CD44 (Calle et al. 2016).

Recently, we demonstrated for the first time that liver CSCs could be generated from iPSCs by culturing iPSCs in the presence of CM of hepatocellular carcinoma cell line (Huh7) cells. As a result, after 4 weeks of culturing miPSCs in the presence of CM, CSCs were induced as miPS-Huh7cm cells, which formed malignant tumors in the liver after 28 days of orthotropic injection into the liver. Primary cells from the malignant tumor of miPS-Huh7cm cells exhibited a similar phenotype to liver CSCs, defined by self-renewal capacity, differentiation potential, and tumorigenicity in vivo. The malignant tumors showed significant expression of the markers mostly common to the liver cancer, such as alpha-fetoprotein (AFP), glypican-3 (GPC3), carcinoembryonic antigen (CEA), and cytokeratin-19 (CK19). The significantly high expression of CD24, CD44, and CD133 was observed in the cells from malignant tumors when compared to miPSCs (Afify et al. 2020).

As we mentioned above, many chemical compounds could change cell epigenetics and assist in the reprogramming process. We assessed the risk of 110 non-mutagenic chemical compounds, most of which are known as inhibitors of cytoplasmic signaling pathways, as potential carcinogens. We treated miPSCs with each compound for 1 week in the presence of a CM of LLC cells. Even a period of 1 week was too short for the CM to convert miPSCs into CSCs. Different compounds showed different potential to accelerate the conversion, where 1 week was enough for induction.

Consequently, PD0325901 (MEK inhibitor), CHIR99021 (GSK-3β inhibitor), and Dasatinib (Abl, Src, and c-Kit inhibitor) were found to confer miPSCs into CSC phenotype in 1 week. The survived converted cells exhibited stemness markers expression, spheroid formation ability, and tumorigenesis in Balb/c nude mice (Du et al. 2020). Finally, several different protocols now exist, investing the iPSCs in creating models for cancer study. These protocols, however, differ from each other depending upon the purpose of the research and the perspective of the researchers. While the primary propose still remains the uncovering and understanding of tumorigenesis mechanisms and exploration of new targets for treatment or new therapeutic strategies, these models are expected to have great impact on cancer prevention, diagnosis, and drug development.

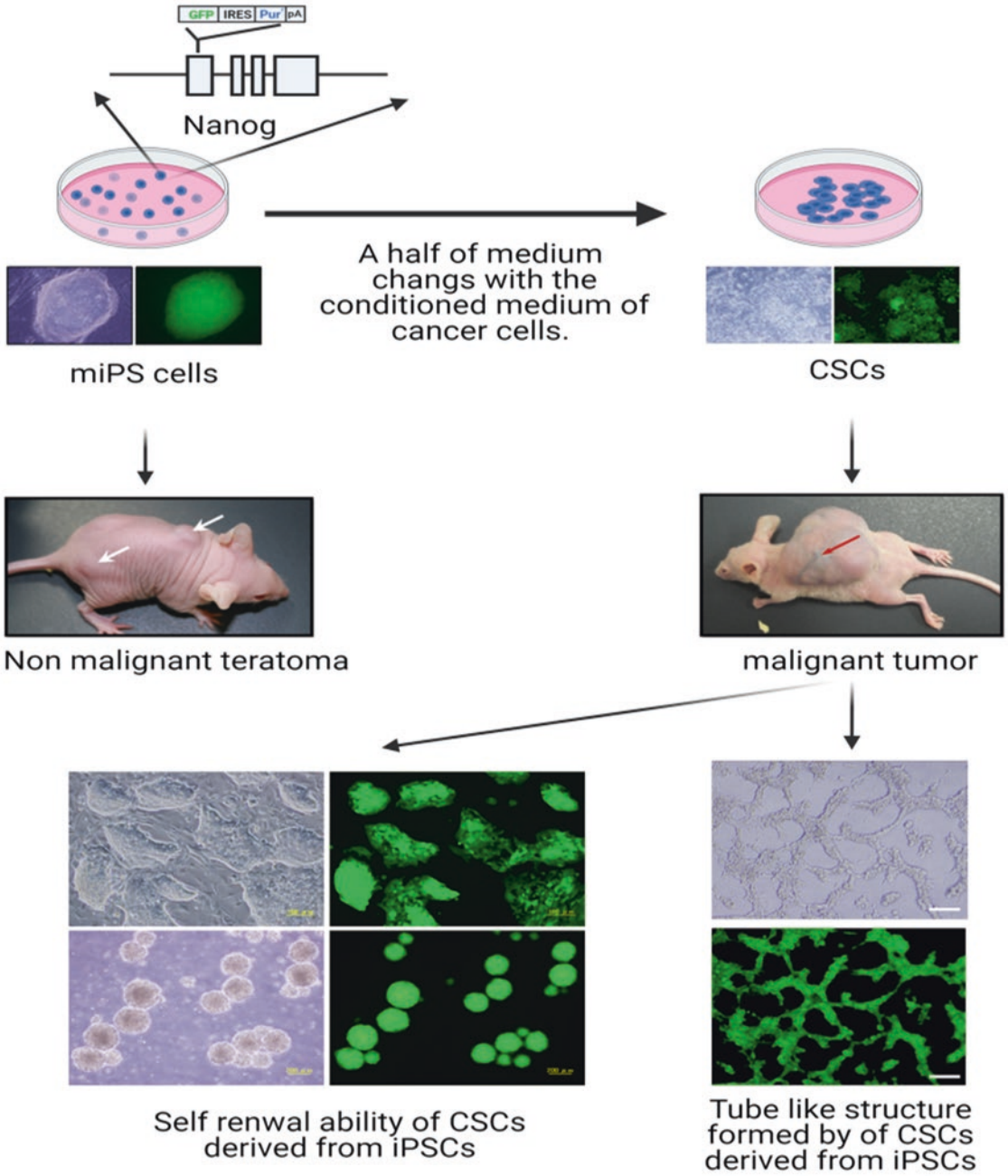

Fig. 15.4 Conversion of iPSCs into CSCs using conditioned media from cancer cells. The green fluorescent protein (GFP) and puromycin resistance genes were introduced into miPSCs under the Nanog promoter. The miPSCs were cultured in media containing 50% conditioned media prepared from cancer cells. One-month treatment converts miPSCs into CSCs. Injection of miPSCs into nude mice resulted in the formation of teratoma; however, injection of CSCs converted from miPSCs produced malignant tumors. Isolated CSCs from primary tumors expressed GFP and could form spheroids in low adherent culture conditions which indicate self-renewal ability. CSCs also demonstrated the ability to form tube-like structures and differentiate into endothelial cells. (A part of this figure was taken from (Chen et al. 2012))

15.4 Investigation of Tumor Initiation Mechanisms with iPSCs

The first stage of cancer development is usually very slow and hence prolonged and stepwise. Besides, it takes for the developing cancer many years to be noticeable. Recently, many changes in the cancer concept have been proposed, and scientists became more flexible in considering different ideas of tumorigenesis. Great efforts have been made to identify the origin of CSCs and explore the potential mechanisms of cancer initiation. During the last half century, research in stem cell biology accumulated information and proposed

that CSCs could be developed from stem cells residing in each tissue. The proposed origin of CSCs from stem cells was explained by either mutation theory or inflammation and epigenetic concepts. Thus, stem cells have attracted cancer researchers to uncover the development process of CSCs and their role in cancer diagnosis, metastasis, and as a novel target in cancer therapeutics.

Recent studies show that iPSCs could be a useful source to uncover cancer initiation and progression (Fig. 15.3). For example, iPSCs reprogrammed from PDAC cells were used to study pancreatic cancer initiation. These iPSCs formed the early stage of invasive ductal adenocarcinoma, pancreatic intraepithelial neoplasia (PanIN), and then developed into invasive ductal adenocarcinoma after injection into immunodeficient mice. Moreover, cells derived from these iPSCs tumors had the same phenotype of human pancreatic adenocarcinoma. This study showed the ability of iPSCs reprogrammed from cancer cells to capture different stages of cancer progression when they are differentiated back into the original phenotype (Kim et al. 2013). The iPSCs were also generated from patients with myelodysplastic syndromes, a bone marrow disorder and a type of blood cancer. In this case, the iPSCs were reprogrammed from both normal cells and cells with chromosome 7q deletion. The comparison between these two types of iPSCs showed a variation in the differentiation ability. The chromosome 7q deletion impairs the iPSCs differentiation potential into hematopoietic cells, which signifies the role of this deletion in immature cell production in the myelodysplastic syndrome (Kotini et al. 2015, 2017).

The pediatric myeloproliferative disorder, juvenile myelomonocytic leukemia (JMML), mainly affects children that make specimens hard to obtain. The availability of JMML specimens for cancer progression studies and drug screening is limited. The iPSCs reprogrammed from cells of those patients are valuable tools to create disease models. Gandre-Babbe et al. developed iPSCs from juvenile myelomonocytic leukemia patients and used them for drug screening and study clonal and differentiation potentials (Gandre-Babbe et al. 2013). Also, iPSCs could be reprogrammed from mutant noncancer cells taken from volunteers with high risk for specific types of cancers, such as women with germline mutations in BRCA1, which is an increased risk for breast cancer. Griscelli et al. successfully generated iPSCs from blood samples taken from a triple-negative breast cancer patient with BRCA1 mutations (Griscelli et al. 2017). In colon cancer, APC mutations are linked to a high risk of cancer incidence. To understand the relationship between APC mutation and colon cancer induction, Sommer et al. established iPSCs from APC mutant cells and normal cells and then compared them to each other. When differentiated into the intestinal progeny, APC mutations dysregulated signaling pathways and changed lipid metabolism causing abnormalities and resulting in the cell phenotype change. Modeling colon cancer with iPSCs could give insights into the earlier stages of tumorigenesis in the colon mediated by APC (Sommer et al. 2018).

CSCs converted from iPSCs are highly useful to study epigenetic alterations affecting normal stem cells to transform themselves into CSCs. In attempts to find epigenetic changes through the conversion of iPSCs into CSCs, we evaluated the levels of methylation in the genome during CSCs development from iPSCs under the tumor microenvironment (Oo et al. 2018). The methylation status of CpG-islands in CSCs was compared with that in iPSCs. The differentially methylated regions (DMRs) showed that hypomethylation significantly appeared in CSCs when compared to hypermethylation. Furthermore, analysis of the hypomethylated genes by the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool coupled with the KEGG pathway database revealed that several cancer-related pathways were enriched in CSCs derived from iPSCs. Among the nominated genes, high expression of modules such as pik3ca, pik3cb, pik3r1, and pik3r5 genes in the PI3K-Akt signaling pathway was detected. Accordingly, Akt phosphorylation was found to be increased in the obtained CSCs. Therefore, the activation of the PI3K-Akt signaling pathway was involved in the conversion of iPSCs into CSCs with high malignancy and metastatic potential. In a similar way, our previous study demonstrated that different chemical compounds such as Dasatinib, PD0325901, and CHIR99021 accelerated the conversion of iPSCs into CSCs in the presence of CM from LLC cells. Taking into account the signaling pathways inhibited by these compounds, the inhibition of Erk1/2, tyrosine kinase, and/or Gsk-3β was indirectly involved in the enhancement of the PI3K-Akt signaling pathway, resulting in the sustained stemness properties and enhancing the malignant transformation of iPSCs (Du et al. 2020).

We also showed that CSCs, which were derived from iPSCs under pancreatic cancerous microenvironment derived from the CM of PK8 cells, exhibited high expression of ErbB2 and ErbB3 genes and those related to PI3K pathway. Moreover, the inhibition of ErbB2 in iPSCs by lapatinib arrested cell proliferation and impaired the conversion process. This study shows the potential role of ErbB2/ErbB3 heterodimer and its related pathway, PI3K, in CSCs generation and could lead to potentially new options for cancer treatment and prevention (Hassan and Seno 2020a).

15.5 Using iPSCs-Derived CSCs to Study Cancer Microenvironment and Heterogeneity

Tumors have heterogeneous structures that contain many different phenotypes of cells. The tumor microenvironment comprises of different cell types such as fibroblasts, endothe-

lial cells, and immune cells. The tumor microenvironment raises many questions about the mechanisms and interactions controlling tumor heterogeneity. The cellular plasticity which may result in tumor heterogeneity was explained by the concept of CSCs. The cellular plasticity of CSC results from the self-renewal and differentiation potential. Therefore, the development of CSC models will assist in understanding the cancer microenvironment and heterogeneity. CSCs developed from miPSCs were shown to have the ability to differentiate into vascular endothelial cells and contribute to the tumor angiogenesis process (Matsuda et al. 2014). In this study, CSCs were confirmed to develop vascular tube-like structures when cultured on Matrigel (Fig. 15.4). The in vitro tube formation capacity in CSCs showed a dependency on autocrine effects of the angiogenic factors expressed from CSCs such as vascular endothelial growth factor A (VEGF-A) and basic fibroblast growth factor (bFGF) during in vitro tube formation assay. These findings are the first to report in literature and provide insights into the ability of CSCs to generate a self-sustaining niche in the presence of tumor-derived soluble and/or paracrine factors.

In a comprehensive in vivo study, CSC developed from miPSCs displayed critical role in the recruitment of host endothelial vessels into a tumor and the differentiation into endothelial linage, including vasculogenic mimicry (Prieto-Vila et al. 2016). These results show that CSCs have a critical role in tumor vasculature, which could be a good target for cancer therapies in the future. In the same context, we demonstrated that miPSCs-derived CSCs could establish their niche by differentiating into fibroblasts. We concluded that CSCs were the potential origin of the cancer-associated fibroblasts (CAFs), an essential cell type in the stromal compartment of the tumor microenvironment. In this study, the CSCs converted from miPSCs were transplanted into the mammary fat pad of nude mice, and the resulting tumor primary cells expressed CSC-specific markers. CSCs exhibited the ability to differentiate into CAF-like phenotype thus suggesting that they had differentiated into subpopulations of cells that support CSC self-renewal. This was confirmed by evaluating the expression of CAF markers such as smooth muscle actin (α-SMA), fibroblast-specific α-protein (FSP1), TGFβ1, stromal-derived factor-1 (CXCL12), and platelet-derived growth factor (PDGFα), collagen type I alpha 1 (Col1α1) and vimentin. These markers were upregulated in the differentiated myofibroblast-like cells derived from CSC spheroids. These results confirmed that CSCs could be the source of CAFs in the tumor microenvironment (Nair et al. 2017).

In the tumor microenvironment, different hematopoietic cells play critical functions in tumor growth and progression (Gajewski et al. 2013; Hassan and Seno 2020b; Pages et al. 2005; Salama et al. 2009). We also described that adherent CSCs could give hematopoietic cells. In our recent study, we observed that the non-adherent cells (NACs) originated from adherent CSCs and expressed different hematopoietic cell markers, such as CD10, CD34, CD38, c-kit, and Runx1. Also, NACs could home into the bone marrow as well as hematopoietic progenitor cells after injection into the tail vein of busulfan-conditioned nude mice. Another study showed that CSCs deriving from human iPSCs had differentiation capacity into macrophages in vivo. These studies opened the door for further investigation of the origin of immune cells in the tumor microenvironment and the role of CSCs in this field (Hassan et al. 2019; Osman et al. 2020).

Unfortunately, 2D culture models limit cells in one environment providing the only cell-to-cell interaction and failing to represent the human body's complexity. In this context, 3D organoids are necessary to mimic the heterogeneity of different cellular niches. Since cancer has been described as heterogeneous tissue, 3D models mimicking tumor tissue, including the microenvironment in its natural habitat, are required. These models could provide more practical and relevant tools than 2D models during the early stages of anticancer drug screening in vitro. Moreover, the availability of the 3D models will minimize the dependency on animal experiments. The 3D models of cancer using iPSCs provide novel tools for cancer research. More recently, cancer organoid technology employing iPSCs has been described with differentiation and gene editing methods such as CRISPR/Cas. In a recent study, iPSCs were reprogramed from somatic c-Met mutant cells taken from a patient with type 1 papillary renal cell carcinoma (PRCC) and then differentiated into kidney cell progenitors in a 3D environment. When differentiated, established organoids expressed PRCC markers indicating that the cancer initiation process was triggered (Hwang et al. 2019). This combination of different technology presents new opportunities to study human cancers by developing wide scale in vitro models (Papapetrou 2016).

15.6 Drug Screening, Precision Medicine, and New Treatment Strategies

Drug resistance is one of the biggest problems in the cancer field, and many researchers are working to develop effective anticancer drugs. Despite the considerable advancements in the cancer science, there are still many patients suffering from untreatable cancers. The drug resistance, complexity of cancer pathology, and the presence of CSCs which have been implicated in cancer progression and relapse are believed to be responsible for the poor prognosis and overall low survival rate in cancer patients (Kim 2015).

Although many anticancer drugs and treatments for patients currently exist, the effective treatment strategy must be determined depending on each type of cancer. Moreover, cancer is hypothesized as a patient-specific disease that sub-

stantiates the heterogeneity of cancer existing between patients. Genetic variability and epigenetic factors are considered responsible for this heterogeneity (Guo et al. 2019). Therefore, the treatment strategy should be determined for each patient, depending on these factors. Precision medicine or personalized medicine selects a treatment strategy based on the genetic information of cancer patients. The recent advances in the genetic analysis technologies have accelerated precision oncology research. In this context, iPSC is an attractive tool providing cancer models specific to each individual. When combined with new genetic technology, one advantage of iPSC technology is the ability to give the models of cancer and CSC for individuals who have risks of cancer even before cancer develops. The prediction of effective treatment for different types of cancer could be possible with these models. The CSCs derived from the patient's iPSCs will enable screening other treatments and selecting the appropriate one depending on personal genetic and epigenetics profiles. Such predictive approaches could also take advantage of genomic, transcriptomic, proteomic, and metabolomic analyzing tools (Kim 2015; Papapetrou 2016). CSCs are considered resistant to drugs because of their stem cell-like properties such as dormancy, drug export, and high survival capacity and their niches. Therefore, current models for drug screening should consider CSC niche and tumor heterogeneity.

CSCs integrated with advanced 3D-culture technologies could form a useful source for drug screening. Since CSCs are hard to be obtained and maintained in culture, CSCs generated from iPSCs could substitute those from patients or their patient-derived xenografts (PDX) models as a renewable source. CSCs construct their niches by differentiating into cancer cells or cancer-associated cells, as mentioned above, producing heterogeneity in the tumor microenvironment. In a recent study, different types of normal neural cells such as neurons, astrocytes, and glial cells were derived from iPSCs and cultured in a 3D environment with glioblastoma tumor cells. This 3D model was used as an anti-glioblastoma drug screening platform and suggested as a tool with the availability to execute several assays simultaneously in the same condition. We also did a drug screening test on CSC deriving from miPSCs using around 200 anticancer drugs from the screening committee of anticancer drugs (SCADS) library. We showed that daunorubicin, a topoisomerase II inhibitor, can eliminate CSCs in a mechanism associated with caspase pathway activation and P53 accumulation (Seno et al. 2019). We also showed that the combination of paclitaxel and sorafenib could be very effective in suppressing CSC's self-renewal ability when tested on CSCs derived from miPSCs. This combination showed a synergistic effect (Nawara et al. 2020).

The iPSC-derived cells can be used to evaluate the toxicity of anticancer drugs toward normal cells. For instance, iPSC-derived cardiomyocytes are used to assess anticancer drugs cardiotoxicity. Assessment of the anticancer drugs on function and morphology is more flexible with iPSC-derived cardiomyocytes than with patient-derived cardiomyocytes (Schwach et al. 2020). Neuronal cells derived from iPSCs were also used to investigate the chemotherapy-induced peripheral neuropathy, which occurs after cancer treatment. Wheeler et al. showed that the effects of neurotoxic drugs differed between patients. This study showed that sensitivity to paclitaxel increased in neurons, while the expression of tubulin beta 2A class IIa (*TUBB2A*) decreased. Therefore, iPSC-derived cells could be more suitable than cell lines to assess drug's neurotoxic side effects, which are different between patients (Rana et al. 2017).

The expression of surface proteins in iPSCs was suggested to be similar to cancer cells. This similarity drove to another idea to use iPSCs as a cancer vaccine. In a recent study, Kooreman et al. prepared iPSCs from mice, impaired their proliferation by irradiation, and injected them into same mice to vaccinate. Then, breast cancer cells were injected into the vaccinated mice with iPSCs. The cells developed tumors, began to shrink, and disappeared compared with those injected into non-vaccinated mice, wherein the newly formed tumors continued to grow. Moreover, T-cells from mice injected with iPSCs were able to suppress cancer and teratoma growth in other unvaccinated mice. This suggests that T-cells activated by iPSCs injection became able to recognize epitopes shared between iPSC and cancer cells (Kooreman et al. 2018). The CSCs derived from iPSCs could also serve as much more active vaccines since the deriving CSCs from iPSCs could reveal other epitopes specific to CSCs. Overall, iPSCs and their derivative cells and models show a wide range of potential applications in drug screening and developing new cancer prevention and treatment strategies.

15.7 Current Challenges and Future Perspectives

The iPSCs provide patient-based models as a novel tool in the bioscience field. The application of iPSCs in cancer science is still relatively new and requires more effort to shape their usage in cancer research. The unique characters of iPSCs make them ideal tools to study tumor initiation and CSCs. On the other hand, the shared characteristics between iPSCs and CSCs bring new insights into the investment of iPSCs in the oncology field.

Subgroups of CSCs are classified depending on specific surface markers showing the different ability of tumor initiation in animal models. The tumorigenicity of CSCs proved to vary between different subpopulations of CSCs. The iPSCs could serve as a starting point to understand the concept of

plasticity in CSCs. Cancer models from iPSCs offer new opportunities to investigate cancer heterogeneity and tumor microenvironment. The map of interactions between CSCs and tumor microenvironment could be illustrated in the future by the use of different approaches such as genetic engineering, iPSCs-derived cells and 3D cell culture models.

Though it has been almost two decades since CSCs were first isolated, a lot of information about regulation mechanisms must be clarified. The comparison of CSCs induced from iPSC with those derived from patients could reveal some novel drug targets, and the origin and fate of CSCs in disrupted tissue microenvironments. In the new era of basic cancer research and precision oncology, iPSCs deriving from cancer patients or healthy individuals will expand our knowledge about cancer, replacing the need for animal experiments in some stages of drug development and accelerating cancer research. The optimized protocols will be necessary to develop iPSC-derived cancer or CSC models that can reflect cancer's heterogeneity and nature. These cells and models will be available for drug screening and deciding treatment strategy. Therefore, interdisciplinary approaches combining cancer researchers, bioinformatic specialists, bioengineers, and drug companies' efforts are needed to make breakthroughs in this direction.

In the end, the iPSC-derived CSCs could be created for a wide range of cancer types and for each individual enabling the collection of big data that reflect the genetic and epigenetic specificity of each individual. This will predict cancer incidence risk, prevention approaches, and personalized drugs and treatment strategies. This could enhance survival rates and decrease the suffering of cancer patients with minimized side effects of cancer treatments. The iPSCs, as a new tool in cancer research, could open the door of different perspectives to be investigated, challenging the old believed concepts about cancer origin and progression.

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