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Kursad Turksen *Editor*

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Stem Cells: Translational Science to Therapy

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Stem Cells: Translational Science to Therapy

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in regenerative medicine. The chapters in this volume cover a range of topics in the area, from biological function of stem cells in translational studies to tissue and organ regeneration and restoration of function in clinical settings. What all of the chapters have in common is a focus on both the advances and the challenges in this rapidly evolving field.

I remain very grateful to Peter Butler, Editorial Director, and Meran Lloyd-Owen, Senior Editor, for their ongoing support of this series that we have embarked upon.

I would also like to acknowledge and thank Sara Germans-Huisman, Assistant Editor, for her outstanding efforts in getting the volume to the production stages.

A special thank you also goes to the production crew for their work in generating the volume.

Finally, I thank the contributors not only for their support of the series but also for their efforts to capture both the advances and remaining obstacles in their areas of research. I am grateful for their efforts, and trust readers will find their contributions interesting and helpful.

Ottawa, CA, Canada — Kursad Turksen

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Therapeutic Cardiac Patches for Repairing the Myocardium

Benjamin W. Streeter and Michael E. Davis

Abstract

The explosion of stem cell research in the past several years has made its presence known in the field of cardiology and has been recently tasked with solving one of the largest health problems to afflict humanity: cardiovascular disease (CVD). Although stem cell therapy has shown glimmers of promise, significant problems remain that need to be addressed if these therapies are to ever find true success. One way to achieve this success is to take engineering principles and apply them to fabricate engineered cardiac tissues, composed of the aforementioned therapeutic stem cells and biomaterials to bolster the tissue's reparative capacity. In this review, the authors examine advancements in cardiac cell therapy and biomaterial research and discuss how their combination has been used to create tissue-engineered patches capable of restoring function to the damaged or failing myocardium.

Keywords

Cardiac patch · Cardiac tissue engineering · Stem cells · Biomaterials · Myocardial repair

Abbreviations

BMMNCs	bone marrow-derived mononuclear cells
CDC	cardiosphere-derived cell
CM	cardiomyocyte
CSC	cardiac stem cell
CTE	cardiac tissue engineering
CVD	cardiovascular disease
EC	endothelial cell
ECM	extracellular matrix
EHT	engineered heart tissue
ESC	embryonic stem cell
HF	heart failure
HSF1	heat shock factor 1
iPSC	induced pluripotent stem cell
Isl-1	Islet-1
LV	left ventricle
MI	myocardial infarction
miRNA	micro-RNA
MSC	mesenchymal stem cells
PCL	polycaprolactone
PGS	poly(glycerol serbate)
PLGA	poly(lactic-co-glycolic) acid
PLLA	poly-(L-lactic) acid
Sca1	stem cell antigen-1
SIS	small intestinal submucosa
SkMBs	skeletal myoblasts
SMC	smooth muscle cell

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1 Introduction

CVD is the number one cause of death in the United States and claims more lives each year than cancer and chronic lower respiratory disease combined. Around 11.5% of American adults (27.6 million) have been diagnosed with some form of CVD, and, despite many advances in cardiovascular research and medical treatment, many of these patients still succumb to the disease (Benjamin et al. 2018). The limited capability of the heart to regenerate following injury remains a major hurdle to effectively restoring heart function in CVD patients. Specifically, the loss and replacement of cardiomyocytes (CMs) by fibrotic scar and CMs' extremely low turnover rate in the endogenous myocardium leaves the heart unable to perform contractile functions and eventually leads to end-stage heart failure (HF) (Bergmann et al. 2009, 2015 Porrello et al. 2011; Eschenhagen et al. 2017). The only current treatment for end-stage heart disease is heart transplantation. However, several complications including infection, rejection, and malignancies can occur following heart transplant procedures (Wilhelm 2015). Further, many HF patients never receive a transplant and die while on the waitlist due to a shortage of donor hearts (Goldstein et al. 2016). Therefore, there is still a significant need to develop new techniques and therapies to combat CVD and repair injured myocardium.

Recently, cell therapy has emerged as an alternative therapeutic option to transplantation. Several preclinical studies have illustrated the potential of injected stem cells to repair injured myocardium, but this potential is limited due to low engraftment into the native myocardium and lack of differentiation to new, functional CMs (Zhang et al. 2001; Terrovitis et al. 2010; Wu et al. 2011; Nguyen et al. 2016). Many current research efforts are geared towards solving these problems and improving cell therapy's therapeutic benefit, specifically using cardiac tissue engineering (CTE). Tissue engineering combines knowledge from physiology and cellular biology with engineering principles to create scaffolds *in vitro* whose purpose is to improve cell survival, retention, and reparative capacity following implantation. Researchers often use native matrix composition and architecture as blueprints for the construction of scaffolds in laboratory conditions. Combining these scaffolds with the appropriate cell source can lead to the creation of functional tissue and other therapeutic products. In the case of CTE, engineered cardiac patches can be generated to help reverse many of the deleterious effects of CVD (Ye et al. 2013; Hirt et al. 2014; Best et al. 2016; Zhang et al. 2018). This article reviews several different recent CTE and cardiac patch advancements, particularly in the context of restoring contractile function of the myocardium.

2 Cell Sources

It is estimated that CM turnover in the human heart is only about 0.5–2% (Eschenhagen et al. 2017). Although this rate may increase following injury (Hsieh et al. 2007), endogenous CM renewal is not sufficient to restore contractile function. Stem cells from many different sources have been investigated to both replace lost CMs in the native heart as well as to galvanize cardioprotective events through paracrine signaling following their implantation (Dimmeler et al. 2007). Cell sources used in cardiac cell therapy include skeletal myoblasts, bone marrow-derived cells, mesenchymal stem cells, embryonic stem cells, induced pluripotent stem cells, and cardiac stem cells (Chavakis et al. 2010; Zhang et al. 2018). In this section, we review previous work that has utilized these different cell sources to treat the failing heart and highlight important preclinical and clinical findings.

2.1 Skeletal Myoblasts

The first clinical study investigating cell therapy for myocardial repair was published in 2001 by Menasché et al. The authors delivered autologous skeletal myoblasts (SkMBs) to a myocardial infarction (MI) patient. Several SkMB injections were administered in and around sites of necrosis on the LV wall, and the patient's contractile

function improved upon a 5-month follow-up (Menasché et al. 2001). The same group performed a much larger clinical trial in MI patients, The Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) Trial, in 2011, but found that SkMB injection showed no improvement in cardiac function compared to a placebo control injection. Additionally, a greater number of arrythmias were reported in patients receiving SkMB patients (Menasche et al. 2008). Further pre-clinical research investigating SkMBs' potential as a therapeutic cell type for myocardial repair has focused on *ex vivo* engineering of SkMBs to prevent arrhythmic events upon implantation and to improve their reparative paracrine signal secretion. SkMBs do not naturally express the gap junction protein connexin 43 that CMs use to propagate electrical signals through the myocardium. Therefore, several studies have investigated overexpressing connexin 43 in SkMBs to enhance electrical coupling and engraftment to host CMs. These studies have shown that this strategy leads to attenuation of arrythmias and improvement in ventricular function following treatment with connexin-43-overexpressing SkMBs (Abraham et al. 2005; Kolanowski et al. 2014; Antanavičiūtė et al. 2015). SkMBs have also been used in tissue engineered constructs to produce functional tissue and production sources of reparative paracrine signals. Both Blumenthal et al and Siepe et al used polyurethane-based scaffolds seeded with SkMBs as a cardiac patch that was implanted onto the epicardium of MI animal models. Further, the SkMBs used in these constructs overexpressed the pro-survival gene *Akt1*. Implantation of these patches led to increased angiogenesis and reductions in infarct size (Blumenthal et al. 2010; Siepe et al. 2010). Recently, scaffold-free SkMB cell sheets have been shown in clinical trials to decrease arrythmias, improve LV injection fraction, and increase angiogenesis in ischemic and dilated cardiomyopathy (Sawa et al. 2015; Yoshikawa et al. 2018). The results from these studies suggest that the use of SkMB cell sheets and SkMB-based tissue-engineered patches may circumvent some of the safety and efficacy issues that plagued earlier injection-based trials and may make them more successful in larger clinical studies.

2.2 Bone Marrow-Derived Cells

Bone marrow-derived mononuclear cells (BMMNCs) are a heterogeneous group of cells composed of several cell types including hematopoietic progenitor cells, bone marrow mesenchymal cells, and monocytes (Yoon et al. 2010). They have been studied extensively as a candidate to regenerate and repair the damaged myocardium and, to date, are the cell type most commonly used in cardiac cell therapy clinical trials. The first clinical application of BMMNCs took place in 2001, when Strauer et al. delivered BMMNCs to an acute MI patient via intracoronary injection. At follow-up 10 weeks later, the patient had a reduced infarct area and improved cardiac function (Strauer et al. 2001). This study set the stage for several other clinical trials that later used BMMNCs to treat acute MI, including the BOOST (Wollert et al. 2004) and TIME (Traverse et al. 2012) trials, and subsequent follow-ups to these trials, BOOST-2 (Wollert et al. 2017) and LateTIME (Traverse et al. 2011). Additionally, BMMNCs have been used in clinical trials to restore myocardial function in other cardiac disorders including ischemic cardiomyopathy (Perin et al. 2003, 2004, 2012; Assmus et al. 2006; Heldman et al. 2014) and dilated cardiomyopathy (Fischer-Rasokat et al. 2009; Seth et al. 2010; Martino et al. 2015; Xiao et al. 2017) along with others focused on addressing acute MI similar to the BOOST and TIME trials (Sürder et al. 2016). Although many of these studies report modest improvement in left ventricle (LV) function following BMMNC injection in certain areas, these results have not often translated to improvement in clinically meaningful outcomes (Simari et al. 2014). Because of this, other research efforts have been driven toward modifying BMMNC therapy through enhanced delivery methods. One previous study by Lin et al. used self-assembling peptide nanofibers to increase BMMNC retention

following injection and showed improved systolic and diastolic function in pig models of MI (Lin et al. 2010). BMMNC populations have also been enriched by sorting cells for the stem cell marker c-kit, and this enriched cell population has been modified via tissue and genetic engineering methods to enhance the bone marrow-derived c-kit$^+$ cells' efficacy (Quijada et al. 2012; Liu et al. 2016b).

2.3 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are multipotent fibroblast-like cells have the ability to differentiate into osteoblasts, adipocytes, and chondroblasts (Dominici et al. 2006). MSCs can be derived from many different organ and tissue sources in the body, but the most common sources are bone marrow and adipose tissue. They are an attractive cell source due to their immunoprivileged nature, as they lack major histone compatibility complex class II markers and due to their many pro-reparative functions including attenuating fibrosis, enhancing angiogenesis, and kickstarting endogenous cardiac repair mechanisms (Golpanian et al. 2016). Because MSCs have limited retention at target sites when injected (Bahr et al. 2012), these functional improvements are often attributed to the secretion of paracrine effectors (Banerjee et al. 2018). MSCs are the most commonly used cell type in preclinical CTE studies, and several clinical trials have been conducted investigating MSCs as a feasible and efficacious cell source for cardiac cell therapy (Zhang et al. 2018). The POSEIDON trial conducted by Hare et al. compared the use of allogenic and autologous MSCs as a therapy for ischemic cardiomyopathy. The authors injected MSCs transendocardially at three different doses: 20 million, 100 million, or 200 million cells. They found that both autologous and allogenic MSCs significantly reduced infarct size and that the lowest dose, 20 million cells, led to the greatest reduction in LV volume and increase in ejection fraction (Hare et al. 2012). MSCs have also been used in combination with other cell types including BMMNCs and cardiac progenitor cells in preclinical and clinical studies (Heldman et al. 2014; Quijada et al. 2015; Bolli et al. 2018). MSCs may be able to enhance the proliferation and cardiac reparative capacity of these cell types, while also improving therapy through immunomodulation. Further, MSCs are being explored as a cell therapy candidate in pediatric patients with hypoplastic left heart syndrome in the ELPIS clinical trial (Kaushal et al. 2017). Other preclinical work has used computational modeling to better understand MSCs' ability to couple to host myocardium and to elucidate the underlying mechanisms of MSC paracrine secretion that leads to functional benefit (Mayourian et al. 2016, 2017). MSCs have been used extensively in combination with biomaterials for CTE applications, and several of these studies will be discussed later in this review.

2.4 Embryonic Stem Cells

Embryonic stem cells (ESCs) are stem cells isolated from the inner cell mass of an embryo and can give rise to any cell type in the body, excluding those in placental tissue (Evans and Kaufman 1981). ESCs have been used both in their undifferentiated, pluripotent state as well as differentiated into many different cell types for cardiac cell therapy and tissue engineering purposes. Kofidis et al. combined undifferentiated mouse ESCs with Matrigel and injected this mixture into a mouse infarct model. Injected ESCs formed colonies in the infarcted area, showed expression of connexin 43 at contact sites with host cells, and improved fractional shortening (Kofidis et al. 2005). Although in this study no teratoma formation was observed, the tumorgenicity of undifferentiated ESCs, along with ethical issues, immunogenicity, and scaling up of ESC isolation and production, remains a major concern for their clinical translation (Zimmermann 2011). To bypass some of these issues as well as produce more cardiac-like cells, ESCs are often differentiated to cardiac progenitors, CMs, and endothelial cells (ECs). A fibrin patch embedded with ESC-derived SSEA1$^+$ progenitor cells was shown to increase

ejection fraction and angiogenesis when implanted onto infarcted rat hearts (Bellamy et al. 2014). ESC-derived CMs have been a highly investigated topic of cardiac cell therapy, and many studies have shown their efficacy in both rodent and non-human primate models (Laflamme et al. 2007; van Laake et al. 2008; Shiba et al. 2012; Chong et al. 2014). ESC-derived ECs and CMs have been used together with one another to form vascularized cardiac muscle, illustrating the versatility and flexibility of ESCs as a cell source (Caspi et al. 2007). Ongoing research focuses on further maturation of ESC-CMs so that they more closely mimic the mechanical properties of natural CMs *in vivo*. A variety of methods including modulating substrate stiffness (Jacot et al. 2010), electrical stimulation (Martherus et al. 2010), and delivering biochemical cues (Földes et al. 2011) are currently being explored to increase ESC-CM maturation.

2.5 Induced Pluripotent Stem Cells

The Yamanaka laboratory first demonstrated the creation of induced pluripotent stem cells (iPSCs) in 2007. The authors of this study retrovirally transduced human dermal fibroblasts with four key transcription factors: Oct3/4, Sox2, c-Myc, and Klf4. These "Yamanaka factors" converted human dermal fibroblasts into pluripotent stem cells with similar characteristics, including gene expression and morphology, to ESCs (Takahashi et al. 2007). This landmark work set the stage for the use of iPSCs throughout the field of regenerative medicine, including CTE. Because iPSCs are patient-derived from adult fibroblasts, they circumvent the immunogenic and ethical concerns associated with the use of ESCs, while still providing the versatility of a pluripotent cell source. iPSCs have a similar differentiation potential to ESCs (Mauritz et al. 2008) and have been differentiated to a variety of different cardiac cells for use in cell therapy. Ye et al differentiated iPSCs into ECs, CMs, and smooth muscle cells (SMCs) and implanted this tri-lineage combination with a fibrin patch into a porcine model of MI. The iPSC-derived CMs integrated into the host myocardium, while the ECs and SMCs contributed to endogenous vessels, leading to improvements in LV function (Ye et al. 2014). This same group later reported a similar tri-lineage cell patch approach with larger, more clinically relevant dimensions and more advanced maturation of iPSC-CMs through dynamic culture on a rocking platform (Gao et al. 2018). Other studies have shown improvements in iPSC-CM therapy and maturation using anisotropic scaffolds (Khan et al. 2015), naturally-derived extracellular matrix (ECM)-based materials (Fong et al. 2016; Wang et al. 2016), and 3D spheroid aggregation (Beauchamp et al. 2015). Despite their promise, issues with iPSCs, including partial reprogramming that can lead to genetic and epigenetic changes, remain a challenge for clinical translation, and are being addressed by ongoing research (Okano et al. 2013).

2.6 Cardiac Stem Cells

The first population of cardiac stem cells (CSCs) was discovered in 2003 when cells marked by the tyrosine kinase c-kit were isolated from the adult mammalian heart. These cells were clonogenic, self-renewing, and multipotent with the ability to differentiate into CMs, ECs, and SMCs (Beltrami et al. 2003). Since this discovery, several other cardiac stem cell populations have been studied including cardiosphere-derived cells (CDCs), stem cell antigen-1^+ ($Sca1^+$) cells, and Islet-1^+ (Isl-1+) cells (Le and Chong 2016). However, the true nature of these stem cells' role in cardiac biology, specifically that of c-kit+ CSCs, and their contribution to the functional cardiomyocyte population *in vivo* has been hotly debated and shown to be very minimal (Ellison et al. 2013; van Berlo et al. 2014; Sultana et al. 2015; Liu et al. 2016a; Vicinanza et al. 2017, 2018; Gude et al. 2018). Nonetheless, CSCs' ability to provide therapeutic benefit has been shown extensively in both preclinical and clinical studies. SCIPIO, a Phase I clinical trial, was the first in-human clinical trial using autologous c-kit^+ CSCs. The CSCs were used to treat patients

with ischemic cardiomyopathy undergoing coronary artery bypass grafting. Results from the trial showed encouraging outcomes including an increase in LV ejection fraction and a decrease in infarct size (Bolli et al. 2011; Chugh et al. 2012). CADUCEUS, another Phase I clinical trial, employed CDCs to treat patients 2–4 weeks following an MI. Although initial results showed no changes in LV ejection fraction with CDC therapy, CDC treatment did lead to a decrease in infarct size and an increase in viable myocardium (Makkar et al. 2012). As is true with other cell therapy, the functional benefit provided by CSCs is thought to be due largely to paracrine factor secretion. Therefore, current research has employed computational methods to elucidate the paracrine factors most important for cardiac repair (Gray et al. 2015; Agarwal et al. 2017; Sharma et al. 2017). Additionally, our laboratory and others have shown an age-dependent decline in the reparative capability of CSCs (Agarwal et al. 2016; Sharma et al. 2017). Other arms of CSC research have focused on *ex vivo* conditioning through hypoxic growth conditions (Gray et al. 2015), genetic manipulation (Fischer et al. 2009), and combinatorial cell therapy (Avolio et al. 2015; Bolli et al. 2018).

3 Material Considerations

While a sufficiently therapeutic cell source is vitally important to the success of CTE strategies, a biomaterial that can effectively act as a carrier of this therapeutic cell source is equally essential. Further, cell-free materials that bolster the function of endogenous cells and tissue may be a similarly effective option to cell-laden patches. Because of this, research uncovering the optimal parameters of a biomaterial's formulation as well as the most therapeutic combination of material and cell source is paramount for advancing CTE. In this section, we review the considerations for cardiac biomaterials, including material source and fabrication technique, that have been explored in literature.

3.1 Material Sources

3.1.1 Natural Materials

Natural biomaterials are those derived from naturally occurring, biological sources, giving them many advantages for their use in CTE. Most notably, because these materials are derived from *in vivo* sources, they retain much of the microenvironmental architecture cells experience in native tissue. Cues from this biomimetic microenvironment can help improve stem cell maturation and therapeutic function. Additionally, natural materials often have superior biocompatibility, allowing them to avoid immune reaction and thrombosis once implanted. Natural materials that have been used in CTE applications include collagen, chitosan, fibrin, alginate, Matrigel, hyaluronic acid, gelatin, and decellularized ECM (Reis et al. 2016). Decellularized ECM has been derived previously from both cardiac sources, including the myocardium (Singelyn et al. 2012; Seif-Naraghi et al. 2013; Dai et al. 2013) and pericardium (Wei et al. 2006, 2008; Rajabi-Zeleti et al. 2014; Vashi et al. 2015), as well as from non-cardiac sources, including small intestinal submucosa (SIS) (Tan et al. 2009; Okada et al. 2010) and urinary bladder matrix (Kochupura et al. 2005; Robinson et al. 2005). Cardiac decellularized matrix was first harvested by Ott et al in 2008 when the authors delivered detergents including PBS, SDS, and Triton X-100 through coronary perfusion to rat cadaveric hearts. This process eliminated virtually all cellular contents in each heart but retained ECM content such as collagens I and III, laminin, and fibronectin and maintained ECM fiber composition and architecture. Further, the researchers were able to repopulate the decellularized hearts with neonatal cardiac rat cells and produced hearts that displayed expected electrical and contractile responses to electrical stimulation (Ott et al. 2008). The Christman group then expanded upon this decellularization principle and used it to process soluble porcine myocardial ECM that gelled at 37 °C, allowing the ECM to be used as an injectable material (Singelyn et al. 2009). Although decellularized ECM and other natural

materials confer better mimicry of the native cell microenvironment, use of these materials still often faces the challenge of being too mechanically weak to successfully function as a cardiac patch. The human myocardium can range in stiffness from 20 kPa (end of diastole) to 500 kPa (end of systole), while many natural materials have stiffnesses in the tens of Pa range (Reis et al. 2016). Moreover, batch-to-batch variation of natural materials is largely dependent on variations in material sources. Engineering natural materials to more closely mimic *in vivo* stiffnesses and creating consistent material processing practices are needed to continue to push these materials towards clinical use.

3.1.2 Synthetic Materials

While natural materials often provide a ready-made cell microenvironment that closely mimics that seen *in vivo*, synthetic materials offer modular building blocks that can be combined in myriad ways to create scaffolds with more clearly-defined physical and mechanical properties. Additionally, use of synthetic materials allows for more reproducible fabrication processes and often have enough mechanical strength for implantation as a patch. Synthetic materials commonly used in CTE include polycaprolactone (PCL), poly(glycerol serbate) (PGS), polyurethane, poly-(L-lactic) acid (PLLA), and poly(lactic-co-glycolic) acid (PLGA), among several others (Reis et al. 2016). PCL has been used in combination with neonatal rat CMs to form cardiac grafts where CM beating was maintained, cell-to-cell contact occurred, and cardiac specific markers such as connexin 43, cardiac troponin I, and α-myosin heavy chain were expressed (Shin et al. 2004; Ishii et al. 2005). PCL has also been combined with other co-polymers, including those from synthetic sources like carbon nanotubes (Wickham et al. 2014) and PGS (Tallawi et al. 2016) and from natural sources such as chitosan (Pok et al. 2013) and gelatin (Kai et al. 2011), to form more complex constructs. PGS is another particularly attractive synthetic polymer due its elastic nature, making it ideal for mimicking the mechanically dynamic environment of contracting CMs. Additionally, mechanical characterization of PGS scaffolds has shown its Young's modulus to more closely mimic that of native myocardium compared to several other synthetic polymers (Chen et al. 2008). PGS has also been shown to provide a viable environment for a variety of cells including fibroblasts and ESC-CMs (Chen et al. 2010). While synthetic materials offer many benefits for CTE, there are also problems with their use that still need to be solved. Synthetic materials provide a great deal of mechanical support, but this often means that their stiffnesses can be orders of magnitude greater than that seen in the native myocardium. This may cause a mechanical mismatch with the heart upon implantation and could induce additional burden on the pumping function of the heart. Additionally, due to many of these materials' bioinert nature, they may not be able to fully propagate electrical signals to the heart and could cause arrythmias when implanted. Continuing research into properly tuning the properties of synthetic polymers and co-polymers is needed to address these issues.

3.2 Material Fabrication Techniques

3.2.1 3D Bioprinting

Several different techniques for fabricating biomaterials exist, and each technique can significantly impact material properties. 3D bioprinting, one such technique, is the process of depositing sequential layers of biological materials (often a biomaterial/cell mixture) on top of one another to form 3D structures. Utilizing this technique allows for precise control of cardiac patch geometry and modulation of properties such as strand diameter and pore size (Murphy and Atala 2014). Further, one major issue with cardiac patches is delivery of a clinically relevant number of cells. Patches may suffer from either low proliferation of cells or insufficient oxygen and nutrient delivery to cells within the patch, leading to a high degree of cell death. 3D printing allows for the creation of complex porous networks that permit efficient nutrient delivery, and ensures a uniform distribution of cells throughout the patch

(Mosadegh et al. 2015). 3D printing has been used with many combinations of materials and different cell types, often termed "bioinks", for cardiac applications. Gao et al. recently printed a methacrylated gelatin-based patch with CMs, SMCs, and ECs, all differentiated from iPSCs. To precisely control the architecture of their patch, the authors used multiphoton-excited 3D printing and mapped the blueprint for the printed scaffold to the distribution of fibronectin within the mouse myocardium (Fig. 1). This technique produced printing with a resolution of <1 μm, giving a highly accurate approximation of native ECM structure. Following printing and seeding with the three cell types, the patch beat synchronously after just 1 day and improved cardiac function, infarct size, and vessel formation when implanted into a mouse model of MI (Gao et al. 2017). Our laboratory has also used gelatin methacrylate as a bioink for 3D printing, and recently combined gelatin methacrylate with porcine-derived cardiac ECM and neonatal c-kit^{+} CSCs to create a cardiac patch for right ventricular heart failure (Bejleri et al. 2018). Another recent study used 3D printing to print gelatin microchannels to improve MSC differentiation and CM alignment and beat synchronicity. MSCs aligned and elongated at a higher rate in microchannel constructs than unpatterned constructs, leading to a higher expression of cardiac specific proteins. CMs also showed more pronounced alignment and display synchronous beating on microchannels (Tijore et al. 2018). These studies highlight the ability of 3D bioprinting to produce complex and highly defined architectures to influence cell behavior and function.

3.2.2 Electrospinning

Electrospinning is another fabrication process that has garnered a great deal of attention recently in CTE. During scaffold fabrication, a high voltage (~1–30 kV) is applied to the needle of a syringe containing a polymer of choice. As the polymer is extruded out of the needle, the electrostatic force at the surface of the polymer droplet and the Coulombic force from the surrounding electric field overcome the surface tension of the droplet, leading to the formation of a polymer jet that is deposited onto a grounded collector (Li and Xia 2004). Electrospinning produces polymer patches with nanoscale fibers and high porosity, mimicking the fiber composition of native ECM and allowing for efficient nutrient diffusion throughout the patch (Liu et al. 2012). Additionally, electrospinning is a highly reproducible, tunable, and cost-effective process. Electrospinning was first used in CTE by Shin et al. to create PCL patches seeded with neonatal rat CMs (Shin et al. 2004). Since this initial study, electrospinning has been used expansively with both natural and synthetic materials for cardiac applications (Kitsara et al. 2017). Notably, electrospinning has been employed to create patches with highly aligned fibers to align cells, more closely mimicking CM morphology and spatial organization in the myocardium. For example, neonatal rat CMs seeded on aligned PGS/gelatin electrospun patches showed greater anisotropic sarcomere formation and synchronized beating compared to random patches (Kharaziha et al. 2013). Electrospun scaffolds are also easily modifiable with other components to improve cell attachment, survival, and reparative function. These components include gold nanoparticles (Shevach et al. 2013; Ravichandran et al. 2014; Fleischer et al. 2014), growth factors such as VEGF (Ravichandran et al. 2015; Chung et al. 2015), and ECM proteins such as fibronectin (Badrossamay et al. 2010; Fleischer et al. 2015) and laminin (Yu et al. 2014).

3.2.3 Engineered Heart Tissues

While 3D bioprinting and electrospinning precisely control material architecture to mimic *in vivo* tissue, creating engineered heart tissues (EHTs) capitalizes on cells' inherent ability to self-assemble and form tissue. The first EHT was created by Eschenhagen et al. in 1997 when CMs from chick embryos were blended with a collagen matrix and seeded between two Velcro-coated glass tubes. The EHT that formed was able to beat in response to electrical pacing and increased contractile strength with increased pacing frequency (Eschenhagen et al. 1997). Since this initial work, EHTs have been produced in a variety of geometries with an abundance of

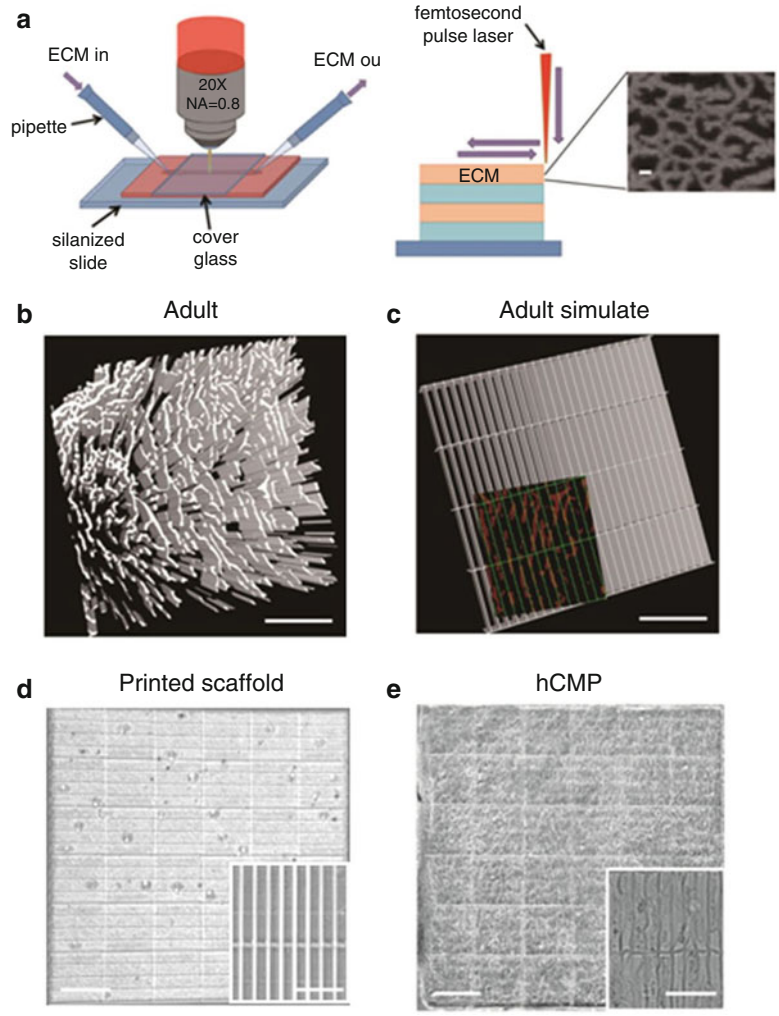

Fig. 1 Human-induced pluripotent stem cell–derived cardiac muscle patch (hCMP) fabrication via 3-dimensional multiphoton excited (3D-MPE) printing. (**a**) The extracellular matrix (ECM) and associated crosslinking solution are passed through the optical interrogation path, although the laser power and dwell time are modulated to deposit ECM at each x, y location in each z plane. The submicron scale features produced in the ECM scaffold are displayed in the inset (scale bar = 1 μm). Three-dimensional structures can be generated by combining multiple layers with the same or different ECM patterns. (**b**) Sections from the heart of an adult mouse were immunofluorescently stained for the presence of fibronectin and scanned via MPE (scale bar = 200 μm); then, (**c**) the distribution of fibronectin in the native tissue was simulated in a template. The simulated channels (green, 100 × 15 μm) are shown overlaying the fibronectin pattern of the native tissue (red) in the inset (scale bar = 100 μm). (**d** and **e**) The simulated template was used to determine the position of crosslinks in a solution of gelatin methacrylate, thereby producing a native-like ECM scaffold (**d**); then, the scaffold was seeded with human-induced pluripotent stem cells (hiPSC)–derived cardiomyocytes (CMs), endothelial cells (ECs), and smooth muscle cells (SMCs) to generate the hCMPs (**e**) The complete hCMP is shown in the larger image (scale bar = 400 μm), whereas the individual channels and incorporated cells are visible in the inset (scale bar = 50 μm). (Reused with permission from Gao et al.)

different cell types. For instance, one study generated EHTs from a combination of Matrigel and a mixed population of cardiac cells including CMs, fibroblasts, ECs, and SMCs. These EHTs were then formed into many different shapes including stars, horizontal tubules, a mesh

network, and a "rope" structure (Fig. 2) (Naito 2006). EHTs have also been investigated as a therapy for chronic MI. Loop-shaped EHTs formed from ESC-CMs and collagen I matrix were mechanically stretched and implanted into a rat model of MI. The EHTs showed high engraftment rates and significantly improved the ejection fraction of rat hearts (Riegler et al. 2015). More recently, a square patch EHT generated from neonatal rat ventricular cells and a gel mixture of thrombin, fibrinogen, and Matrigel was formed using a large (18 mm X 18 mm) PDMS mold. The patches were epicardial implanted onto rat ventricles and electrically coupled to healthy host myocardium without altering any electrophysiology of the heart (Jackman et al. 2018). Because it is very difficult to couple EHT patches to unexcitable, damaged myocardium, ongoing research will continue to work towards more effectively forming effective cell-cell contacts between patch and native cells to allow for more functional integration of patches into hearts.

3.2.4 Hydrogels

Hydrogels are water-insoluble polymers that are formed through crosslinking of synthetic and/or natural precursor polymers. These gels are often at liquid phase *in vitro* but, following injection to a site *in vivo*, will gel and can help replace damaged ECM and deliver therapeutic cells (Sun and Nunes 2015). Hydrogels used in CTE have been both in cell-free and cell-laden forms. Cell-free alginate hydrogels were implanted into rat MI models and were shown to replace up to 50% of the scar tissue area. Additionally, alginate gel injection attenuated LV dysfunction and achieved similarly therapeutic outcomes to neonatal rat CM injection (Landa et al. 2008). Another acellular approach used a cell-free collagen I patch and found that when implanted, the collagen patch attenuated remodeling and fibrosis and enhanced angiogenesis in infarcted LVs (Serpooshan et al. 2013). Hydrogels have also been utilized to facilitate differentiation of stem cells into more mature cardiac phenotypes through methods such as

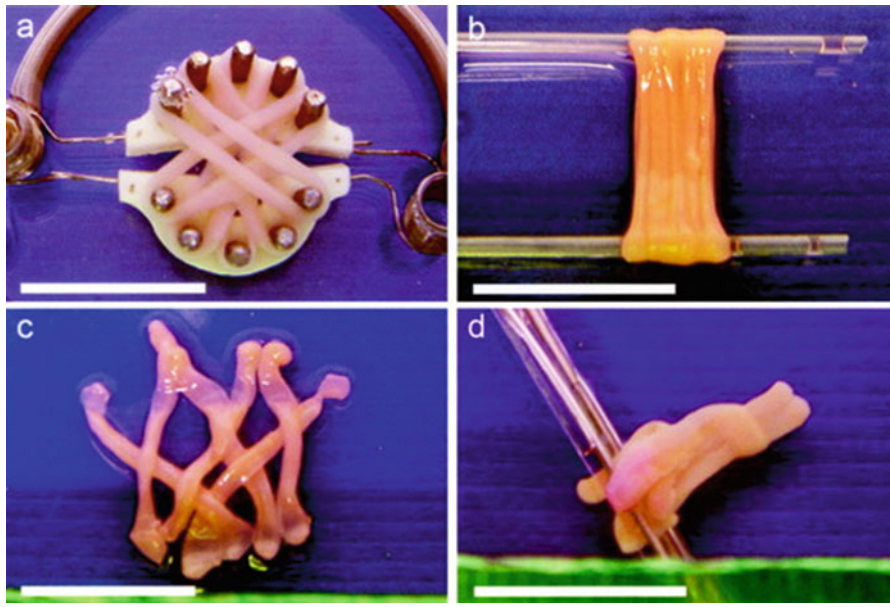

Fig. 2 Generation of different EHT geometries. EHTs fuse after sustained contact to form in-unison contracting complex cardiac muscle constructs. Star-shaped EHTs (**a**) were generated by stacking 5 EHTs on a custom-made holder. Single-unit EHTs fused in the center. 5 EHTs (**b**) were grown on horizontal glass pipettes. Adjacent EHTs fused to form a tubular construct. 6 EHTs (**c**) were cut open and layered to form a contracting network. 3 EHTs (**d**) were twirled together to form a longitudinal "rope" structure. Bars: 10 mm. (Reused with permission from Naito et al.)

cellular aggregation (Kerscher et al. 2016) and incorporation of important signaling molecules such as Notch1 (Boopathy et al. 2014). Continuing research focuses on finding the right combination of cells and hydrogel polymer and introducing components to hydrogels to modulate their mechanical and bioactive properties.

4 Cardiac Patch Clinical Trials

Previous sections of this review discussed several cell therapy clinical trials. These trials used cell injections, separate from any supportive material. However, there are other previous clinical trials have been carried out using biomaterials, both with and without cells. The first such clinical study, the MAGNUM trial, was accomplished by Chachques et al. and used a patch consisting of BMMNCs seeded onto a collagen I matrix to treat infarct patients. Specifically, 250 ± 28 million BMMNCs were injected into several infarcted sites on the heart, and then a patch consisting of the same number of BMMNCs and a collagen I matrix was sutured on the epicardium on top of the scarred area. Results showed that the patch therapy enhanced ejection fraction, increased scar thickness, and improved LV filling (Chachques et al. 2007). The same group later compared their injection/patch procedure to BMMNC injection alone and found that injection/patch group significantly improved ventricular filling compared to cell injection alone (Chachques et al. 2008). The next cardiac patch clinical study investigated the use of CorMatrix®, a cell-free, porcine SIS-derived ECM. The first in-human studies used CorMatrix® to repair cardiac and vessel defects in 37 congenital heart patients (Scholl et al. 2010). It was then shown to be a clinically feasible option for repairing LV complications following MI in 11 patients (Yanagawa et al. 2013). CorMatrix® has also been studied as an epicardial patch in both rat and pig models of MI and shown to improve myocardial recovery following MI (Mewhort et al. 2014; Mewhort et al. 2016). A clinical trial applying the CorMatrix® epicardial patch to 8 MI patients undergoing coronary artery bypass grafting has also taken place, although results have not been made available (Fedak 2017). Another acellular therapy was used in the AUGMENT-HF trial, in which Algisyl, an injectable calcium alginate hydrogel, was delivered to patients with advanced heart failure. Algisyl was administered to the LV wall via 12 to 15 injections during left anterior limited thoracotomy. At 12-months follow-up there was statistical improvement in VO_2 and 6-minute walk test distance (Anker et al. 2015; Mann et al. 2016). Finally, the PRESERVATION clinical trial used a bioabsorbable scaffold made up of sodium alginate and calcium gluconate, IK-5001, to treat ST-segment-elevation MI patients. A pilot study in 27 patients showed that use of the IK-5001 was safe and feasible in ST-segment-elevation MI patients and reported no IK-5001-related adverse events upon 6 months follow-up (Frey et al. 2014). A larger study involving 201 similar patients was later undertaken, but results showed that implantation of IK-5001 did not improve LV function or attenuate LV remodeling at 6 months follow-up (Rao et al. 2016). These clinical studies illustrate the feasibility of tissue-engineered constructs to be used to improve cardiac function in the failing heart and show the opportunities for promising preclinical work to be effectively translated to clinical settings.

5 Challenges and Outlook

5.1 Current Issues

Although significant progress in creating efficacious cardiac patches has been made, several issues persist that ongoing research is working to address. Extensive loss of CMs following cardiac injury and low CM renewal means that a high rate of cell engraftment upon patch implantation is necessary to effectively restore the loss of contractile function. However, it is estimated that only 0.1–10% of cells engraft into the host myocardium following injection (Zhang et al. 2018). This loss of cells can be attributed to cell leakage at the injection site, which is pushed further out as the heart contracts and transported away from the injection site due to blood flow (Terrovitis et al.

2010). Biomaterials, along with methods such as genetic modification and *ex vivo* preconditioning, certainly improve cell engraftment, but present issues of their own that must be addressed (Wu et al. 2011). Even with adequate cell engraftment, the engineered tissue of a cardiac patch must be able to both electrically and mechanically couple to the host myocardium. Perhaps the biggest concern with lack of integration of cardiac patches is formation of arrythmias. Previous studies have shown that implantation of cell types such as MSCs can lead to significant development of arrhythmias and adversely affect the electrophysiology of the heart (Zheng et al. 2013). Further, in contrast to small-animal models, implantation of ESC-CMs in non-human primates led to ventricular arrythmias (Chong et al. 2014). Beyond integration with the native myocardium, immunological issues with implanted cardiac patches may also hamper any therapeutic benefit achieved. Implanted materials can cause both innate and adaptive immune responses and may exacerbate acute inflammation that is occurring at the site of injury (Crupi et al. 2015). Further, xenogeneic materials and allogeneic stem cell sources can often illicit damaging immune responses (Papalamprou et al. 2016). All these issues must be addressed in future research to engineer a successful cardiac patch.

5.2 Future Directions

5.2.1 Strategies for Advanced Cardiac Maturation

While maturation of cells on cardiac patch constructs has improved greatly over the years, more techniques to further advance this maturation are needed to produce true-to-form CMs *in vitro*. Research has focused on subjecting CM precursor cells, such as CSCs, and immature pluripotent cell-derived CMs to similar physical forces native CMs experience in the myocardium, namely mechanical strain and electrical stimulation. French et al. cultured c-kit^{+} CSCs on various ECM proteins and subjected the CSCs to cyclic strain. The CSCs aligned more efficiently when subjected to higher strain, and this led to an increase in angiogenic paracrine factor production on many of the ECM substrates (French et al. 2016). Similar maturation of CSCs and induced calcium handling was also seen following electrical stimulation (Maxwell et al. 2016). This phenomenon has also been seen in neonatal rat CMs, where electrical stimulation induced calcium handling once again and increased CMs' expression of cardiac differentiation markers, independently of contractile effects (Martherus et al. 2010). While both of these maturation techniques can have profound effects on their own, they are often used in combination with one another (Ruan et al. 2016) and with other techniques, including perfusion culture (Lux et al. 2016) and cellular coculture with cell types such as ECs and fibroblasts (Tulloch et al. 2011). Importantly, these techniques not only induce increased maturation and cardiac differentiation, but also confer enhanced cardiac benefit *in vivo*. Tissues engineered from hESC-CMs and ECs and pre-conditioned with mechanical stress showed increased engraftment into the hearts of athymic rats (Tulloch et al. 2011). A similar patch using the elastic polymer poly(lactide-co-caprolactone) and hESC-CMs preconditioned with cyclic strain attenuated fibrosis in a rat infarct model (Gwak et al. 2008). Following mechanical preconditioning, SkMBs were also shown to electrically couple to host myocardium upon implantation and expressed the cardiac-specific gap junction protein connexin 43 (Treskes et al. 2015). More recently, Ronaldson-Bouchard et al. achieved the greatest degree of maturation in engineered cardiac tissue to date using iPSC-CMs and physical conditioning with increasing intensity. Specifically, tissues formed from early iPSC-CMs, just after the cells began to beat, were subjected to electrical stimulation from 2 Hz to 6 Hz, with the stimulation increasing 0.33 Hz each day (Fig. 3). Following 4 weeks of stimulation, the engineered tissues showed sarcomere length and mitochondria density at physiological levels, had formed transverse-tubules, and had functional calcium handling (Ronaldson-Bouchard et al. 2018). Contuining optimization of electrical and mechanical stimulation techniques and fabrication of complex bioreactors

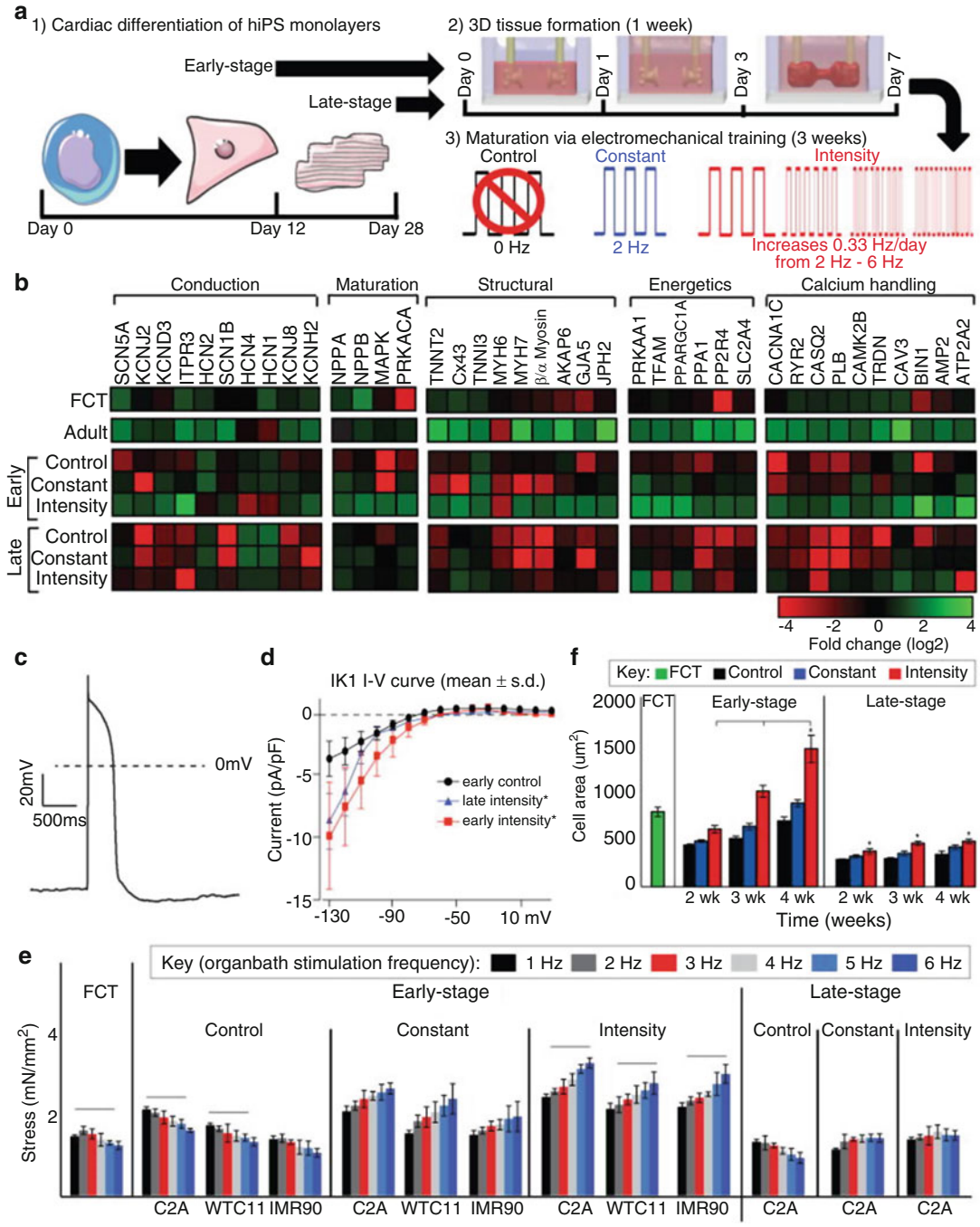

Fig. 3 Intensity training of cardiac tissues derived from early-stage hiPS-CMs enhances maturation. (**a**) Experimental design: early-stage or late-stage hiPS-CMs and supporting fibroblasts were encapsulated in fibrin hydrogel to form tissues stretched between two elastic pillars and made to contract by electrical stimulation. Gradual increase in frequency of stimulation to supra-physiological levels (intensity regime) was compared to stimulation at constant frequency (constant regime), unstimulated controls and human adult and fetal heart ventricles. (**b**) Gene expression data for six groups of cardiac tissues, and adult and fetal heart ventricles. (**c**) Action potential for the early-stage intensity-trained group. (**d**) IK1 current–voltage (I–V) curves (mean ± s.d.). (**e**) Early-stage intensity-trained tissues from all three iPSC lines (C2A, WTC11, IMR90), but not the other groups, developed a positive force–frequency relationship after 4 weeks of culture. Line above graph indicates $P < 0.05$ versus other timepoints using two-way ANOVA followed by Tukey's HSD test; *P < versus control group using one-way ANOVA followed by Tukey's HSD test. (Reused with permission from Ronaldson-Bouchard et al.)

combing these techniques may be needed to more effectively mature stem cell-derived CMs and tissue-engineered grafts.

5.2.2 Cell-Free Products

While cell therapy has been shown to provide functional benefit in the injured heart, exogenous cell engraftment into host myocardium is very low (Zhang et al. 2018). These observations have led researchers to believe that the benefit exhibited following cell therapy must be attributable to paracrine mechanisms from implanted cells, prior to the cells being washed away from the site of injection. Therefore, a recent push has been made to both define the secretory factors responsible for cardiac improvement and to use these reparative factors as their own therapy, separate from the cells that produce them. Informatics and systems biology methods have proved to be powerful tools for elucidating the functional units of many different cell types. Sharma et al. used informatics techniques to identify upregulated growth factors and signaling pathways in the secretome of c-kit$^+$ CSCs from adult and neonatal patients. Using this analysis, the authors were able to pinpoint heat shock factor 1 (HSF1) as a crucial regulator of the secretome of CSCs and demonstrated that knockdown of HSF1 led to decreased secretion of important pro-reparative factors such as VEGF, ANG1, and SDF1 (Sharma et al. 2017). While growth factors such as these certainly play an important role in the reparative secretome, other research has focused on characterization of another important player in the secretome: exosomes. Exosomes are small (30–120 nm in diameter) extracellular vesicles that form within larger multivesicular bodies and release from the cell upon fusion with the cell membrane. Exosomes can contain proteins, lipids, RNA, and/or DNA, and it is this cargo that can provide reparative effects to the heart (Garikipati et al. 2018). Both Gray et al and Agarwal et al used systems biology methods to distinguish what exosomal cargo from rat and human pediatric c-kit+ CSCs, respectively, correlated most strongly with improvement in functional outcomes such as angiogenesis, fibrosis, and ejection fraction (Gray et al. 2015; Agarwal et al. 2017). Specifically, these studies correlated the presence of different micro-RNAs (miRNAs) with these functional changes. It has been shown that miRNAs alone improve heart function, making them one of the most important pieces of exosomal cargo. A recent study identified miR-21-5p as the functional miRNA of the MSC secretome and showed that delivery of miR-21-5p alone was enough to increase expression of calcium handling genes and, consequently, contractility in EHTs (Mayourian et al. 2018). To avoid the issues with implantation of cells, other cell-free delivery methods have been explored to deliver reparative secretome products. In one study, the complete secretome from adipose-derived stem cells was loaded into a gelatin and Laponite® hydrogel and injected into a rat acute MI model and shown to reduce scar area, increase angiogenesis, and improve several cardiac functional parameters (Waters et al. 2018). Another innovative method to deliver therapeutic secretome components is the use of "synthetic stem cells", a method created by Tang et al. Synthetic stem cells are PLGA microparticles loaded with stem cell conditioned media and coated with stem cell membranes. They have been fabricated using the secretomes and membranes from both MSCs and CDCs, and both sets of synthetic stem cells have been shown to repair the heart in mouse models of MI (Tang et al. 2017; Luo et al. 2017). Future work on cell-free products will continue to identify the functional units of different therapeutic cells' secretomes and will work to find efficient ways to deliver and scale up the production of these factors.

6 Conclusion

CVD has been and continues to be the number one cause of death in the world. Even with the technologies at the disposal of modern medicine, there remains an immensely significant need to treat those with CVD and to restore the pumping force of failing hearts. CTE holds promise as the missing piece to the puzzle of treating CVD.

Significant advances have been made in recent years to both identify therapeutic cell types and to combine these cells with supportive materials to further enhance their therapeutic potential. Because current cell-based therapies suffer from a lack of engraftment into the host myocardium, paracrine effectors are currently the main source of providing functional benefit for these therapies. As the CTE field continues to progress, engineered cardiac tissues will become more and more complex and will more closely mimic the native myocardial structure. It will be important in the future to understand how to properly strike a balance between working towards integrating functional, lab-engineered tissue into host tissue and modulating tissue-engineered constructs to maximize their paracrine effects. If paracrine effects prove to be the most beneficial route of research, further research into the therapeutic potential of various cell-free secreted factors alone could prove to be the future of cardiac repair. Other practical considerations including implantation method, manufacturing concerns, and scalability of new patches and therapies will also need to be considered. As CVD continues to plague the world, CTE research will continue to harness the power at the intersection of engineering and cardiac biology and use it to tackle the complex problems that CVD presents.

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Human Induced Pluripotent Stem Cells in the Curative Treatment of Diabetes and Potential Impediments Ahead

Nidheesh Dadheech and A. M. James Shapiro

Abstract

The successful landmark discovery of mouse and human inducible pluripotential stem cells (iPSC's) by Takahashi and Yamanaka in 2006 and 2007 has triggered a revolution in the potential generation of self-compatible cells for regenerative medicine, and further opened up a new avenue for "disease in dish" drug screening of self-target cells (Neofytou et al. 2015). The introduction of four 'Yamanaka' transcription factors through viral or other transfection of mature cells can induce pluripotency and acquired plasticity. These factors include transduction with octamer-binding transcription factor-4 (*Oct-4*), nanog homeobox (*Nanog*), sex-determining region Y-box-2 (*Sox-2*) and MYC protooncogene (*cMyc*). Such cells become iPSC's (Takahashi and Yamanaka 2006). These reprogrammed cells exhibit increased telomerase activity and have a hypomethylated gene promotor region similar to embryonic stem cells (ESC's). These milestone discoveries have generated immense hope that diseases such as diabetes could be treated and effectively cured by transplantation of self-compatible, personalized autologous stem cell transplantation of β-cells that release physiological insulin under glycemic control (Maehr et al. 2009; Park et al. 2008) (Fig. 1). Diabetes is a profligate disease of disordered glucose metabolism resulting from an absolute or relative deficiency of insulin, the consequences of which lead to immense socio-economic societal burden. While there are many different types of diabetes, the two major types (type 1 diabetes (T1DM) and type 2 diabetes (T2DM) are caused respectively by immune-mediated destruction (T1DM) or malfunctioning (T2DM) insulin-producing β-cells within the endocrine pancreas, the islets of Langerhans (Atkinson et al. 2011; Holman et al. 2015; You and Henneberg 2016). Almost 425 million people are affected by the global burden of diabetes, and this is predicted to increase by 48% (629 million) by 2045 (International Diabetes Federation Atlas 8th Ed 2018). Whole pancreas or islet cell transplantation offer an effective alternative to injected insulin, but both require lifelong potent immunosuppression to control both allo-and autoimmunity. Whole pancreas transplantation involves invasive complex surgery and is associated with greater morbidity and

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occasional mortality, while islet transplantation involves a minimally invasive intraportal hepatic infusion. Generally, whole pancreas transplantation provides greater metabolic reserve, but this may be matched by cumulative multiple islet infusions to achieve insulin independence. An additional challenge of islet transplantation is progressive loss of complete insulin independence over time, which may be multifactorial, the dominant factor however being ineffective control of autoimmunity. Both whole pancreas and islet transplantation are restricted to patients at risk of severe hypoglycemia that cannot be stabilized by alternate means, or in recipients that are already immunosuppressed in order to sustain a kidney or other solid organ transplant. The risks of chronic immunosuppression and the scarcity of human organ donors mean that both of these transplantation therapies cannot presently be extended to the broader diabetic population (Shapiro 2011; Shapiro et al. 2006). Recent progress in xenotransplantation of multiple knock-out 'humanized' pig islets could offer one potential solution, perhaps aided by clustered regularly interspaced short palindromic repeats/CRISPR associated-9 (CRISPR/Cas-9) gene editing approaches, but this remains to be proven in practice. Human stem cell derived new β-cell products could effectively address the global supply challenge for broad application across all forms of diabetes, but recurrent autoimmunity may still remain an insurmountable challenge. Considerable progress in the generation of human stem cell derived SC-β cells from ESC, iPS and other adult cell sources such as mesenchymal stem cells (MSCs) offer huge hope that a personalized, 'syngeneic' cell could be transplanted without risk of alloimmunity, thereby securing sufficient supply to meet future global demand (Cito et al. 2018).

Keywords
Pluripotential stem cell · Diabetes · Transplant

Cythera Inc. (subsequently Novocell Inc., and now Viacyte, Inc.) were the first to report a reproducible and efficient *in-vitro* protocol for differentiation of hESC into polyhormonal insulin-expressing 'stage 4' cells that remained glucose unresponsive and immature with 7% insulin content (D'Amour et al. 2006). A period of 2–3 months of *in-vivo* maturation in immunodeficient mice or rats was required to generate complete differentiation and glucose-responsive regulated insulin production to human set-points. These positive findings replicated in over 2,000 mice and rats laid foundation for a first-in-human phase I/II clinical trial initiated in 2014 by ViaCyte Inc., in partnership with the University of San Diego and the University of Alberta in Edmonton, Canada (ClinicalTrials.gov identifier: NCT02239354) (Cito et al. 2018). Later, Rezania and colleagues described a multi-step differentiation protocol that efficiently converted hESC's into glucose-responsive insulin-producing 'stage 7' cells *in-vitro*. The newly generated cells showed an insulin secretion pattern more similar to human islets and reversed experimental induced diabetes by 2 months after transplantation (Rezania et al. 2014). Melton and colleagues applied a parallel strategy and 3-dimensional cell culture to generate mature, mono-hormonal and functional SC-β cells that ameliorated hyperglycemia within 2 weeks from transplantation in NRG-Akita mice (Millman et al. 2016). Currently, it remains unknown whether immature 'stage 4' or more mature 'stage 7' or 'stage 8' cells provide superior cell products for clinical transplantation. Transplantation of more immature cells may carry a survival advantage as their lower metabolic state and reduced oxygen consumption may allow them to tolerate the initial hypoxia until neovascularization and engraftment is complete. Theoretically more immature 'stage 4' cells might be more susceptible to teratoma or other unregulated growth, but in practice thus far this has not been borne out experimentally.

The de-differentiation, trans-differentiation and maturation protocols for handling hESCs may be readily modified for generation of

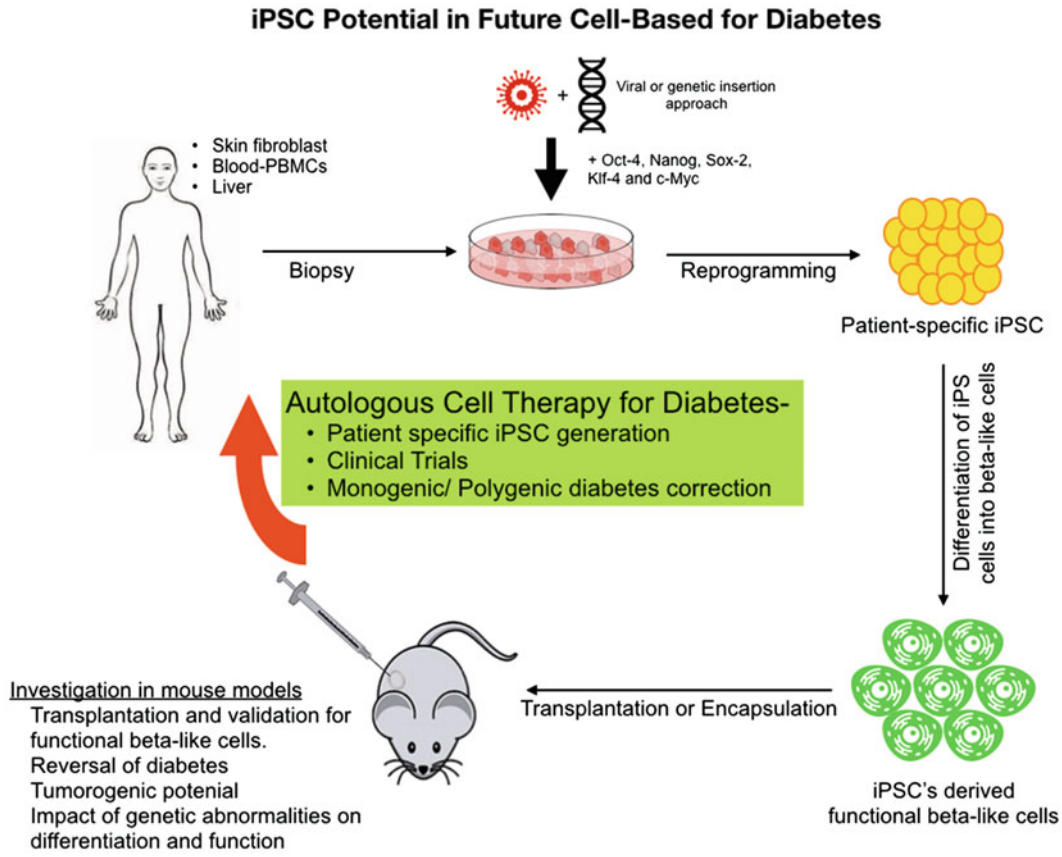

Fig. 1 Schematic representation of inducible pluripotent stem cell (iPSC) generation from somatic cells and their application in the patient-specific iPSC-based personalized-medicine based diabetes therapy. Disease-free iPSCs may be potentially generated from healthy individual- or diabetic patient-derived somatic cells using reprogramming with viral, DNA-, RNA-, protein-, miRNA-, or small molecule-mediated reprogramming systems. iPSC's derived from patients can be further differentiated into insulin-secreting pancreatic for transplantation into patients with diabetes for cell-based therapy

β-cells from MSCs or iPSCs. The first protocol completely differentiating fibroblast-derived hiPSC's into insulin-producing cells reported a four-stage differentiation protocol following stages of definitive endoderm, pancreatic endoderm, endocrine tissue and maturation of insulin-producing cells (Zhang et al. 2009).

1 Generation, Validation, and Pancreatic Differentiation of Murine iPSCs

Ten years following the discovery of iPSCs, several murine studies have highlighted promising implications for their potential as cell therapy in diabetes. Alipio et al in 2010 described effective reversal of diabetes in streptozotocin chemically-induced and type 2 ($Lepr^{db}$) diabetes in mice using iPSC-derived cells. Skin fibroblast-derived iPSC's were converted efficiently into insulin-producing cells in a 3-stage differentiation protocol. These differentiated iPSC's were glucose responsive *in-vitro* and corrected hyperglycemia *in-vivo* upon transplantation. Although implanted iPS cells restored insulin secretion and normalized glucose levels within 2 days post-implantation, limitations included high rates of mortality and recurrence of diabetes in a fraction of mice, but with no evidence of iPSC-derived tumor formation. Despite implantation of a substantial iPSC-derived cell mass, serum insulin levels were not

elevated, and the transplants did not modulate body weight in *Lepr*^{db} mice (Alipio et al. 2010).

Ssang-Goo Cho and colleagues further implanted differentiated iPSC-derived endocrine cells into autoimmune nonobese diabetic (NOD) spontaneously diabetic mice with reversal of hyperglycemia. Two separate NOD-iPSC lines were generated from skin fibroblast and pancreatic lineage epithelial cells (PE) in a four-step differentiation protocol using retinoic acid. Only PE-iPSC's were shown to convert into functional and glucose-responsive insulin-producing endocrine cells capable of reversing diabetes after renal-subcapsular transplantation in NOD/SCID mice. The skin fibroblast-derived iPSCs failed to demonstrate an endocrine differentiation gene signature and did not secrete insulin (Jeon et al. 2012). Both the Ssang and Alipio data raise concern relating to the maturation capacity in generating sufficiently potent mature β cells for transplantation, but support proof of concept for the future potential application of patient self-derived iPSC's for autologous β cell transplantation, once further kinks are worked out.

Interestingly, due to potential pitfalls in the chemical differentiation protocols, two separate studies confirmed that differentiation of mouse embryonic fibroblast (MEF) iPSC lines with genetic reprogramming or protein transduction of pancreatic endocrine differentiation transcription factors with pancreatic and duodenal homeobox-1 (*Pdx-1),* neurogenic differentiation factor-1 *(NeuroD1),* and V-Maf avian musculoaponeurotic fobrosarcoma oncogene homologue-A *(Maf-A)* along with chemical induction (Kaitsuka et al. 2014; Wang et al. 2014). In an elegant study by Wang et al., MEF derived iPSC's were transduced by adenovirus for enforced genetic expression of *Pdx-1, NeuroD1,* and *Maf-A* to differentiate into endocrine cells. Insulin-producing cells from three-genes modified iPSC's acquired comparable gene expression signatures and functionally responsive to glucose as good as mature β cells and MIN6 cells. Liver implantation of these iPSC's into diabetic mice demonstrated improved ability to dispose of glucose load and showed glucose tolerance curve comparable to normal mice (Wang et al. 2014). Although this approach was more robust in generating functional and mature insulin secretory cells, several issues persist with these genetic manipulation approaches. Firstly, the safety of exogenous gene insertion could lead to off-target gene mutation or inflammation. Secondly, the ability to reliably generate reproducible β cell differentiation *in-vivo* is challenging as these three genes may be insufficient to fully promote differentiation and maturity. Indeed, other factors for endocrine functions such as insulin vesicle formation and docking may also be required. Thirdly, the kinetics of longevity and survival of transformed cells is incompletely understood *in-vivo*, and requires further extensive research. Surprisingly, the above limitations were not observed in a protocol described by Kaitsuka et al. where targeted protein transduction of *Pdx-1*, *NeuroD1* and *Maf-A* was combined with a series growth factors and inhibitors for accelerated endocrine differentiation. In their study, the murine iPS cell line 20D-17 was differentiated into insulin-producing cells in a 17-day stepwise protocol using an 804G extracellular matrix and small molecules including activin-a, fibroblast growth factor-10 (FGF10), retinoic acid and glucagon-like peptide-1 (GLP-1) followed by protein transduction of the same pancreatic development genes, which transformed iPSC's to become glucose responsive and secrete insulin *in-vitro*, with mitigation of experimental induced diabetes in mice after transplantation (Kaitsuka et al. 2014). Hebrok et al., used doxycycline (Dox)-inducible secondary mouse embryonic fibroblasts (MEFs) to induce iPSC lines to test iPSC-factor based lineage specific reprogramming paradigm to endoderm (Li et al. 2014). They screened more than 400 small molecules to develop definitive endoderm like cells (DELCs) and pancreatic progenitor like cells (PPLCs) and compared MEF cells with iPS cells. This study postulated that providing lineage specification signals at the initials step of pluripotency induction improves the efficacy of endodermal reprogramming without reprogramming into iPSC. More recently, Stepniewski et al characterized mouse iPSC's as a model for diabetes investigation (Stepniewski et al. 2015). Their study described an efficient differentiation protocol for generation of iPSC's from both wildtype and

Lep$^{db/db}$ (db/db) mice into functional insulin-producing cells. However, in comparison to the wild-type iPS cells, the *Lep*$^{db/db}$ derived iPSC's demonstrated impaired angiogenic potential and failed to differentiate into endothelial progenitor-like cells. It should be noted that endothelial dysfunction is an important complication associated with type 2 diabetes and has been shown to negatively affect a heterogeneous population of endothelial progenitor cells (Stepniewski et al. 2015). Hence, impaired angiogenesis from type-2 derived iPSC's challenged their ability to integrate and function as part of new pancreatic islet mass function post-transplantation.

2 Human iPSC Generation, Validation, and Pancreatic Differentiation

Human iPSCs represent a rich and reliable source for potential cell therapy in diabetes due to their ready accessibility, and the clear future potential of 'personalized medicine' based patient-specific autologous cell replacement therapy, circumventing the need for immunosuppression. Avoidance of allogeneic barriers will be a key step in promoting wide acceptance of this approach across the diabetes population. However, especially in T1DM it remains to be seen what further modifications may be required to overcome a potentially insurmountable autoimmune response. Potentially, additional gene editing with CRISPR/Cas-9 may be required to overcome additional autoimmune targets. The generation of human iPSC's currently requires retroviral or lentiviral-mediated transduction approaches which may or may not raise additional safety concerns. The risk of insertional mutagenesis and off-target consequences including malignant transduction remains a real concern, and alternative approaches are desirable to facilitate faster translation of these approaches to the clinic. Alternative strategies include non-integrating methods of gene delivery involving plasmid, episomal, adenoviral and piggyBac transposons transfection systems. Cre-excisable viral vectors and more recently direct transduction of reprogramming proteins with membrane soluble peptides or protein transduction domains provide a potential way forward (Teo et al. 2013). There has been substantial progress in our understanding of hiPSC's differentiation toward pancreatic β cells. Despite these advances, generation of pure β-like cell populations remains challenging as the process hiPSC transformation results in contamination of elements at various stages of development and therefore heterogeneous including polyhormonal and other endocrine cell types. Reproducibility and standardization remain difficult but essential barriers to overcome if regulatory authorities are to accept and approve future therapies. Most differentiation protocols attempt to mimic developmental events either by modifying the dose and timing of activators, repressors, and small molecules, or the variability of hiPS cell lines. In the developing embryo growth factors vary in a continual and pulsatile fashion which is difficult to replicate *in vitro* simply by media exchange on a daily basis. More precise and local replication of physiologic development factor events, contact inhibition and other presently ill-defined factors may be required to further perfect this process.

Several lines of study have demonstrated the generation of pancreatic cell lineage cells from human pluripotent stem cell conversion. ViaCyte Inc. were the first to lead first-in-human ongoing trials of their pancreatic progenitor cells (ClinicalTrials.gov identifier: NCT02239354) derived from hESC's (pancreatic progenitor cells (PEC-01, trials VC-01 and VC-02). These immature Pdx-1 and Nkx6.1 co-expressing cells spontaneously differentiated into mature, human β-like cells after transplantation in diabetic mice. Substantial data from our own studies using hPSC's differentiated into functional beta cells and transplanted into a 'device less' prevascularized capsule site further provided proof of concept for the use of human pluripotent stem cells (hPS) derived PEC-01 cells for β cells transplantation (Pepper et al. 2015; Pepper et al. 2017). A year later, Dang et al., modified this protocol and reported highly efficient differentiation of both hESC's and hiPSC's into β-like cells and detected 25% insulin+ cell population compared to 7.3% as described previously by ViaCyte. The generated cells deciphered high transcripts of endocrine

reprograming and mature β cell fate genes with comparable insulin/c-peptide values to those of normal human islets. In their study, the use of epidermal growth factor (EGF) significantly enhanced the expression of *Pdx-1* and supported *in-vitro* human iPSC cell differentiation. Cell transplanted in immunodeficient mice correlated similar results, as previously reported without evidence of teratoma formation (Zhang et al. 2009). To investigate the factors influencing the differentiation potential of hiPSC's, Rezania et al., developed a seven-stage protocol describing efficient conversion of human ES and ND-iPSC with importance of thyroid hormone, retinoic acid, EGF and gamma-secretase, transforming growth factor-β (TGF-β), sonic hedgehog (Shh), Axl-receptor tyrosine kinase inhibitor (Axl) and bone morphogenetic protein (BMP) inhibition, under attached/air-liquid interface culture (ALI) conditions. Their protocol was able to promote 40% insulin+ cell generation (Rezania et al. 2014). In parallel, Pagliuca et al. used a similar approach targeting human ES and non-diabetic (ND)-iPSC differentiation with a spinner flask method and resulted in 33% insulin+ population. Alternatively, Noguchi et al showed that epigenetic memory inherited from parental lineage influences the capacity and differentiation potential of iPS cells into β-like cells. A microarray comparison of whole transcripts of the pancreas and fibroblast-derived iPS cells revealed a significant difference in expression of key genes (Gremlin1 (*GREM1),* GATA binding Protein-6 *(GATA6),* and Early growth response-1 *(EGR1)*) resulting in varying β cell phenotype reprogramming with similar chemical stimulation (Tsugata et al. 2015). Matthias Hebrok et al., demonstrated efficient conversion of human fibroblasts cells towards an endodermal cell fate by employing a non-integrative episomal reprogramming factors in combination with specific growth factors and chemical agents. Human fibroblast cells transduced with *Oct4*, *Sox2*, Kruppel-like factor-4 (*Klf4)* and a short hairpin RNA for *p53* were converted to pluripotent cells with EGF and bFGF growth factors. Their data demonstrated more efficient conversion of hPS cells into definitive endodermal cells compared with ES cells using a combination of growth factors like- TGF-β ligand activin-A and Wnt siganling activator CHIR99021 (Zhu et al. 2016). Millman et al used a spinner flask culture format to scale-up β cell population from ND-iPSC donor cells, however, only 22% β cell population was achieved (Millman et al. 2016). These results were later verified in a study displayed lineage tracing approach by generating green fluorescent protein (GFP) tagged NK6 homeobox-1 (*Nkx-6.1*) reporter line in hiPSC' derived from healthy donor using a CRISPR/cas9 aided homologous recombination and confirmed β cell differentiation from GFP-hiPSC line using the seven-step protocol as described by Rezania et al. (2014). The study showed that *Nkx6.1*$^{+}$ cells contribute significantly to β-like cells generated from hiPS line, and may thereby provide a valuable resource for implementation and optimization of differentiation protocols, and thereby a better understanding of specific cell differentiation approaches (Gupta et al. 2018). Very recently, the generation of a xenogenic human pancreatic β-like cell within a neonatal mouse pancreas was described by Rudolf Jaenisch's group (Ma et al. 2018). This elegant study showed orthotropic transplantation of hiPSC's derived β cell may engraft within a mouse pancreas. The engrafted human cells showed endothelial cell recruitment with mature differentiation of SC-β-like cells, and resulted in the glucose-regulated release of human insulin detectable in mouse blood. This study also compared the establishment of human pancreatic cells in chimeric mice by in-utero injection of definitive endoderm cells at the gastrulation stage embryo (E8.5) which failed to produce functional engraftment of the human donor cells (Ma et al. 2018).

3 iPSC Derivation from Diabetic Patients and Autologous β-Like Cells Generation

In the case of clinical diabetes, patient-specific iPSC lines potentially offer a promising alternative for autologous cell transplantation, although only a small number of the lines are currently reported and currently do not necessarily represent the broad spectrum of phenotypes and genetic anomalies observed in the clinic. These cells can

be generated by either reprogramming somatic cells using direct reprogramming with pluripotent genes or somatic cell nuclear transfer (SCNT). Up to the present, hiPSC lines have been generated from several patient populations with diabetes, including those with cystic fibrosis, T1D, T2D and other monogenic diabetes including maturity onset diabetes of the young (MODY), Wolfram syndrome and mitochondrial diabetes (Teo et al. 2013). A major advantage of generating diabetic-iPSC's (DiPSC's) will provide a more complete understanding of underlying genetic abnormalities, and thereby more directed targets for personalized therapeutics and autologous transplantation. Where β cell dysfunction persists after cell expansion, the opportunity for further genetic correctional maneuvers may be applied (zinc finger nuclease enzyme (ZFN), transcription activator-like effector nuclease (TALEN) or CRISPR/Cas9 technologies) through repair of the mutated gene sequences before transplantation into patients with diabetes (Teo et al. 2013). The Melton group have derived DiPSC line from two type-1 diabetic patients' fibroblast cells using three key reprogramming genes and demonstrated efficient generation of insulin-producing cells with similar efficacy as ND-iPS lines (Maehr et al. 2009). Thatava et al used multiple iPS clones from type-1 diabetic patients to screen for a battery of developmental markers for differentiation propensity and proficiency in yielding functional insulin (INS)-producing progeny (Thatava et al. 2013). Although, noticeable intra-patient variation was evident, this was largely confined to the differential dynamic expression of transcriptional players such as hepatocyte nuclear factor 4*α* (*HNF4α*) and hepatocyte nuclear factor 1*β* (*HNF1β*) and other islet-specific genes, in generating islet-like cells. The study concluded that successful generation of functional β cells from IPS cells requires feedback regulation of reprogramming genes inducing pluripotency, as well as the induction of stage-specific pancreatic transcription factors. The Melton group recently demonstrated that similar to ND-hiPSC donors, β-like cells from T1D patient-derived hiPSC's may also be created. They developed three T1D and three ND donor iPSC lines, each of which displayed generation of β-like cells without noticeable difference in *in-vitro* and *in-vivo* endocrine gene expression, and importantly were able to reverse alloxan-induced diabetes in mice (Millman et al. 2016). The proof of concept for the patient-specific hiPSC lines from T2D patients was demonstrated by two independent investigators, but the hiPSC lines so-generated have yet to be validated for β- cell differentiation (Kudva et al. 2012; Ohmine et al. 2012).

Among the monogenic forms of diabetes, several gene mutation-specific iPSC lines have been created. iPSC derived from MODY patients and infants with neonatal diabetes may be used to understand the biological impact of genetic mutations in human pancreas development and β cell function. MODY mutation affects the secretory functions of β cells leading to reduced basal and stimulated insulin secretion. The availability of MODY1-iPSC lines provide a unique opportunity to investigate the role of *HNF4α* in β cell function. Similarly, MODY2-iPSC derived pancreatic β-like cells provide important insight in unraveling the impact of specific *GCK* mutations. Moreover, MODY9-hIPSCs serve to explore the impact of various pancreatic endocrine differentiation genes including *Pdx-1, Neurod-1,* and paired box-4 (*Pax4)* in producing hormone-positive β cells. Another, mutated line MODY10-iPSC was used to investigate insulin structural defects caused by insulin (*INS*) gene mutations (Griscelli et al. 2018; Teo et al. 2013). Also other studies have described DiPSC lines generated from inherited diabetes associated genetic mutations, including hiPSC lines from Wolfram patients (WFS1) derived from c.103+ CISD2 mutation and hiPSC lines from patients with *Pdx-1* mutations at P33T and C18R sites (La Spada et al. 2018; Wang et al. 2016a, b). There was minimal effect upon reprogramming and acquired pluripotency of iPSC's, but formal testing of pancreatic endocrine cells differentiation was not explored.

4 Pathways to the Clinic and Targeting Patient Populations

Conventional models to study diabetes generally involve rodents since humans are genetically diverse, this could account in part for the failure

and shortfalls of clinical trials to date. Application of human cells is ideal for targeted studies of human disease states. Therefore, patient-specific hiPSC's may serve as a valuable resource for clinical trials. ViaCyte launched the first-in-human phase I/II clinical trial evaluating the efficiency of Encaptra Device combined with PEC-01 cells in T1D patients in 2014. More recently, in 2017, the U.S. Food and Drug Administration (FDA) permitted clinical testing for PEC-Direct product, and ViaCyte initiated two further clinical trials to test new "open Encaptra Device" allowing direct vascularization of their PEC-01 cells (ClinicalTrials.gov identifier: NCT02239354; NCT03162926 and NTC03163511) (Cito et al. 2018). In a separate clinical trial in Japan, autologous hiPSC derived differentiated PE cells were transplanted into a diabetic patient. The data with dermal fibroblast-derived hiPSC have showed no safety concerns to date. Nonetheless this trial has demonstrated the feasibility of autologous cell replacement therapy in patients with diabetes (Millman and Pagliuca 2017).

5 Aging as a Critical Regulator of iPSC Generation in Diabetes

If autologous transplantation of iPSC-derived cells is to hold a major future role in the cellular treatment of diabetes, donor age of the diabetic patient may become a critically determining factor for success, and this issue merits careful examination. Growing evidence has emphasized aging as a critical determinant for iPSC generation and its potency (Strassler et al. 2018). For the successful implementation of iPS cell therapy in diabetes, key remaining questions include: (a) Does donor age influence reprogramming efficiency and iPSC potency?; (b) Which impediments influence reprograming of senescent somatic cells?; and (c) Does donor age affect iPSC derived-ß cells survival and function?

Limited reprogramming efficiencies from old donors compared with young ones have been reported. At least in mice, several studies have established that donor age significantly and negatively impacts reprogramming efficiency (Strassler et al. 2018). Although cells from centenarians have been reprogrammed successfully to iPSC's, donor age remains an independent negative obstacle to cellular reprogramming. Underlying factor include: (a) The time-frame for somatic cells culture before iPSC derivation; (b) Prolonged passaging of fibroblasts that decrease reprogramming efficiency by upregulating p21, a marker of cellular senescence, (c) The length of telomerase genes; (d) Mitochondrial function of senescent cells, (e) Epigenetic modifications; and (f) Pre-existing genetic mutations (Strassler et al. 2018).

In diabetes especially, only limited evidence has documented competitive iPSC functionality and stemness from the oldest donor compared with neonatal donors. Prigione et al showed four iPSC lines generated from an 84 years old woman with T2D were compared to iPSC line from cells young neonatal donor, and with hESC's (Prigione et al. 2011). Despite the existence of chromosomal anomalies, all aged-iPSC's acquired pluripotency and were able to differentiate into multi-lineage cell types, but failed to generate ß-like cells compared to iPSCs from young donors and hESC's (Prigione et al. 2011). Subsequently, Ohmine et al compared pluripotency of iPSC lines derived from keratinocytes of elderly T2 diabetic patients (56–78 years) and reported ß-cell differentiation potentials for aged IPS cells. Importantly, the authors demonstrated that the cyclin dependent kinase inhibiotor-2A ($p16^{INK4a}$) gene served as a major regulator of reprogramming efficacy and stemness in aging patients. Suppression of the $p16^{INK4a}$ gene further improved the derivation of iPSC lines (0.01% efficiency iPS clone generation reported with aged keratinocytes) and efficient ß-like cell differentiation compared to young iPSC's donors (Ohmine et al. 2012). In a separate study, the same group compared iPSC lines from 85 years old T2D female and 67 years old T2D male with neonatal foreskin-derived iPSC lines. Again, the suppression of the $p16^{INK4a}$ gene led to efficient iPSC derivation and ß-cell differentiation (Kudva et al. 2012).

Although only few investigations for iPSC's generation from elderly T2D have been conducted to date, their derivation and potency compared with patients with T1D or neonatal diabetic iPSC lines remain incompletely investigated. There is thus a

pressing demand to more thoroughly investigate the underlying mechanisms and determinant factors affecting iPSC cell generation, stemness, genomic variability and differentiation into ß-like cells, if iPS cells therapy is to be successfully translated into the clinic, especially for elderly diabetes patients.

6 Future Outlook

iPS cell therapy in diabetes will hopefully become reality over the next several years for increasing number of patients, with the aim being to promote health and quality of life (Fig. 1). We believe that evolving protocols and technologies for patient-derived robust iPSC generation merit careful consideration, especially taking into account potentially negative factors such as intra-patient variability and underlying genetic abnormalities. Furthermore, dependency of β-cell differentiation agents/growth factors upon established protocols for ES cells require further optimization if consistent endocrine cell populations are to be generated reliably from iPS derived cells. Ultimately, iPSCs will need to be gene edited to make them more resistant to immune attack, and this critical step may be the breaking point for determining how this therapy is accepted and taken up in the clinic (Fig. 2). Scaling up personalized iPSC-based medicine approaches in a good manufacturing

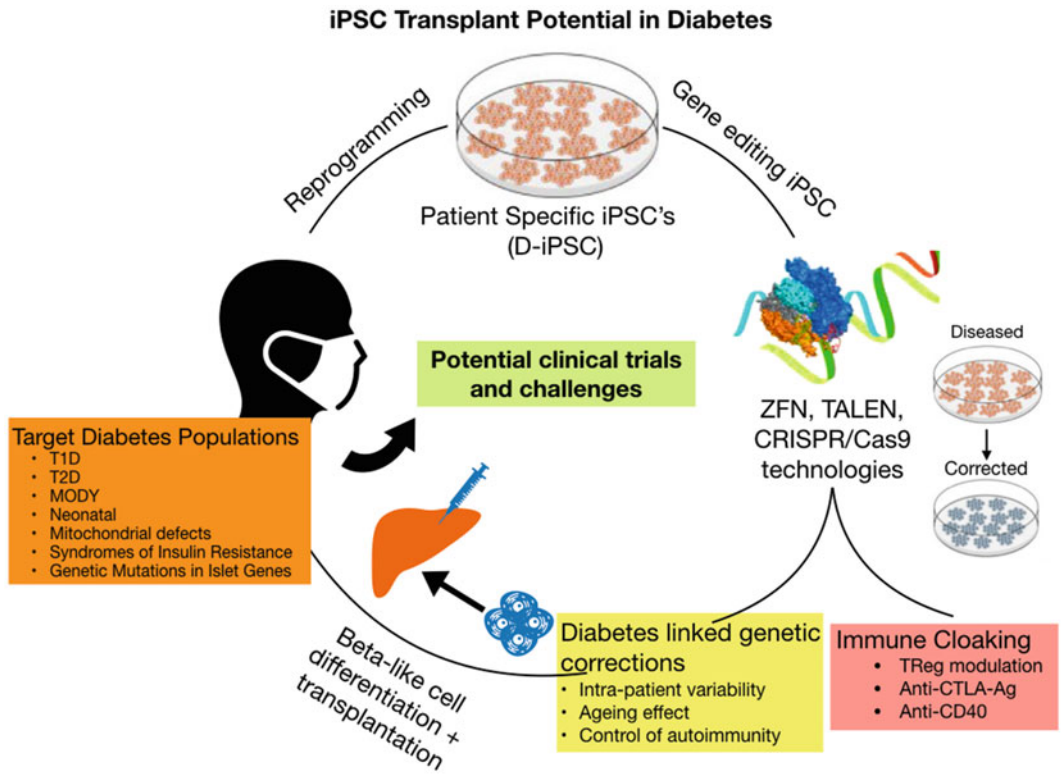

Fig. 2 Schematic diagram dipicting prospects of inducible pluripotent stem cell (iPSC) technologies in the treatment of various forms of diabetes. Patient specific iPSCs or diabetic iPSCs (DiPSCs) may be obtained from somatic cells of patients with monogenic/polygenic diabetes. DiPSCs may be repaired by gene correction and then provide an excellent source for transplantation of self-compatible cell therapy. Additional genetic manupulations will certainly be required to prevent recurrent autoimmune destruction of the self-expanded new beta cells. Personalized medicine-based corrected DiPSCs may be differentiated into functional insulin-secreting pancreatic beta cells, which will circumvent any need for immunosupression provided autoimmunity or the stem cell targets of autoimmunity are eliminated. Systematic evaluation of disease specific iPSC will contribute to clinical trials and disease modeling, and thereby establish innovative therapeutics for multiple forms of diabetes

clinical grade environment will also be a substantial barrier that may be difficult for industry to overcome. Nonetheless, autologous stem cell therapies will certainly transform future clinical practice for patients with diabetes and will provide an invaluable gift to humanity.

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CRISPR/Cas9 for Sickle Cell Disease: Applications, Future Possibilities, and Challenges

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Abstract

Sickle cell disease (SCD) is an inherited monogenic disorder resulting in serious mortality and morbidity worldwide. Although the disease was characterized more than a century ago, there are only two FDA approved medications to lessen disease severity, and a definitive cure available to all patients with SCD is lacking. Rapid and substantial progress in genome editing approaches have proven valuable as a curative option given plausibility to either correct the underlying mutation in patient-derived hematopoietic stem/progenitor cells (HSPCs), induce fetal hemoglobin expression to circumvent sickling of red blood cells (RBCs), or create corrected induced pluripotent stem cells (iPSCs) among other approaches. Recent discovery of CRISPR/Cas9 has not only revolutionized genome engineering but has also brought the possibility of translating these concepts into a clinically meaningful reality. Here we summarize genome engineering applications using CRISPR/Cas9, addressing challenges and future perspectives of CRISPR/Cas9 as a curative option for SCD.

Keywords

Gene editing · Gene therapy · Hematopoietic stem cell transplantation · Hemoglobinopathies · Programmable endonucleases

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Abbreviations

AAV	Adeno-associated virus
BM	Bone marrow
Cas9	CRISPR associated protein 9
CRISPR	Clustered regularly interspaced short palindromic repeats
DSB	Double strand breaks
dCas9	Dead Cas9
ddPCR	Droplet digital PCR
eSpCas9	Enhanced specificity *Streptococcus pyogenes* Cas9
GVHD	Graft-vs-host disease

HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HbS	Hemoglobin S
HDR	Homology directed repair
HLA	Human leukocyte antigen
HPFH	Hereditary persistence of fetal globin
HPLC	High performance liquid chromatography
HRI	Heme-regulated inhibitor
HSCT	Hematopoietic stem cell transplantation
HSPCs	Hematopoietic stem/progenitor cells
HU	Hydroxyurea
INDELs	Insertions/deletions
iPSCs	Induced pluripotent stem cells
LCR	Locus control region
MUD	Matched unrelated donor
NHEJ	Non-homologous end-joining
OTEs	Off-target effects
PACE	Phage-assisted continuous evolution
PAM	Protospacer-adjacent motif
QTL	Quantitative trait loci
RBCs	red blood cells
ScCas9	*Streptococcus canis* Cas9
SCD	Sickle cell disease
$shRNA^{miR}$	MicroRNA-adapted small hairpin (sh) RNAs
SpCas9-HF1	high fidelity *Streptococcus pyogenes* Cas9
TALENs	TAL-effector nucleases
UCBT	Umbilical cord blood transplantation
ZFNs	Zinc finger nucleases

1 Introduction

Sickle cell disease (SCD) is an inherited monogenic disorder characterized by a single substitution on chromosome 11 where glutamic acid is replaced by valine in the sixth codon of the β-globin gene. Whether inherited either in a homozygous state or with another abnormal β-globin gene, SCD encompasses a group of disorders with variable clinical phenotypes yet share a common pathophysiologic consequence derived from a single monogenic change. The modified β-globin gene produces an abnormal hemoglobin S (HbS) which rapidly polymerizes in the deoxygenated state altering red blood cell (RBC) rheology and lifespan. This single substitution leads to multiple downstream effects and devastating clinical complications including chronic anemia, chronic inflammation, recurrent vaso-occlusion, acute and chronic pain, stroke, organ failure, and early mortality (Paulukonis et al. 2016).

SCD is the most common inherited hemoglobinopathy worldwide, and despite knowledge of the disorder for over 100 years, it remains a life-limiting disease with few therapeutic options to reduce disease severity. Unlike other more recently identified molecular disorders that have benefited from higher federal, foundational, and per person funding (Smith et al. 2006; Lobner et al. 2013), there are only two FDA approved medications to lessen disease severity, hydroxyurea (HU) (approved for adults in 1998; children in 2017) and L-glutamine (approved in 2018). There remains misinformation, poor adherence, and a reluctance to prescribe HU despite benefit (Wang et al. 2011; Zimmerman et al. 2007; Steinberg et al. 2003; Ware 2010), while insurance companies will often not cover the cost of the highly purified form of L-glutamine approved by the FDA. Whereas the two mainstay treatments for SCD, blood transfusions and HU do not fully eliminate the consequences of the disease, simple public health measures such as newborn screening, penicillin prophylaxis, and vaccinations have significantly reduced early childhood mortality. Between 1979 and 2005, childhood mortality for children with SCD decreased by 3% per year; however, a 1% per year increase during the same period was observed for adults (Lanzkron et al. 2013). As more than 94% of children with SCD in well-resourced countries now survive until age 18, and with an expected rise in birth rate for babies with severe hemoglobin disorders to be over 400,000 by 2050 (Piel et al. 2013), disease management needs to shift to a two-tiered system

addressing acute and chronic disease needs while simultaneously searching for curative options to address the global burden and public health issues of the disease. Hematopoietic stem cell transplantation (HSCT) and gene therapy offer a way to reduce disease burden, improve outcomes and quality of life for patients with SCD, and potentially reduce health care costs over the long term (Ballas 2009; Arnold et al. 2017; Saenz and Tisdale 2015; Bhatia et al. 2015).

Since the first HSCT in 1984 for a pediatric patient with SCD and acute myelogenous leukemia, numerous patients have successfully undergone bone marrow (BM) HSCT with a human leukocyte antigen (HLA)-matched sibling donor. Whether using a myeloablative or non-myeloablative preparative regimen, greater than 90% of all patients are cured of SCD with a BM HSCT (Walters et al. 1996; Hsieh et al. 2014; Walters et al. 2001; Gluckman et al. 2017). Between 1986 and 2013, over 1,000 patients have received an HLA-matched sibling HSCT with a 5-year event free survival and overall survival of 91.4% and 92.9%, respectively (Gluckman et al. 2017). HSCT should be considered standard of care when a patient has a clinical indication and an HLA-matched sibling donor, yet less than 15% of patients with SCD have an appropriately matched donor (Walters et al. 2001). Furthermore, only 10% of eligible patients have undergone curative HSCT despite patient willingness to consider HSCT morbidity and mortality at the chance for cure (Chakrabarti and Bareford 2007). HLA-matched unrelated donor (MUD) transplantation, umbilical cord blood transplantation (UCBT), and haploidentical transplantation offer more patients the chance for cure, though high rates of complications currently limit the broad use of these therapies. Such complications, including graft rejection and graft-vs-host disease (GVHD), are addressed in gene therapy models where a patient's autologous hematopoietic stem and progenitor cells (HSPCs) are modified thereby eliminating such complications.

The premise of gene therapy either by gene editing or insertion into autologous HSPCs raises the promise of a safer cure for SCD that is available to all patients. Such methodology eliminates two major barriers in the cure of SCD: the lack of suitable donors, and the morbidity and mortality associated with GVHD. After decades of scientific progress, gene therapy for the cure of SCD is currently in multiple clinical trials with promising initial results. Potential methods for gene therapy in SCD are multiple: (i) addition of therapeutic globin such as β-globin or β^{T87Q}-globin to make adult hemoglobin (HbA), or γ-globin to enhance fetal hemoglobin (HbF) levels, (ii) HbF induction by editing of globin regulatory elements or knockdown of HbF repressors, or (iii) direct gene correction of the SCD mutation with programmable nucleases. Here we focus on the challenges of CRISPR-Cas9 editing, it's implications, and future possibilities as a curative option for SCD.

2 Genome Editing in SCD

Given the prospect for genotypic and therefore phenotypic correction in a monogenic disorder like SCD, significant effort has been devoted to find critical genes/chromosomal areas contributing to the pathophysiology of the disease. Antisickling genes such as wild type β-globin, modified β-globin (T87Q) which confers additional antisickling properties, γ-globin or β/γ hybrids have been transferred to sickle HSPCs using various viral constructs; of those, some are currently being tested in clinical trials for both safety and efficacy (reviewed in (Demirci et al. 2018)).

Genome editing is desirable as it leads to permeant removal or correction of a detrimental mutation, or by the creation of protective insertions or deletions. Theoretically, programmable nucleases create double strand breaks (DSB) at a specific genomic locus followed by recruitment of DNA repair mechanism through either non-homologous end-joining (NHEJ) or homology directed repair (HDR) (using homologous sequences found in sister chromatids, homologous chromosomes or extrachromosomal donor DNA sequence provided for correction purposes) to the DSB site. Until 5 years ago, three major nucleases including meganucleases also known as homing endonucleases (reviewed

in (Stoddard 2011)), zinc finger nucleases (ZFNs, reviewed in (Urnov et al. 2010)), and TAL-effector nucleases (TALENs, reviewed in (M Scharenberg et al. 2013)) were introduced for various genome editing purposes. These tools have been successfully used *ex vivo* to correct the SCD mutation and induce fetal globin by editing regulatory sequences such as promoters or other regulatory sequences including *BCL11A*, *KLF1* and *MYB* to circumvent the severity of the mutation in sickle HSPCs (reviewed in (Tasan et al. 2016)). While these nucleases are highly specific thereby diminishing off-target effects (OTEs), programming of these enzymes is difficult, time consuming, and requires significant expertise.

In 2012, Doudna et al. presented a new genome editing technology (Wiedenheft et al. 2012; Jinek et al. 2012), referred to as Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9), in which a specific RNA (guide RNA) sequence recognizes the target DNA region of interest and directs the effector Cas protein there for editing. This strategy not only revolutionized genome editing strategies but also brought forth the improved possibility of translation of genome editing approaches to the clinical setting due to its advantages: easy to design, highly efficient, and inexpensive. Once introduced into target cells, CRISPR/Cas9 directed DSBs result in activation of DNA repair mechanisms. This machinery would lead to either some insertions/deletions (INDELs), which ideally results in loss-of-function for a given gene, or would repair the DNA break using homology strands if HDR is activated. In this manner, CRISPR/Cas9 technology can target correction of the SCD mutation or induce fetal hemoglobin expression by editing chromosomal areas controlling its expression (Fig. 1), yet challenges in the use of this technology remain surrounding efficiency, safety, and delivery.

2.1 HbF Induction

HbF is the predominant globin type after the first trimester of gestation and is replaced by HbA by 6 months after birth. Both HbA and HbF are maintained on chromosome 11, with switching from HbF to adult globin mainly controlled by a powerful upstream enhancer known as the locus control region (LCR) that loops to each globin promoter to activate their expression (Li et al. 2002). After the switch to HbA, HbF is not entirely suppressed, though it is not evenly distributed among RBCs. When there is not a genotypic cause for persistence of HbF in all RBCs, HbF can be minimal in some cells or concentrated in specific cells referred to as F-cells (Demirci et al. 2018).

After the initial observation by Janet Watson and colleagues that newborn babies do not show SCD complications for a certain period due to high levels of HbF in the infant's blood (Watson et al. 1948), more work has been devoted to increase HbF levels in the adult body. The important role of elevated HbF for SCD protection was further confirmed with the reports showing asymptomatic patients with SCD with elevated HbF as a result of coinheriting hereditary persistence of fetal globin (HPFH) mutations (Forget 1998; Stamatoyannopoulos et al. 1975). Such mutations occur either in the form of large deletions in the β-globin gene, or smaller deletions/single nucleotide polymorphisms (SNPs) in γ-globin promoter or HbF regulating quantitative trait loci (QTL) (Paikari and Sheehan 2018). In line with these reports, deletion or inversion of 13.6 kb chromosomal region to obtain a HPFH-like phenotype in SCD patient derived HSPCs resulted in elevated levels of HbF in erythroblast and ameliorated the *ex vivo* sickling (Antoniani et al. 2018). Similarly, point mutations created by CRISPR/Cas9 approach in the −115 and − 200 clusters of the γ-globin promoter, inhibiting the binding of validated HbF transcriptional repressors *BCL11A* and *LRF* (also known as ZBTB7A), respectively (Wang and Thein 2018), de-repressed the expression of HbF (Martyn et al. 2018; Liu et al. 2018). To show the applicability of these approaches, animal models are required for *in vivo* evaluations prior to human studies. Immunodeficient mice are generally used for human cell engraftment studies but are not proper for *in vivo* erythropoiesis. To

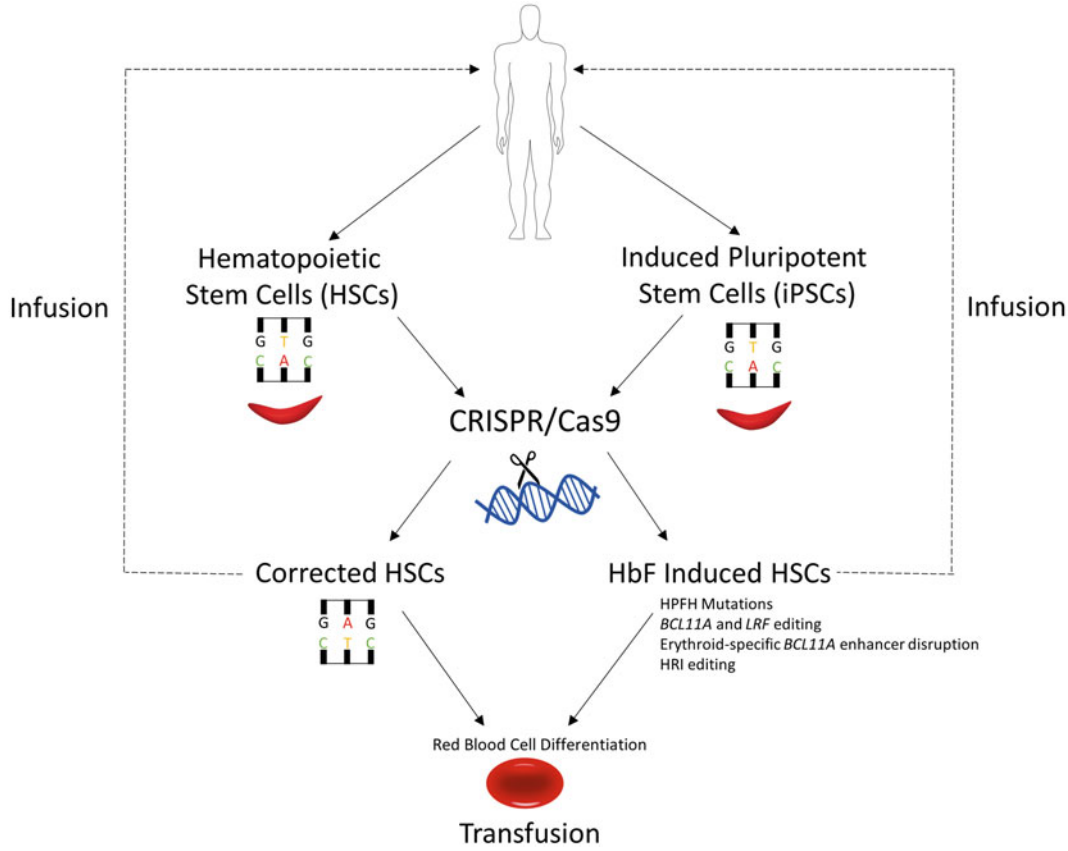

Fig. 1 Potential CRISPR/Cas9 applications for sickle cell disease (SCD). The proof-of-principle experiments have proven the possibility of SCD mutation correction and fetal hemoglobin (HbF) induction in SCD derived HSCs and iPSCs, and subsequent normal red blood cell derivation for transfusion purposes. However, these advances are waiting to be addressed by clinical trials to explore the full potential

overcome this problem, Li et al. used a human β-globin locus transgenic (β-YAC) mice model to study the *in vivo* effect of disruption of the repressor binding region within the γ-globin promoter (Li et al. 2018). Along with significant target site distribution which was sustained in the secondary transplantation experiments, no hematological abnormality was seen and pronounced switch from human β to γ globin expression in RBCs of adult mice was noted.

Gene edition of transcriptional regulators is an alternative methodology to stimulate rare naturally occurring HPFH mutations to control HbF expression. Several transcription factors including *SCA/TAL1*, *GATA1* and *KLF1* are reported to be involved in HbF regulation (Sankaran and Orkin 2013). While all of them could be considered potential candidates, direct targeting of these factors for HbF induction is challenging as all of them have either broader roles in non-erythroid linages or have significant roles in normal erythropoiesis. Significant candidates, LFR and BCL11A, are validated HbF silencers (Uda et al. 2008; Menzel et al. 2007), and have been edited in the erythrocyte progenitor cell line (HUDEP-2) leading to robust HbF expression (Masuda et al. 2016). BCL11A is important for HSPC function (Tsang et al. 2015) and normal lymphoid development (Liu et al. 2003), with only one paper demonstrating very low level indels and a slight increase in γ-globin expression in a non-human primate model using TALE nuclease mRNA targeting the *BCL11A* coding sequence with respect to control transplants

(Humbert et al. 2018). The safety and feasibility of the *BCL11A* knockdown is still awaiting to be addressed by large animal models with high indel ratios and subsequent clinical trials with large patients cohorts. The first clinical trial launched in February 2018 uses a lentiviral gene transfer vector encoding a microRNA-adapted small hairpin (sh) RNAs ($shRNA^{miR}$) targeting *BCL11A* in patients with severe SCD is currently ongoing with the first patient demonstrating 23% HbF (NCT03282656, Shim et al. 2017; Esrick et al. 2018). Recently, Daniel Bauer and colleagues have presented a different approach in which they induce comparable levels of HbF in CD34+ cells by targeting the +58 intronic site of the *BCL11A* gene that acts as an erythroid specific enhancer (Bauer et al. 2013; Canver et al. 2015). They were able to show that while guide RNA directed disruption of the enhancer site provided substantial reduction in *Bcl11a* expression in erythrocyte cells leading to elevated HbF expression in mice, it did not affect the expression in non-erythroid lineages (Smith et al. 2016). The results were extended to erythroid cells derived from progenitor cells of patients with β-Thalassemia major (Psatha et al. 2018), supporting that this enhancer disruption strategy would be favorable for clinical use if it is proven safe with preclinical and clinical studies.

With the establishment of guide RNA screening models, it has become possible to discover novel genomic sites/genes controlling HbF expression. In a recent paper, protein kinase domain–focused CRISPR/Cas9–based genetic screening revealed that heme-regulated inhibitor HRI (also known as EIF2AK1), an erythroid-specific kinase that controls protein translation as an HbF repressor, could be used as a potential candidate for the treatment of hemoglobinopathies (Grevet et al. 2018). Using similar methodology, the same group also identified that SPOP, a substrate adaptor of the CUL3 ubiquitin ligase complex, as a HbF repressor in both HUDEP-2 and CD34+ cells (Lan et al. 2018). Extending these guide RNA screening strategies to non-coding regions and epigenetics would allow identification of stronger candidates or gene combinations to enhance HbF expression to clinically meaningful levels that reverse the sickling of RBCs and reverse the disease phenotype as seen in patients with HPFH.

2.2 SCD Mutation Correction

As the pathologic mutation for SCD is already clearly identified, correction of the SCD mutation seems the most difficult but potentially the most feasible and promising approach as Cas9 cuts sickle β-globin and this break can be repaired if a normal β-globin sequence flanked with homology arms to the DSB is supplied. Genotypic correction appears possible by targeting the specific locus at the genome and providing the correct sequence for β-globin without the necessity of exogenous transgene activation.

To ensure proper correction, an increasing number of researchers are using gene editing technologies for correcting the SCD mutation in different cell types (Table 1). Most of these works use the CRISPR/Cas9 system as it has shown better correction efficiency and lower OTEs than other gene editing tools such as TALENs (Bak et al. 2018; Hoban et al. 2016a). The HSPC source is historically bone marrow derived $CD34^+$ HSPCs, currently used in the majority of genome editing studies, though recently peripherally mobilized $CD34^+$ HSPCs using plerixafor has shown promise in patients with SCD given safety concerns regarding granulocyte colony stimulating factor use in these patients. These CD34+ cells can be modified to be infused back into the patient. However, differences in the cell cycle or the presence of specific nucleases that might disrupt the correction pathways used by the cells after the DSBs offer overall resistance to successful gene editing (Lomova et al. 2018). In order to maximize success, the preferred delivery method in these studies is electroporation with an Adeno-associated virus (AAV)-6 viral vector for the delivery of the CRISPR/Cas9 system with the donor DNA. For evaluating the correction of the SCD mutation, several studies analyzed gene editing at the DNA level using either targeted deep sequencing

Table 1 Selected sickle cell disease (SCD) mutation correction studies using CRISPR/Cas9

Gene	Cell types	Genome editing tool	Outcomes/comments	Mouse transplantation experiments	References
HBB	iPSCs	CRISPR/Cas9	Correction of the SCD mutation detectable at RNA expression and western blot	ND	Huang et al. (2015)
HBB	BM $CD34^+$	TALEN CRISPR/Cas9	Correction of SCD mutation with higher performance (7.3% HbA production evaluated by HPLC) using CRISPR/Cas9	ND	Hoban et al. (2016a)
HBB	BM $CD34^+$ Mobilized $CD34^+$	CRISPR/Cas9	Correction of SCD mutation using an anti-sickling β-globin cDNA donor with a 29% efficiency at the RNA expression level	Long term engraftment of enriched $CD34^+$ edited population measured by flow cytometry in femur BM (4–30%)	Dever et al. (2016)
HBB	Mobilized $CD34^+$	CRISPR/Cas9	Correction of SCD mutation up to 33% correction evaluated by RNAseq and up to 29.3% HbA production evaluated by HPLC	Long term engraftment ability of non-enriched $CD34^+$ edited cells evaluated in BM (2.3 ± 1.8%) and Spleen (3.7 ± 1.4%)	DeWitt et al. (2016)
HBB	iPSCs	CRISPR/Cas9	Correction of SCD mutation evaluated by sequencing. No functional studies	ND	Park et al. (2017)
HBB	$CD34^+$ selected from PBMCs	CRISPR/Cas9	9.4–9.6% genome editing efficiency evaluated by sequencing. Correction also evaluated at protein level by HPLC	ND	Wen et al. (2017)
HBB	iPSCs	CRISPR/Cas9	Up to 67.9% correction efficiency evaluated by sequencing. No functional studies	ND	Li et al. (2016)
HBB	Mobilized $CD34^+$	CRISPR/Cas9	20% SCD mutation correction evaluated by RNAseq	Long term engraftment of enriched $CD34^+$ edited cells evaluated in BM (23.4% average)	Magis et al. (2018)
HBB	ESCs iPSCs	CRISPR/Cas9	63% SCD mutation correction evaluated by nested ddPCR	ND	Martin et al. (2018)
HBB	$CD34^+$	CRISPR/HiFiCas9	More than 50% HbA evaluated by HPLC and nested ddPCR	ND	Vakulskas et al. (2018)
HBB	$CD34^+$	CRISPR/Cas9	Evaluation of the SCD mutation correction by High-throughput sequencing	Levels of long-term engraftment of non-enriched CD34+ edited cells up to 2.5% in BM	Lomova et al. (2018)

(Lomova et al. 2018; Wen et al. 2017) or nested droplet digital (dd)PCR (Vakulskas et al. 2018), while others used more functional studies like RNAseq or RNA expression levels (Dever et al. 2016; DeWitt et al. 2016; Chung et al. 2018; Magis et al. 2018) with only three studies using High performance liquid chromatography (HPLC) for measuring protein levels after the correction of the SCD mutation in the β-globin gene (Hoban et al. 2016a; Vakulskas et al. 2018; DeWitt et al. 2016).

Since the publication in 2008 of a protocol for generating human iPSCs from somatic cells, many groups have developed protocols for the differentiation of iPSCs into different cell lineages such as hematopoietic cells to become another viable source

of autologous HSPCs (Fujita et al. 2016; Ferreira et al. 2018; Sugimura et al. 2017). Currently however, the hematopoietic cells derived from iPSCs are primitive rather than definitive hematopoietic cells and are therefore unable to engraft in a xenograft mouse model. The available protocols for the differentiation of iPSCs towards HSPCs mainly mimic primitive hematopoiesis, which can be noticed when the generated HSPCs are differentiated into erythroid cells containing mainly ε-globin and γ-globin, with very low amounts of β-globin if present at all. In order to realize the available gene editing tools to correct the SCD mutation in iPSCs for therapeutic purpose, a proper differentiation protocol is needed to produce engraftable HSPCs from iPSCs. Such therapy, as with other autologous modification strategies, would ultimately eliminate two major hurdles in allogeneic transplantation; rejection and GVHD in transplantation therapies. In addition, as low efficiency of correction is a problem for HSPC studies, cloning corrected cells from a bulk iPSC population would allow derivation of a population with 100% of cells corrected.

In addition to the difficulties differentiating iPSCs towards HSPCs, only one study has presented the correction of the SCD mutation in SCD-derived iPSCs at the RNA and protein levels by qPCR and Western blot analyses, respectively (Huang et al. 2015), though several groups have shown the correction of the SCD mutation at the DNA level using nested ddPCR or DNA sequencing (Park et al. 2017; Li et al. 2016; Martin et al. 2018). Correction of underlying mutation in both $CD34^+$ and iPSCs seems promising, yet while significant correction rates are reported in *ex vivo* conditions, limited corrected human cell engraftment are reported in immunodeficient mouse models (Table 1). While immunodeficient mice transplantation models for human cell engraftment studies are being widely accepted, it is not clear that whether these results completely reflect the clinical outcome of these approaches. After optimization of the correction methodologies, larger animal models are necessary to explore the potential of the application.

Though editing of CD34+ cells is possible, multiple genotypic outcomes are possible and editing of long-term engrafting HSPCs are not yet fully explored. Treating cells with CRISPR/Cas9 and a β-globin donor might result with cells in their native state (uncorrected), as sickle trait (one allele corrected), as healthy (both alleles corrected), as β-thalassemia major (both alleles disrupted), as β-thalassemia trait (one allele corrected and other disrupted), and/or sickle/β-thalassemia (one allele disrupted) due to NHEJ/HDR machinery of the cells (Esrick and Bauer 2018). As precise correction in long-term HSPCs is not yet efficient and editing results in reduction in engrafting HSPCs (Hoban et al. 2016a; Dever et al. 2016), transplantation of mixed culture could be clinically problematic and possible unintended consequences should be addressed before clinical trials.

3 Challenges

Genome editing has been the most attractive tool for scientists seeking to correct genetic mutations either as gene knockout or knock-in. Conventional methods for genome engineering, however, are costly, time-consuming, labor-intensive, and require expertise in protein engineering to design specific nucleases (Roy et al. 2018). On the contrary, CRISPR/Cas9 genome editing is a system that is relatively easier, cheaper and more efficient, and is being used in a large variety of model cells and species. It has not only led to easier and cheaper development of knock-out animal models but has also contributed to the establishment of whole-genome screening libraries that identify therapeutic genes/chromosomal regions that may directly affect a targeted phenotype. While there is a huge international interest in CRISPR/Cas9-based editing approaches, there is still much to improve upon such as the efficiency of cutting and editing (both NHEJ and HDR), improving specificity, and improving delivery methods. Lastly, there is a world-wide concern about safety, particularly as it relates to OTEs, that needs to be clarified and addressed before transferring this approach into routine clinical care.

3.1 Efficiency of Editing

The limiting factor for diverse application of a given CRISPR/Cas9 system has been the dependency on a protospacer-adjacent motif (PAM) sequence flanking the target. For instance, as SCD mutation correction studies need to target a specific chromosomal area, there are not many guide RNA options for different Cas proteins. Therefore, substantial effort has been made to engineer various Cas effector proteins for the recognition of different PAM sequence (Kleinstiver et al. 2015a; Nishimasu et al. 2018; Kim et al. 2017). While the introduction of 19 subtypes of CRISPR systems with various Cas effector proteins recognizing different PAM sites have extended targetable genomic loci (Leenay and Beisel 2017), not all of them have been widely studied in terms of efficacy and safety. Therefore, scientists still tend to use well-established Cas types (*i.e Streptococcus pyogenes* Cas9-SpCas9 or Cpf1-Cas12a) in their research. SpCas9 has a PAM recognition of 5' NGG 3′, while some other Cas9 orthologs have been reported to require longer PAM sites (Fonfara et al. 2013; Ran et al. 2015). While these have some advantages over classical SpCas9, their longer PAM sites restrict their use despite potentially more efficient delivery. For example, smaller Cas effector proteins such as *Staphylococcus aureus* derived Cas9 (SaCas9) with a PAM site of NNGRRT, are more efficient for viral delivery systems (Kleinstiver et al. 2015b). To extend the boundaries of targeting range for Cas9 proteins, PAM preference can successfully be altered by targeted mutations to residues near the PAM DNA duplex (Anders et al. 2016; Hirano et al. 2016).

Understanding the subunits of Cas effector proteins have allowed the modification of PAM specificity. In a recent report, Chatterjee *et al.* characterized *Streptococcus canis* Cas9 (ScCas9) displaying 5′-NNG-3′ PAM, reporting an 89.2% sequence similarity to SpCas9 (Chatterjee et al. 2018). Structural analysis showed that two distinct mutational areas [a positive-charged insertion in the REC domain (at 367–376) and a KQ insertion in the PAM-interacting domain (at 1337 and 1338)] are responsible for having the specificity for a minimal PAM sequence. Another group has recently generated Cas9 variants with various PAM compatibilities (including NG, GAA and GAT) using phage-assisted continuous evolution (PACE) approach (Hu et al. 2018). But more intriguingly, although extending PAM recognition capacity of Cas9 variants would be assumed to augment OTE (Hu et al. 2018; Tsai et al. 2015), they reported greater DNA specificity for Cas9 variants with respect to canonical SpCas9 along with lower genome-wide off-target. In a different approach, Sniper-Cas9 (F539S/M763I/K890 N variant) was successfully obtained using directed evolution, and characterized with high on-target and reduced OTEs (Lee et al. 2018). These studies illustrate the potential and the need for further improvements in targetable loci on the genome for various Cas effector proteins. While improving the efficiency, safety should also be parallelly taken into account to realize the approaches in routine clinical applications.

3.2 Potential Immunogenicity of Editing Tools or Edited Cells

The ultimate goal of CRISPR technology is to edit mutations related with disorders or control disease associated gene expressions in patient-derived specific stem/progenitor cells. However, *in vivo* effects of CRISPR/Cas9 systems have a lot of unanswered questions. In 2019, there are open clinical trials in the United States and abroad using CRISPR/Cas9 for a potential treatment of SCD, Thalassemia, HIV-1, and several cancer types (https://clinicaltrials.gov/keyword CRISPR). Though hope remains for these clinical trials, e*x vivo* work conducted thus far, demonstrate preliminary data pointing toward possible adverse effects of the technology. The first question is whether guide RNAs or Cas9 itself has any effects on the immune system. To partially address this uncertainty, Kim *et al*. demonstrated that *in vitro* transcribed guide RNAs with a 5-′-triphosphate group (5′-ppp) leads to cytotoxicity due to the activation of innate immune system in human and mouse cells (Kim et al. 2018). The authors also reported that removal of triphosphate resulted in high mutation rate in primary human

CD4+ cells thus avoiding the innate immune system. In a recent pre-print article, Charlesworth et al. showed pre-existing antibodies against Cas9 derived from *Staphylococcus aureus* (79%) or *Streptococcus pyogenes* (65%) in a small group of healthy volunteers (Charlesworth et al. 2018). In a follow up work performed with 200 blood samples, prevalence of antibodies against SaCas9 and SpCas9 were reported to be 10% and 2.5%, respectively (Simhadri et al. 2018). While these results are not unexpected, triggering of the immune system by CRISPR/Cas9 is potentially problematic and harmful in vivo. While these observations and potential immune response are awaiting to be addressed by large animal models and clinical studies, Cas9 expression levels, delivery methods, vector types in case of transduction routes, and target cells populations should be optimized in any capacity to diminish a severe immune response.

3.3 Specificity of Editing

Other than a potential immune response, OTEs are one of the biggest challenges of CRISPR/Cas9 system. As Cas9-guide RNA complex can recognize sequences with up to 5 mismatched bases (Fu et al. 2013), the possibility of OTE for a given guide RNA cannot be ignored. A number of advances have been taken to increase the specificity of CRISPR/Cas system, but the guide RNA design is the first critical process for reduction of OTEs. There are vast guide RNA design tools available; of those, newer ones include supplementary algorithms evaluating on-target cutting efficiency other than selectivity for the target. During the synthesis of guide RNA, additional modifications on the guide RNA structure including truncation of spacer RNA (Fu et al. 2014) and chemical modifications (Cromwell et al. 2018) have been reported to increase Cas9 endonuclease specificity. In addition, chemical modifications with 2′-O-methyl 3′ phosphorothioate (Hendel et al. 2015) and 2′-fluoro-ribose (Rahdar et al. 2015) improve the editing efficiency via increasing the stability of guide RNAs in cells.

The second important aspect to reduce OTEs is to enhance Cas9 specificity. A mutated variant of Cas9, nickase (Cas9n), can only cut a single DNA strand such that two close recognition sites in the DNA are required for a double strand break and thus OTEs are drastically reduced (50–1500 fold in human cells) (Ran et al. 2013). However, as some single nicks can be converted to double strand breaks, this approach was further improved with introduction of a catalytically inactive Cas9 (dead (d)Cas9) and Fok1 fusion protein (Tsai et al. 2014). In this approach, recognition of guide RNAs by dCas9 brings Fok1 enzyme the close proximity that is required for active dimerized Fok1 nuclease. While these approaches provided significant reduction in the off-target issues, requirement for a double recognition site might result in less editing efficiencies, and the necessity for double guide RNA usage might limit viral delivery approaches. To therefore keep editing efficiency high enough for clinical application, active nucleases are being engineered for higher specificities. The initial idea for high specificity nucleases was to decrease the interactions of Cas9 with its DNA target to lessen OTEs while keeping enough energy for on-target recognition. With the introduction of high fidelity Cas9 (SpCas9-HF1, N497A/R661A/Q695A/Q926A) (Kleinstiver et al. 2016) and enhanced specificity Cas9 (eSpCas9 (1.1), K848A/K1003A/R1060A) (Slaymaker et al. 2016), there are no or significantly reduced OTEs compared to wild type nucleases while maintaining robust on-target activities. Recently, Doudna *et al*. has published that both SpCas9-HF1 and eSpCas9(1.1) are trapped in an inactive state when bound to mismatched targets and that the non-catalytic domain of Cas9, REC3, is responsible for target recognition and direction of nuclease activity (Chen et al. 2017). Using these observations, they were able to create hyper-accurate Cas9 variant (HypaCas9) with wide-range genome specificity without compromising any detectable OTEs.

Recently, several publications have raised appropriate concern about the CRISPR/Cas system showing unintended consequences such as large deletions, insertions, and rearrangement of the chromosome when used in clinical trials

(Kosicki et al. 2018; Shin et al. 2017; Adikusuma et al. 2018). It is not clear that this uncertainty is going to be elucidated, or is clinically relevant, but it is sensible to urge more pre-clinical studies addressing these valid safety concerns.

3.4 Delivery

To apply CRISPR/Cas9 system to a given cell type/organism, the structure and vehicle of the components should be determined based on requirements for protein amount, exposure time, efficiency, and restrictions for OTEs and other safety issues. For the structure of the system, it could be (i) integrating/non-integrating viral vectors/plasmids expressing both mRNAs for guide RNA and Cas9, (ii) Cas9 mRNA and guide RNA, or (iii) ribonucleoprotein complex (RNP) constituting Cas9 protein and guide RNA. A short time after the discovery that CRISPR/Cas9 system could be used in human cells for genome editing purposes, viral constructs providing continuous expression of Cas9 and guide RNAs were used to explore this potential. However, while it might be advantageous for gene editing approaches requiring long-term expression, it was also recognized that sustained expression of guide RNAs and Cas9 augmented the possibility of mismatch bindings and OTEs (Pattanayak et al. 2013). For precise temporal control of expression, several inducible systems have been presented (Nihongaki et al. 2015; Zetsche et al. 2015). Using vectoral delivery in the lab is stable and cheap; however, there is still ongoing debate about the problems of viral systems with the immune system (Yin et al. 2014) and insertional mutagenesis (Hoban et al. 2016b). An alternative method to plasmids/vectors carrying Cas9 sequence is the introduction of mRNA for Cas9 that is translated to active protein once it is transferred to the cell. While this system avoids the time needed for transcription for Cas9 transferred with plasmids, it is also applicable only for genome editing approaches doable with transient Cas9 expressions. In addition, as mRNAs are not as stable as DNAs, delivery time of RNAs for Cas9 and guide RNA would be critical. Jiang *et al.* showed that Cas9 protein was at maximum level 6 h after delivering Cas9 mRNA and not detectable after 24 h in mice (Jiang et al. 2017). One way to optimize efficiency would be different delivery times or chemical modifications to provide the stability of RNAs as was mentioned earlier (Safety section).

The RNP complex is another alternative in which native Cas9 protein and guide RNA form a single complex that is readily active once it is in the cell. Other than the question of whether native foreign protein to the human cells is significantly immunogenic to hinder the potential of RNP usage, the main drawback of this application is that Cas9-guide RNA structure is a relatively large complex. Non-viral delivery systems including electroporation, encapsulation, and delivery by modification are trending for not only transferring this large cargo but also for other DNA and RNA systems (reviewed in (Glass et al. 2018)). Electroporation, a non-selective delivery method, has been used for a long time for various DNA, RNA, and protein transfers through the cell membrane by enlarging the pores on the cell membrane via a strong electric field. While this method is highly efficient in transferring Cas9 and guide RNA to HSPCs for an aim of correction of SCD mutation (Hoban et al. 2016a; Dever et al. 2016; Magis et al. 2018), toxicity and the long-term viability issue of electroporation for a clinical setting is still being questioned. From a clinical point of view, huge quantities of Cas9 protein might be required for a clinical setting, and purification of endotoxin-free Cas9 protein is not economically feasible at this time. More industrial work is warranted to explore feasible ways for GMP grade Cas9 production in order for this technique to be practical in a clinical setting.

4 Future Perspective and Directions

While SCD was characterized more than century ago, definitive treatment for all patients is not currently available given a lack of suitable donors for curative HSCT. As monogenic disease, SCD is

one of the most important candidates for programmable nucleases, particularly CRISPR/Cas9 due to being cost-effective, easily applicable, and highly efficient. Proof-of-principle studies have shown that CRISPR/Cas9 can efficiently be used to correct the SCD mutation or induce HbF expression in *ex vivo* cell culture conditions and mouse models. However, there is still concerns about the safety due to random off-target effect and subtherapeutic efficiency. More work should be conducted in larger animal models to demonstrate the safety of the approach along with optimization studies in *ex vivo* conditions.

Clinical trials investigating the prospective of CRISPR/Cas9 for SCD are in progress or are starting soon, which will certainly direct the future of this approach. The application itself is promising but it is not currently feasible for translation into routine use especially for less developed counties such as Africa where prevalence of SCD is high. Additional cost-effective manufacturing processes for clinical grade guide RNAs and Cas9 proteins should be implemented to extend the use, and ensure a safer, more efficient product. The premise of gene therapy for the cure of SCD is moving closer to reality, though questions and challenges remain to ensure this as a feasible, safe, and lifelong curative strategy.

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Photoresponsive Hydrogels with Photoswitchable Stiffness: Emerging Platforms to Study Temporal Aspects of Mesenchymal Stem Cell Responses to Extracellular Stiffness Regulation

David Richards, Joe Swift, Lu Shin Wong, and Stephen M. Richardson

Abstract

An extensive number of cell-matrix interaction studies have identified matrix stiffness as a potent regulator of cellular properties and behaviours. Perhaps most notably, matrix stiffness has been demonstrated to regulate mesenchymal stem cell (MSC) phenotype and lineage commitment. Given the therapeutic potential for MSCs in regenerative medicine, significant efforts have been made to understand the molecular mechanisms involved in stiffness regulation. These efforts have predominantly focused on using stiffness-defined polyacrylamide (PA) hydrogels to culture cells in 2D and have enabled elucidation of a number of mechano-sensitive signalling pathways. However, despite proving to be a valuable tool, these stiffness-defined hydrogels do not reflect the dynamic nature of living tissues, which are subject to continuous remodelling during processes such as development, ageing, disease and regeneration. Therefore, in order to study temporal aspects of stiffness regulation, researchers have developed and exploited novel hydrogel substrates with *in situ* tuneable stiffness. In particular, photoresponsive hydrogels with photoswitchable stiffness are emerging as exciting platforms to study MSC stiffness regulation. This chapter provides an introduction to the use of PA hydrogel substrates, the molecular mechanisms of mechanotransduction currently under investigation and the development of these emerging photoresponsive hydrogel platforms.

Keywords

Biophysical regulation · Cell adhesion · Cell-matrix interaction · Hydrogel substrates ·

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Mechanotransduction · Mesenchymal stem cells · Photoresponsive · Photoswitchable stiffness · Polyacrylamide · Regenerative medicine · Stiffness regulation · Temporal

Abbreviations

MSC	Mesenchymal stem cell
ECM	Extracellular matrix
BMPs	Bone morphogenic proteins
TGF-β	Transforming growth factor beta
PA	Polyacrylamide
IAC	Integrin adhesion complex
FA	Focal adhesion
MAPK1	Mitogen-activated protein kinase 1
LIM	LIN-11, ISl1 and MEC-3
NMII	Non-muscle myosin-II
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
YAP	Yes-associated protein
TAZ	Transcriptional coactivator with PDZ-binding motif
LINC	Linker of nucleo- and cytoskeleton
MKL1	myocardin-like protein 1
RUNX2	Runt-related transcription factor 2
SRF	Serum response factor
PEG	Poly(ethylene glycol)
PEGdiPDA	Photodegradable PEG diacrylate

1 Introduction

Half a century of cell-matrix interaction studies has laid the foundations for our understanding of matrix-stiffness-mediated cellular regulation. Initial research in the late 1970s first identified matrix 'flexibility' as a potential regulator of cellular morphology and behaviour (Emerman et al. 1977). Work in the early 1980s later confirmed this role for matrix 'flexibility' in regulating functional cell phenotypes (Shannon and Pitelka 1981). Subsequent work over the following three decades has been able to confirm a role for matrix 'flexibility' or stiffness in the regulation of cell morphology (Pelham et al. 1997), contractility (Discher et al. 2005), motility (Lo et al. 2000), apoptosis (Wang et al. 2000), proliferation (Klein et al. 2009), growth and differentiation (Engler et al. 2004). Matrix stiffness is now well understood to be a potent regulator of cell and tissue homeostasis, with implications in development, ageing, disease (e.g. cancer and fibrosis) and regeneration (Jaalouk and Lammerding 2009; Mammoto and Ingber 2010; Mammoto et al. 2013; Tschumperlin et al. 2013; Pickup et al. 2014). Furthermore, following a seminal study in 2006, research has repeatedly demonstrated matrix-stiffness-mediated regulation of mesenchymal stem cell (MSC) phenotype (e.g. morphology, growth and secretome) and lineage commitment (Engler et al. 2006; Rowlands et al. 2008; Park et al. 2011; Shih et al. 2011; Yang et al. 2016b).

Such MSCs are a heterogeneous population of self-renewing multipotent (i.e. potential for osteogenic, adipogenic and chondrogenic *in vitro* differentiation) mesenchymal stromal cells, typically isolated from adipose tissues or the bone marrow and characterised against a combination of positive and negative cell markers (e.g. positive for CD105, CD73 and CD90, and negative for CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR), with the capacity for plastic-adherent culture and rapid expansion *in vitro* (Friedenstein et al. 1970; Dominici et al. 2006; Caplan 2007; Marquez-Curtis et al. 2015). Further to their capacity for self-renewal and multipotent differentiation, MSCs have been extensively reported to have immunomodulatory properties, making them a very promising cell source for regenerative medicine (Pittenger et al. 1999; Barry and Murphy 2004; Richardson et al. 2010; Gao et al. 2016). Indeed, a growing number of MSC-based regenerative medicine therapies are continually progressing through clinical trials (Trounson and McDonald 2015). However, in order to effectively exploit the therapeutic potential of MSCs for regenerative medicine, it is necessary to improve our understanding of how their phenotype and lineage commitment is regulated *in vivo*, both in health and disease, and furthermore how this knowledge can be translated for their regulation and manipulation *in vitro* (Bara et al. 2014; Richardson et al. 2016).

The local extracellular matrix (ECM) microenvironment or 'stem cell niche' is known to be a potent regulator of MSC phenotype and lineage commitment *in vivo* (Reilly and Engler 2010). Soluble (e.g. cytokines) and insoluble (e.g. ECM) signalling factors combine within the niche to maintain MSC potency or stimulate differentiation (O'Brien and Bilder 2013). Notably, the insoluble ECM provides both a biochemical (e.g. matrix composition) and biophysical (e.g. matrix stiffness) regulatory environment (Schofield 1978; Discher et al. 2009). Recent research has highlighted the importance of the regulatory interplay between these soluble and insoluble signalling factors during MSC maintenance and lineage commitment, both *in vivo* and *in vitro*, with particular emphasis on the cross-talk between traditionally studied biochemical (both soluble and insoluble) stimuli and more recently appreciated biophysical stimuli (i.e. ECM stiffness) (Dingal and Discher 2014; Ivanovska et al. 2017; Assis-Ribas et al. 2018). For instance, matrix stiffness has been shown to modulate the MSC secretome, with increased stiffness significantly increasing expression of bone morphogenic proteins (BMPs) and other key factors of the transforming growth factor beta (TGF-β) superfamily known to modulate MSC phenotype and lineage commitment (Wang et al. 2004; Engler et al. 2006; Dingal and Discher 2014). This indicates the potential for matrix-stiffness-mediated autocrine/paracrine regulation and could be the driving factor behind ectopic bone formation following MSC injection and engraftment into infarcted hearts, where post-infarct tissue stiffening and scar formation may upregulate BMP expression and subsequently stimulate osteogenesis (Berry 2006; Breitbach et al. 2007; Dingal and Discher 2014). Furthermore, matrix stiffness has been shown to regulate MSC osteogenesis synergistically with soluble retinoids (both *in vivo* and *in vitro*), which could be responsible for driving a fibrogenic phenotype known to contribute to fibrosis within perivascular niches (Kramann et al. 2015; Dingal et al. 2015; Ivanovska et al. 2017).

In contrast to traditional *in vitro* culture substrates (e.g. glass or polycarbonate), which have stiffnesses in the GPa range, physiological stiffnesses experienced by MSCs *in vivo* (e.g. within the bone marrow niche) vary within a range approximately two orders of magnitude lower (i.e. from 'soft' ~0.2 kPa marrow to 'stiff' ~35 kPa precalcified bone) (Discher et al. 2005; Engler et al. 2006; Winer et al. 2009; Ivanovska et al. 2017). These physiological tissue stiffnesses are known to change with ageing, and pathophysiological stiffnesses induced by fibrosis (i.e. tissue stiffening) can prime MSCs toward pro-fibrotic phenotypes (Sherratt 2009; Humphrey et al. 2014; Li et al. 2016). In light of the physiological importance and role of ECM stiffness in regulating MSCs, significant research over recent years has focused on developing novel culture platforms with physiologically relevant stiffnesses in order to enable in-depth study of matrix-stiffness-mediated MSC regulation and elucidate the relevant molecular mechanisms of mechanotransduction (i.e. conversion of biophysical stimuli into biochemical signals and processes) involved in sensing and responding to changes in stiffness within the local microenvironment (Ivanovska et al. 2015; Gilbert and Swift 2016).

The following chapter will provide the reader with an insight into the emergence of photoresponsive hydrogels with photoswitchable stiffness as novel platforms to study temporal aspects of MSC responses to extracellular stiffness regulation. An introduction to the use of polyacrylamide (PA) hydrogel substrates to study MSC stiffness regulation and the molecular mechanisms of matrix stiffness mechanotransduction currently under investigation will be provided, along with brief perspectives on the role of mechanosensitive adhesions in mediating matrix stiffness mechanotransduction and the modification of PA hydrogel substrates to study spatial aspects of MSC stiffness regulation [readers are referred elsewhere for more in-depth discussions (Horton et al. 2016; Brown and Anseth 2017)], before concluding with a more in-depth perspective on photoresponsive hydrogels as an emerging platform to study temporal aspects of MSC stiffness regulation.

2 Utilising Polyacrylamide Hydrogel Substrates to Study MSC Stiffness Regulation

Inspired by work in the early 1980s using deformable silicone rubber substrates to study cellular traction, efforts to understand matrix-stiffness-mediated MSC regulation have predominately utilised PA hydrogel substrates first developed in the late 1990s (Harris et al. 1980; Pelham et al. 1997). This approach involves the fabrication of covalently cross-linked water-swollen networks, formed via free radical polymerisation of acrylamide and bis-acrylamide, bound to functionalised (e.g. silanized) glass support surfaces (Fig. 1) (Tse and Engler 2010).

By carefully controlling both the ratio of acrylamide to bis-acrylamide and hydrogel thickness, the mechanical properties of these PA substrates can be systematically defined in order to expose cells to a range of specific physiological stiffnesses (Buxboim et al. 2010). While the high water content and porosity of these PA hydrogel substrates ensures cells are exposed to a relatively biomimetic surface, as the network is bioinert, covalent attachment of ECM molecules (e.g. fibronectin, collagen or laminin) is also required in order to provide binding sites for cell adhesions, essential to support the survival, migration, proliferation and differentiation of anchorage dependant cells such as MSCs (Geiger and Yamada 2011).

2.1 Mechanosensitive Adhesions Mediate Matrix Stiffness Mechanotransduction

Cell adhesions are primarily mediated by integrins, a family of 24 αβ-heterodimeric transmembrane receptors (composed from a combination of 18 α and 8 β subunits), which physically bind and link various extracellular matrix components (e.g. fibronectin, vitronectin, laminin and collagen), via common recognition motifs (e.g. Arg-Gly-Asp: RGD), to the actomyosin cytoskeleton (Brakebusch and Fässler 2003; Humphries 2006). Integrins mediate this connection via a dynamic array of integrin adhesion complexes (IACs) which form a continuum of highly dynamic adhesion structures (i.e. nascent adhesions, focal complexes, focal adhesions (FA) and fibrillar adhesions) with a rich composition of cytoskeletal, adaptor and signalling proteins (known as the integrin adhesome) responsible for transducing adhesion-dependant signals and subsequently mediating cytoskeletal tension, regulating adhesion dynamics, and modulating a wide range of cellular properties and behaviours, such as morphology, migration, apoptosis and differentiation (Horton et al. 2016).

Integrins and IACs have been implicated in matrix stiffness mechanotransduction following identification of mechanoresponsive elements (e.g. force-dependent integrin bond kinetics and vinculin, talin and p130Cas conformational changes) and links to diseases associated with

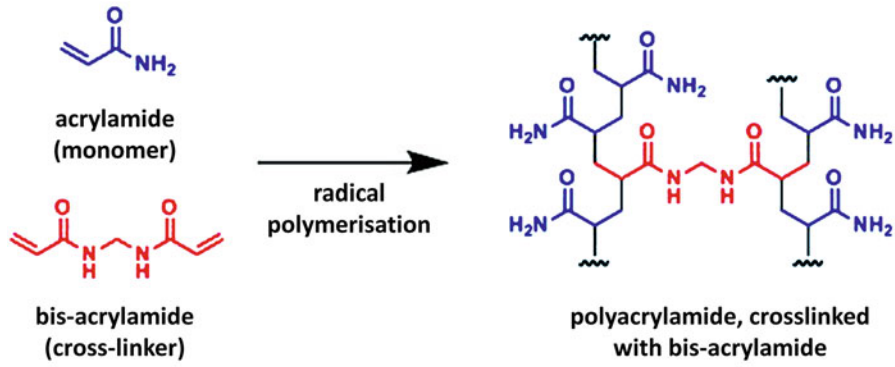

Fig. 1 Radical polymerisation of acrylamide (monomer) and bis-acrylamide (cross-linker) to form polyacrylamide

stiffness sensing (Horton et al. 2016). Specifically, a set of four interconnected axes (i.e. talin-vinculin, α-actinin-zyxin-VASP, FAK-paxillin, and kindling-ILK-PINCH) have been identified and suggested to form part of a consensus adhesome responsible for structurally linking ECM-bound integrins (e.g. fibronectin-bound α5β1 and αVβ3) to the actomyosin cytoskeleton and mediating matrix stiffness mechanotransduction (Horton et al. 2015). Indeed, kinase binding site screening and knockdown of the prominent adhesome protein vinculin in MSCs cultured on collagen-I coated PA hydrogel substrates implicated a force-sensitive cryptic binding site in vinculin for regulating mitogen-activated protein kinase 1 (MAPK1)-mediated stiffness-induced MyoD expression and myogenic lineage commitment (Holle et al. 2013). Furthermore, analysis of the α5β1-integrin-adhesome of MSCs cultured on fibronectin-coated PA hydrogel substrates identified IAC enrichment of adhesion associated adaptor and actin regulator proteins, as well as the stiffness-dependent recruitment and localisation of LIN-11, Isl1 and MEC-3 (LIM) domain-containing proteins, likely to be involved in mediating connection to the actin cytoskeleton and nucleation of signalling hubs for downstream regulation (Ajeian et al. 2016).

2.2 Molecular Mechanisms of Matrix Stiffness Mechanotransduction

A seminal study of MSCs cultured on collagen coated PA hydrogel substrates demonstrated matrix-stiffness-mediated morphology regulation and lineage-commitment, with substrate stiffness in the range of muscle (~10 kPa) promoting a spindle-shaped morphology indicative of myogenesis, while substrate stiffnesses in the range of precalcified bone (~35 kPa) promoted a spread polygonal morphology similar to differentiated osteoblasts (Engler et al. 2006). This study also demonstrated that matrix-stiffness-mediated regulation was dependant on non-muscle myosin-IIA (NMII-A) generated cytoskeletal tension (regulated by phosphorylation of NMII-A light and heavy chains), previously implicated in regulating MSC linage commitment via the downstream regulators RhoA (Ras homolog gene family, member A) and ROCK (Rho-associated protein kinase), which increased with increasing substrate stiffness and promoted FA growth (i.e. IAC regulation) (McBeath et al. 2004; Vicente-Manzanares et al. 2009). NMII-A mediated cytoskeletal tension has also been shown to be regulated by geometric features and surface topographies (e.g. 'nano-pit' arrays), which similarly modulate MSC morphology, contractility, IAC regulation (i.e. FA maturation and turnover), and subsequent lineage commitment (e.g. geometries/topographies which increase contractility and promote FA maturation stimulate osteogenesis over adipogenesis), indicating a potential core mechanotransduction pathway for biophysical stimuli (Dalby et al. 2007; Kilian et al. 2010).

Cytoskeletal tension generated by the actomyosin machinery (i.e. NMII-A cross-linked actin filaments) regulates IACs and associated adhesion structures, providing contractile forces which allow the cell to deform the surrounding matrix and migrate, while matrix resistance to deformation (i.e. stiffness) in turn modulates the levels of cytoskeletal tension generated within the cell and thus feeds back into the regulation of IACs, downstream signalling and ultimately cell phenotype (e.g. morphology and lineage commitment) (Discher et al. 2005; Choi et al. 2008; Vicente-Manzanares et al. 2009). NMII-A mediated cytoskeletal-tension-dependant IAC regulation has since been repeatedly implicated in mediating matrix stiffness mechanotransduction in MSCs (Ivanovska et al. 2015).

For instance, MSCs cultured on collagen coated PA hydrogel substrates prepared with gradient stiffnesses from 'soft' (~1 kPa) to 'stiff' (~14–40 kPa) demonstrated stiffness-dependant migration (i.e. 'durotaxis' from 'soft' to 'stiff' regions) and lineage commitment (Tse and Engler 2011; Raab et al. 2012). Interestingly, this durotaxis was dependant on the assembly of NMII-A into oriented stress fibres which were subsequently polarised by NMII-B, and certain

cell populations maintained 'soft' lineage markers even after migration from 'soft' to 'stiff' regions, indicating the potential for a 'mechanical memory' (i.e. temporally persistent reprogramming induced by mechanical inputs) which polarises and maintains cell phenotype even as biophysical ECM stimuli (i.e. matrix stiffness) change.

Moreover, analysis of MSCs cultured on 'soft' (~0.7 kPa) and 'stiff' (~40 kPa) fibronectin coated PA hydrogel substrates identified a cytoskeletal-tension-dependent role for the transcriptional co-activators YAP (Yes-associated protein) and TAZ (transcriptional coactivator with PDZ-binding motif) as molecular regulators of matrix-stiffness-dependent MSC lineage commitment (Dupont et al. 2011). Translocation of YAP/TAZ was shown to be dependent on matrix stiffness, with nuclear localisation and accumulation increasing on 'stiff' (~40 kPa) osteoid-like matrix, subsequently promoting osteogenesis. Interestingly, analysis of MSCs sequentially cultured on 'stiff' (~3 GPa) tissue culture polystyrene and then 'soft' (~2 kPa) PA hydrogel substrates indicated a further role for YAP/TAZ as a mechanical rheostat (i.e. influencing sensitivity to biophysical stimuli) responsible for modulating temporal aspects of matrix stiffness mechanotransduction and imparting MSCs with their previously identified mechanical memory (Yang et al. 2014).

Furthermore, proteomic analysis of MSCs cultured on 'soft' (~0.3 kPa), 'intermediate' (~10 kPa) and 'stiff' (~40 kPa) PA hydrogel substrates demonstrated a cytoskeletal-tension-dependent regulation of the nuclear lamina (particularly expression levels of lamin-A), implicated in the enhancement and co-regulation of YAP/TAZ-mediated stiffness-dependent lineage commitment (Swift et al. 2013). Composition of the nuclear lamina (i.e. ratios of A-type to B-type lamins) was also shown to scale with tissue stiffness *in vivo*, increasing with increasing stiffness (e.g. from 'soft' fat to 'stiff' bone). Lamin-A levels were further shown to be synergistically modulated by the retinoic acid (RA) pathway, with increased substrate stiffness and lamin-A expression promoting increased nuclear accumulation of RA receptor-γ (RARγ) and subsequent RA-antagonist-dependent enhancement of osteogenic differentiation (potentially via upregulation of runt-related transcription factor 2 (RUNX2)) (Swift et al. 2013; Dingal and Discher 2014; Ivanovska et al. 2017). Furthermore, lamin-A knockdown was shown to suppress the serum response factor (SRF) pathway, potentially implicating a role for lamin-A in co-regulating the SRF-mediated expression of actomyosin cytoskeletal components involved in stiffness-dependant differentiation (Swift et al. 2013; Dingal and Discher 2014; Buxboim et al. 2014; Ivanovska et al. 2017).

Modulation of the nuclear lamina is thought to occur in response to the transduction of mechanical signals (i.e. cytoskeletal tension) through direct connection to the actomyosin cytoskeleton via the linker of nucleo- and cytoskeleton (LINC) complex, potentially influencing global chromatin organisation and gene transcription (Crisp et al. 2006; Wang et al. 2009; Lombardi et al. 2011; Iyer et al. 2012). Indeed, analysis of MSCs cultured on defined 'osteogenic' nanotopographies similarly demonstrated cytoskeletal-tension-dependant morphology and FA regulation but also indicated remodelling of the nuclear lamina (i.e. lamin-A/C density) and chromosomal organisation during osteogenic differentiation, indicating the potential for direct biophysical regulation of gene transcription (Tsimbouri et al. 2014).

Recent analysis of both primary and immortalised MSCs cultured on 'soft' (~2 kPa) and 'stiff' (~25 kPa) collagen-I coated PA hydrogel substrates further confirmed the stiffness-dependant regulation of morphology, lamin-A/C expression and the translocation of mechano-sensitive transcription factors (Fig. 2) (Galarza Torre et al. 2018). MSCs cultured on 'stiff' substrates exhibited greater spread area, increased lamin-A/C expression and upregulation of osteogenic over adipogenic (as seen on 'soft' substrates) lineage markers. Furthermore, nuclear localisation and accumulation of the mechano-sensitive transcription factors YAP1 and myocardin-like protein 1 (MKL1) was increased

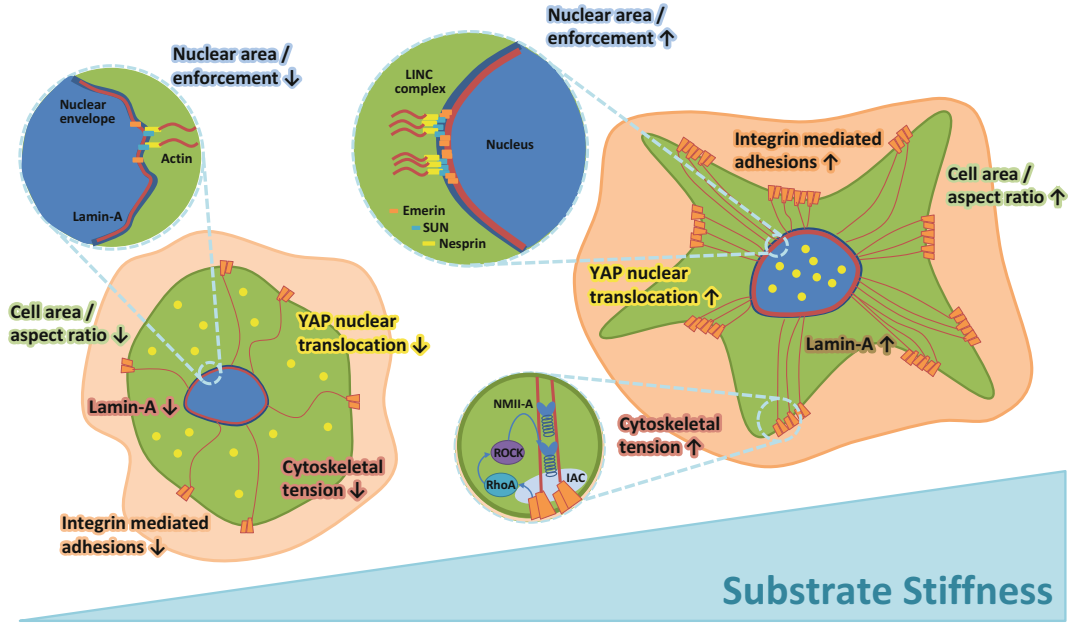

Fig. 2 Schematic detailing general MSC responses to substrate stiffness. As substrate stiffness increases (from left to right) cell area/aspect ratio, integrin mediated adhesions and cytoskeletal tension (e.g. RhoA/ROCK dependant NMII-A contraction) increase (McBeath et al. 2004; Discher et al. 2005; Choi et al. 2008; Vicente-Manzanares et al. 2009; Ivanovska et al. 2015). In addition, YAP nuclear translocation, Lamin-A expression and nuclear area/enforcement increase with increasing substrate stiffness (Dupont et al. 2011; Swift et al. 2013; Galarza Torre et al. 2018)

in MSCs cultured on 'stiff' substrates, further implicating a role for the SRF pathway (co-activated by MKL1) in mediating matrix stiffness mechanotransduction downstream of lamin-A/C regulation via direct transmission of cytoskeletal tension through the LINC complex (Connelly et al. 2010; Ho et al. 2013; Buxboim et al. 2014).

2.3 Modification of Traditional PA Hydrogel Substrates to Study Spatial Aspects of MSC Stiffness Regulation

These studies clearly illustrate the utility of stiffness-defined PA hydrogel substrates for studying and elucidating the molecular mechanisms of matrix stiffness mechanotransduction in MSCs. Moreover, it is apparent that researchers have begun to modify traditional PA hydrogel substrates, for instance utilising photopolymerisation methods in combination with photomasks to produce PA substrates with novel stiffness gradients pertinent for studying spatial aspects of MSC durotaxis, differentiation and putative mechanical memory (Fig. 3) (Tse and Engler 2011).

However, photo-patterned substrates, including those that present stiffness gradients, are limited by their static mechanical properties and thus are not best suited for studying temporal aspects of stiffness regulation such as mechanical memory or during inherently dynamic processes, such as development, ageing, disease and regeneration (Burdick and Murphy 2012). Research has therefore begun to focus on the development and exploitation of similarly motivated photoresponsive hydrogel substrates with *in situ* tuneable stiffness (Kloxin et al. 2009; Yang et al. 2014; Rosales et al. 2015, 2017, 2018; Brown and Anseth 2017; Lee et al. 2018).

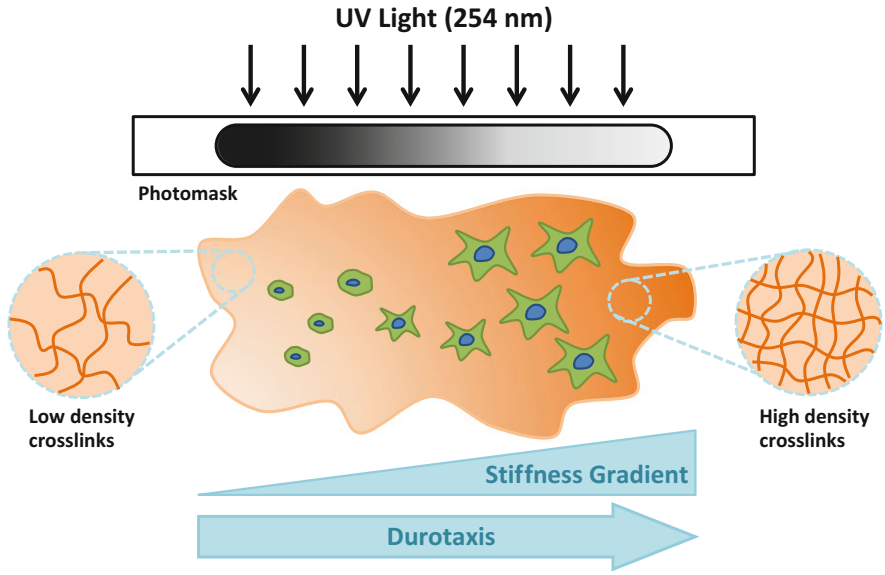

Fig. 3 Schematic detailing the preparation of a PA hydrogel substrate with stiffness gradient (increasing left to right) via photopolymerisation, resulting in directed MSC migration (durotaxis from left to right) towards increasing substrate stiffness (Tse and Engler 2011). A free radical photoinitiator was used to polymerise PA hydrogel substrates via exposure to UV light (254 nm) and gradient stiffnesses were then achieved via the use of patterned nitrocellulose photomasks which create a patterned UV exposure and subsequent hydrogel cross-linking gradients via selective photoinitiator activation

Alternative novel hydrogel substrates with phototuneable mechanical properties, exploiting similar photoirradiation methods to produce submicrometre spatially patterned stiffness gradients, have been developed and utilised in order to demonstrate a role for the spatial organisation of matrix stiffness in modulating mechanotransduction and subsequent regulation of MSC phenotype and lineage commitment, with potential implications for understanding how mechanotransduction occurs over different length scales (Fig. 4) (Di Cio and Gautrot 2016; Yang et al. 2016a; Norris et al. 2016). For instance, a novel poly(ethylene glycol) (PEG)-based hydrogel substrate with phototuneable mechanical properties was prepared via the copolymerisation of PEG monoacrylate (PEGA) with a photodegradable PEG diacrylate (PEGdiPDA) (Fig. 5) (Yang et al. 2016a). A photomask was then used to direct UV light exposure (at a wavelength (λ) of 365 nm; irradiation intensity (I_0) of 10 $mW\ cm^{-2}$; and irradiation time (t) 360 s) and subsequent degradation of the photolabile cross-linker resulting in spatially patterned softening (from ~10 kPa to ~2 kPa) of the hydrogel. MSCs cultured on hydrogels with higher concentrations of 'stiff' (~10 kPa) regions exhibited greater spread area and nuclear YAP accumulation. However, this response was only found on 'regularly organised' stiffness patterns and not maintained on 'randomised' patterns which exhibited reduced nuclear YAP accumulation and cell spread area, potentially implicating the importance of both the magnitude and spatial organisation of matrix stiffness on MSC phenotype and lineage commitment.

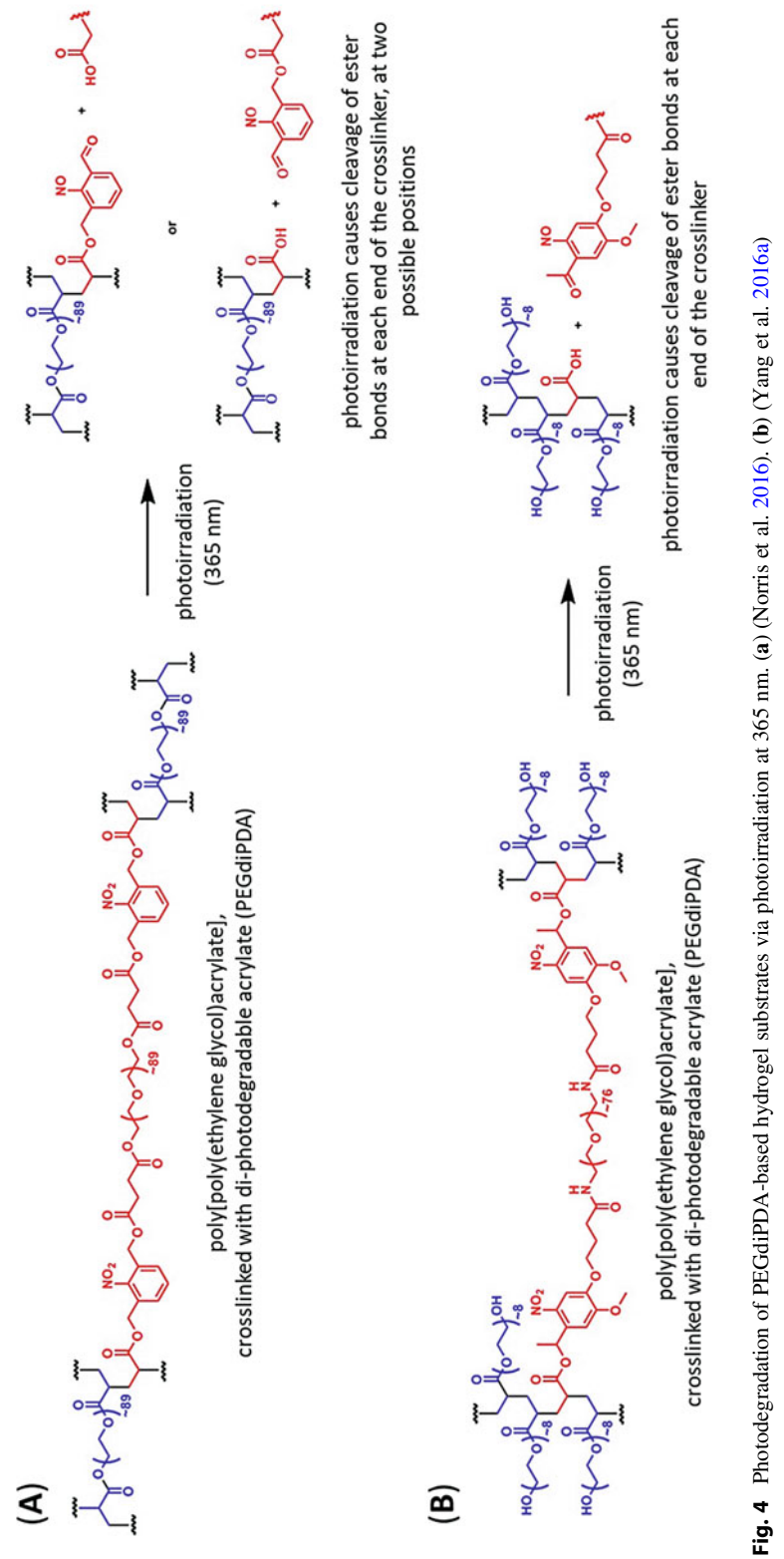

Fig. 4 Photodegradation of PEGdiPDA-based hydrogel substrates via photoirradiation at 365 nm. (**a**) (Norris et al. 2016). (**b**) (Yang et al. 2016a)

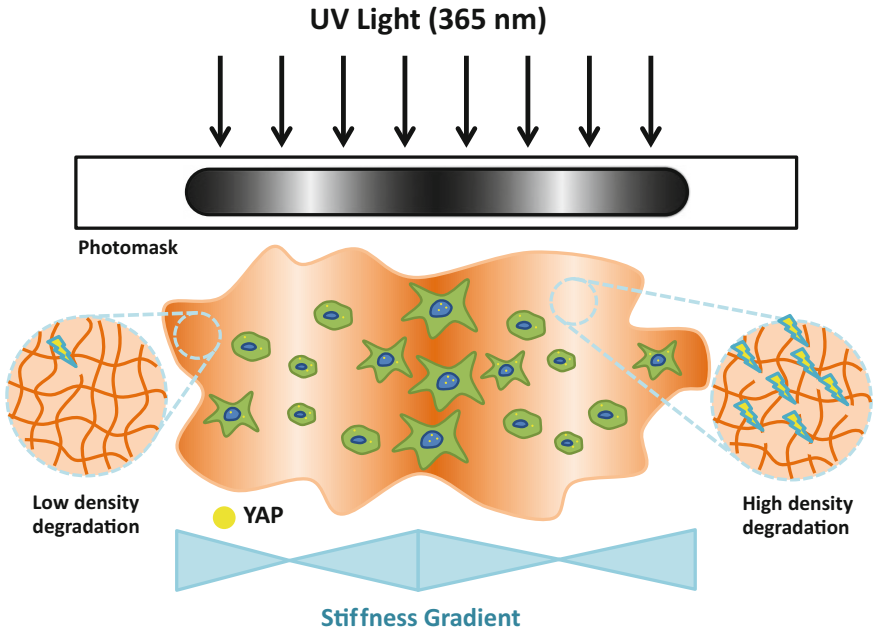

Fig. 5 Schematic detailing patterned stiffness gradient preparation via directed UV (365 nm) light exposure and spatially patterned degradation of photolabile cross-linkers resulting in isolated substrate softening and modulation of MSC spread area and YAP nuclear translocation (increasing with increasing substrate stiffness) (Yang et al. 2016a)

3 Photoresponsive Hydrogels as an Emerging Platform to Study Temporal Aspects of MSC Stiffness Regulation

Photoresponsive hydrogels that exploit rapid cytocompatible light-based chemistries for tuneable stimulation of photoswitchable stiffness *in situ* (i.e. in the presence of cells) are emerging as novel platforms for studying temporal aspects of matrix stiffness mechanotransduction in MSCs (Rosales and Anseth 2016). In comparison to alternate systems based on external triggers and stimuli (e.g. pH, temperature, Ca^{2+} concentration, competitive binding partners or magnetic fields) light-based chemistries provide a broadly biocompatible and acute, 'reagent free' stimulus (Gillette et al. 2010; Davis et al. 2011; Yoshikawa et al. 2011; Abdeen et al. 2016).

Indeed, research focusing on the putative mechanical memory of MSCs confirmed the mechanical rheostat function for the molecular regulators YAP/TAZ by utilising a PEGdiPDA-based photodegradable hydrogel substrate in order to expose MSCs to periods of *in situ* mechanical dosing on initially 'stiff' (~10 kPa) and then 'softened' (~6/4/2 kPa) substrates (Kloxin et al. 2009; Yang et al. 2014). The photodegradable hydrogel could be softened *in situ* via exposure to cytocompatible UV light ($\lambda = 365$ nm; $I_0 = 10\ mW\ cm^{-2}$) for varying amounts of time in order to achieve defined stiffnesses (e.g. from ~10 kPa to ~6/4/2 kPa with increasing exposure times). Nuclear localisation of YAP and the pre-osteogenic transcription factor RUNX2 was observed in MSCs cultured on initially 'stiff' (~10 kPa) substrates, with both subsequently translocating to the cytoplasm following substrate exposure to UV light ($\lambda = 365$ nm; $I_0 = 10\ mW\ cm^{-2}$; $t = 360$ s) and resultant softening to ~2 kPa.

Furthermore, research reporting the development of a photoresponsive hyaluronic acid-based hydrogel substrate with photoswitchable stiffness based on sequential photodegradation and

photoinitiated cross-linking reactions, demonstrated the reversible modulation of MSC morphology and YAP/TAZ translocation in response to *in situ* substrate softening and subsequent stiffening (Fig. 6) (Rosales et al. 2017). In this study, the hyaluronic acid-based hydrogel was functionalised with RGD binding domains to support MSC adhesion, *o*-nitrobenzyl acrylates to enable photodegradation, and methacrylates to enable subsequent photoinitiated cross-linking. This material was therefore able to 'reversibly' alter its stiffness from 'stiff' to 'soft' upon cleavage of the *o*-nitrobenzyl groups, and back to 'stiff' again upon methacrylate cross-linking. Specifically, initial exposure to dithiothreitol stimulated preferential cross-linking of the photodegradable *o*-nitrobenzyl acrylates and the formation of a 'stiff' (~14.8 kPa) hydrogel. Subsequent exposure to UV light ($\lambda = 365$ nm; $I_0 = 10\ mW\ cm^{-2}$; $t = 600$ s) stimulated degradation of the *o*-nitrobenzyl containing cross-links and softening of the hydrogel to ~3.5 kPa. Stiffening (from ~3.5 kPa to ~27.7 kPa) was then achieved by incubating the softened hydrogel in photo-initiator and subsequently exposing the system to visible light (400–500 nm) in order to initiate chain polymerisation of the remaining methacrylate groups. MSCs exhibited spread morphologies and nuclear YAP/TAZ localisation on initially 'stiff' substrates, which decreased and then subsequently increased following substrate softening and subsequent stiffening, confirming the robust mechanosensitivity of YAP/TAZ as molecular regulators of matrix stiffness mechanotransduction in MSCs. While this material enables *in situ* analysis of MSC responses to a relatively large range of reversible stiffness modulation, enabling real-time analysis of mechanotransduction mechanisms and mechanical memory, the system requires the use of a small molecule photoinitiator with potential confounding effects.

In addition to systems based on photodegradation and photoinitiated cross-linking, photoresponsive hydrogels with photoswitchable stiffness have been demonstrated utilising azobenzene-containing cross-linkers which do not require the use of any potentially cytotoxic chemical initiators or suspected carcinogenic by-products (e.g. nitrosobenzyl-containing products) (Zhu et al. 2014; Rosales et al. 2015, 2018; Lee et al. 2018). Azobenzene undergoes reversible *trans-cis* isomerisation, upon exposure to cytocompatible wavelengths of light (e.g. 350–550 nm), which results in a structural change suitable for modulating network mechanics without effecting connectivity (Beharry and Woolley 2011; Rosales et al. 2015). Poly(ethylene glycol) and hyaluronic acid-based systems with reversibly tuneable stiffnesses (~ <500 Pa range) have been demonstrated for potential use in mechanobiology studies focusing on alternate cell types (valvular interstitial cells and NIH 3 T3 fibroblasts), utilising azobenzene within peptide cross-linkers and noncovalent host-guest interactions with cyclodextrin, respectively (Rosales et al. 2015, 2018). These systems demonstrate the potential for achieving reversible stiffness modulation without the use of small molecule photoinitiators but are limited by their relatively small range.

Building on this work, recent research confirming the phenotypic response of MSCs to *in situ* substrate stiffening via morphometric analysis reported the development of a photoresponsive PA-based hydrogel substrate with intrinsically photoswitchable stiffness (~4 kPa range) based on the incorporation of a photoisomerisable azobenzene-based cross-linker (Fig. 7) (Lee et al. 2018). MSCs cultured on 'stiff' (~10 kPa) fibronectin-coated substrates exhibited spread morphologies, while those cultured on pre-UV irradiated ($\lambda = 365$ nm;$I_0 = 32.5\ mW\ cm^{-2}$; $t = 1800$ s) and therefore softened substrates were smaller and more rounded. Furthermore, MSCs cultured on softened substrates for 24 h and then exposed to blue light irradiation ($\lambda = 365$ nm; $I_0 = 32.5\ mW\ cm^{-2}$; $t = 3600$ s) and subsequent substrate stiffening over a further 24 h exhibited a significant increase in cell area and decrease in circularity.

These studies set the stage for the development and exploitation of novel photoresponsive hydrogels with temporal regulation of photoswitchable stiffness, in order to study mechanisms of matrix stiffness mechanotransduction in MSCs and inform methods of regulating their therapeutic potential for regenerative medicine. In particular, these emerging platforms enable time-resolved analysis of

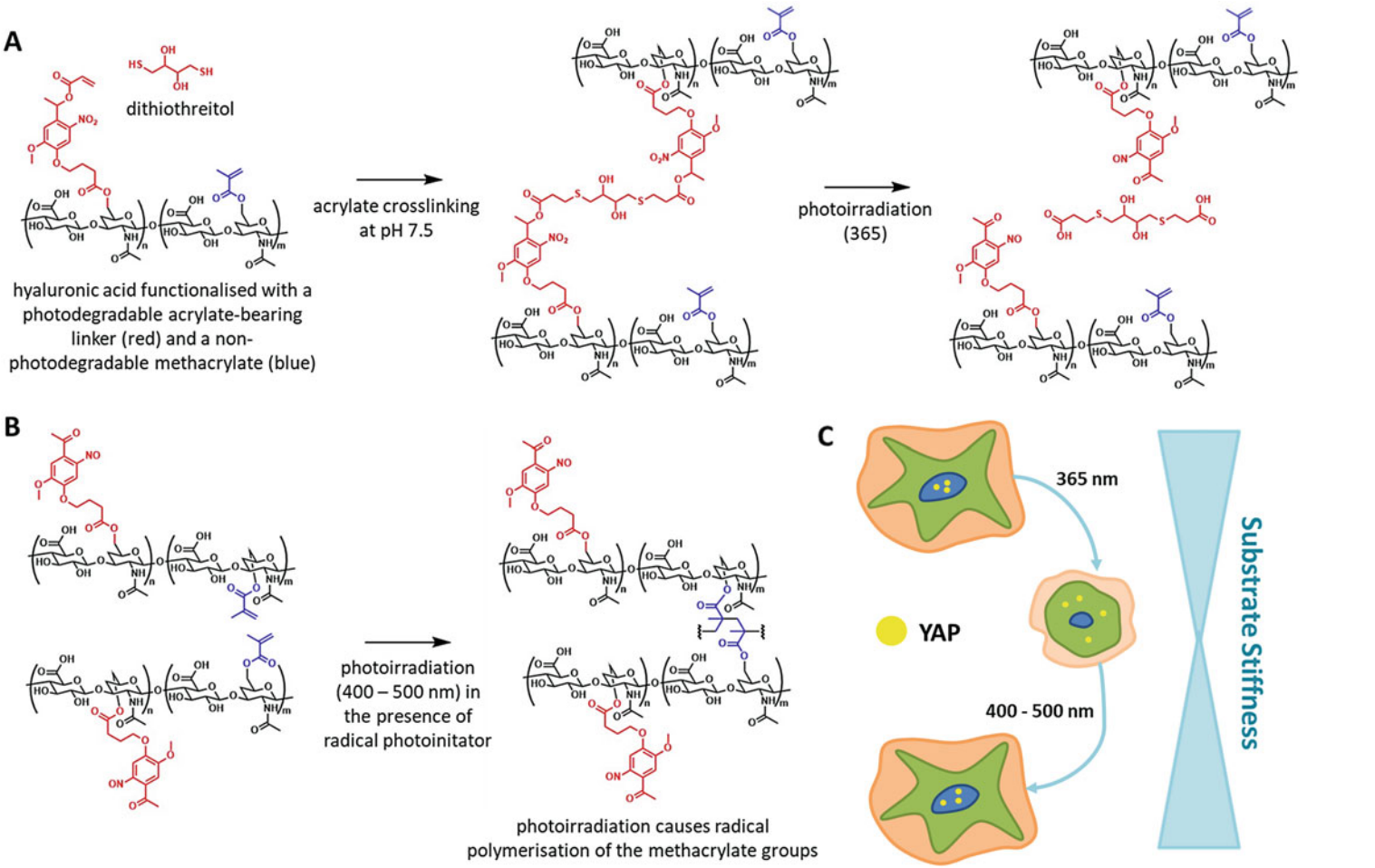

Fig. 6 (**a**) Dithiothreitol stimulated cross-linking of photodegradable acrylate-bearing linkers followed by photodegradation of o-nitrobenzyl containing cross-links via irradiation at 365 nm (Rosales et al. 2017). (**b**) Radical polymerisation of non-photodegradable methacrylate groups via photoirradiation (400–500 nm) in the presence of radical photoinitiator (Rosales et al. 2017). (**c**) Schematic detailing the modulation of MSC spread area and YAP nuclear translocation during substrate softening and subsequent stiffening (Rosales et al. 2017)

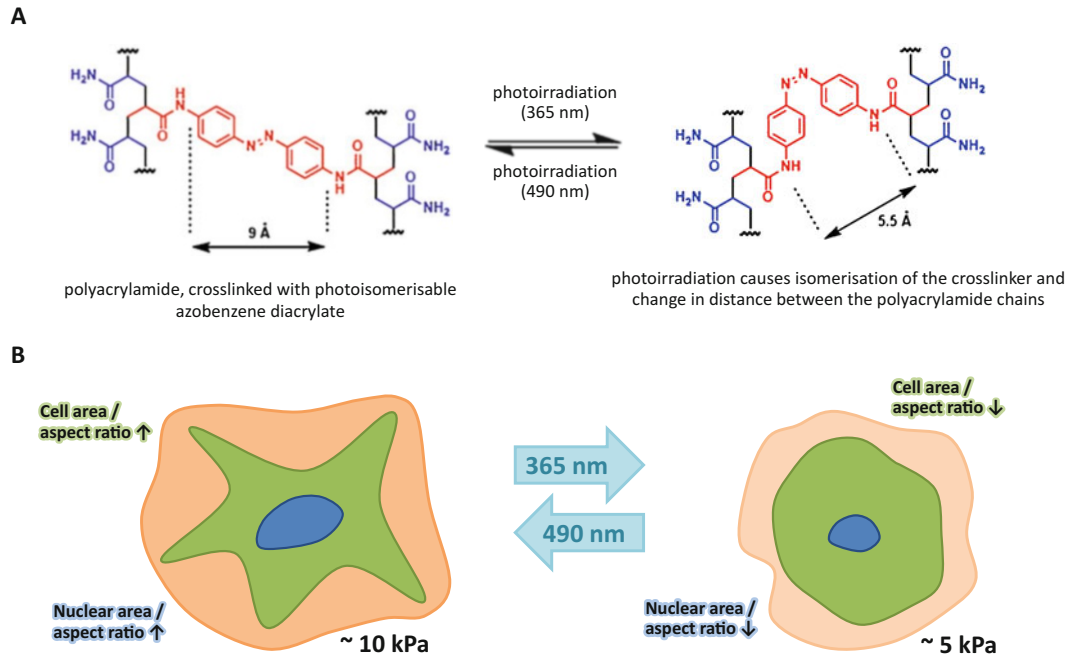

Fig. 7 (**a**) Reversible photoisomerisation of azobenzene diacrylate, used to crosslink polyacrylamide, via photoirradiation at 365 nm (*trans* – *cis*) or 490 nm (*cis* – *trans*), resulting in modulated network structure. (**b**) Schematic detailing MSC morphology (cell and nuclear area/aspect ratio) in response to substrate stiffness modulation from 'stiff' (~10 kPa) to soft (~5 kPa) (Lee et al. 2018)

MSCs providing a novel method to study matrix stiffness mechanotransduction and mechanical memory, particularly in the context of dynamic biological processes with implicit changes in matrix stiffness and tissue mechanics, such as development, ageing, disease (e.g. cancer and fibrosis) and regeneration.

4 Conclusions and Future Prospects

Seminal cell-matrix interaction studies have identified and confirmed a role for matrix stiffness in regulating MSC phenotype and lineage commitment (Pelham et al. 1997; Engler et al. 2006). An era of subsequent research utilising the methods developed in these initial studies has expanded our understanding of matrix-stiffness-mediated MSC regulation and elucidated several mechanisms of mechanotransduction (Du et al. 2011; Dupont et al. 2011; Holle et al. 2013; Swift et al. 2013; Yang et al. 2014; Ajeian et al. 2016). However, these formative methods utilising stiffness-defined PA hydrogel substrates have limited potential for probing spatiotemporal aspects of MSC stiffness regulation. Therefore, recent research has begun to modify traditional PA hydrogel substrates with photopolymerisation techniques and develop novel alternatives with phototuneable stiffnesses in order to create spatially patterned stiffness gradients and begin to study their effects on MSC phenotype and lineage commitment (Tse and Engler 2011; Yang et al. 2016a; Norris et al. 2016). However, not all of these modified platforms are suitable for studying both spatial and temporal aspects of MSC mechanotransduction (Tse and Engler 2010; Norris et al. 2016). Therefore, researchers have begun to develop novel photoresponsive hydrogels with *in situ* tuneable stiffness (Kloxin et al. 2009; Yang et al. 2014; Rosales et al. 2015,

2017, 2018; Brown and Anseth 2017; Lee et al. 2018). Hydrogel substrates with photoswitchable stiffness in particular are emerging as superior platforms for studying temporal aspects of MSC stiffness regulation and are therefore well placed to help develop our understanding of matrix stiffness mechanotransduction and inform the effective use of MSCs within regenerative medicine therapies (Rosales et al. 2017; Lee et al. 2018).

Future development of these substrates will likely involve the combination of spatial and temporal stiffness modulation, through the advancement of new photopatterning technologies (e.g. Alvéole), in order to model matrix interfaces and design systems which robustly direct multiple cell fates *in situ*, as well as the design of photoswitchable chemistries with tuneable photoabsorption (i.e. visible or near-infrared wavelengths) in order to enable long-term cytocompatibility and integration with live-cell fluorescent imaging techniques for the real-time modulation and investigation of spatiotemporal stiffness-dependant MSC regulation (Rosales and Anseth 2016; Wu et al. 2016; Calbo et al. 2017; Dong et al. 2017; Stoecklin et al. 2018; Lee et al. 2018).

Furthermore, combining these platforms with high-throughput '-omics' analyses will rapidly improve our ability to elucidate and unpick the mechanotransduction mechanisms mediating MSC stiffness regulation.

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Cellular Complexity at the Interface: Challenges in Enthesis Tissue Engineering

Isabel Calejo, Raquel Costa-Almeida, and Manuela E. Gomes

Abstract

The complex heterogeneous cellular environment found in tendon-to-bone interface makes this structure a challenge for interface tissue engineering. Orthopedic surgeons still face some problems associated with the formation of fibrotic tissue or re-tear occurring after surgical re-attachment of tendons to the bony insertion or the application of grafts. Unfortunately, an understanding of the cellular component of enthesis lags far behind of other well-known musculoskeletal interfaces, which blocks the development of new treatment options for the healing and regeneration of this multifaceted junction. In this chapter, the main characteristics of tendon and bone cell populations are introduced, followed by a brief description of the interfacial cellular niche, highlighting molecular mechanisms governing tendon-to-bone attachment and mineralization. Finally, we describe and critically assess some challenges faced concerning the use of cell-based strategies in tendon-to-bone healing and regeneration.

Keywords

Biomarkers · Bone · Cell Biology · Cell-based therapies · Enthesis healing/repair · Regeneration · Tendon

Abbreviations

3D	Three-dimensional
ACL	Anterior cruciate ligament
ALP	Alkaline phosphatase
ASCs	Adipose-derived stem cells
Bmp/BMP	Bone morphogenetic protein
Bmpr1a	Bone morphogenetic protein receptor 1a
BMU	Basic multicellular unit
BSP	Bone sialoprotein
ECM	Extracellular matrix
GDF	Growth and differentiation factors

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Hh	Hedgehog
IBSP	Integrin binding sialoprotein
Ihh	Indian hedgehog
MMP	Matrix metallopeptidase
MSCs	Mesenchymal stem cells
OCN	Osteocalcin
PTHrp	Parathyroid hormone-related protein
RUNX2	Runt-related transcription factor 2
Scx/ SCX	Scleraxis
Smo	Smoothened
Sox9	Sex-determining region Y-box 9
TCs	Tenocytes
TDSCs	Tendon-derived stem cells
TgfbrII	Transforming growth factor beta receptor II
TGFß	Transforming growth factor beta
TSPCs	Tendon stem/progenitor cells

1 Introduction

Tendon- (or ligament-)to-bone interface, also known as enthesis, is a musculoskeletal structure that allows a smooth transition between two widely different tissues. This soft-to-hard tissue is prone to acute and overuse injuries, being the rotator cuff and anterior cruciate ligaments (ACL, ligament-to-bone insertion) the most common and debilitating injury sites.

In trauma and orthopedic surgery, these injuries are very common and affect both young people, from professional athletes to active workers, and elderly patients (Yamaguchi et al. 2006; Docheva et al. 2015; Gans et al. 2018). Commonly, orthopedic surgeons are faced with partial or complete rupture of Achilles and rotator cuff tendons, which are very difficult to heal due to the complexity of interface tissue structure and the formation of scar tissue (Lu and Thomopoulos 2013). Even though some patients are asymptomatic, the damage caused in these sites significantly affects the daily life due to associated morbidity. Besides conservative therapies, which frequently fail or are not appropriate, current surgical treatments rely on the use of grafts, but it has already been reported that these procedures present structural and mechanical failure rates of 20–94% at the surgical site (Cho and Rhee 2009; Øiestad et al. 2009). Therefore, it is crucial to explore easy and suitable strategies to improve tendon-to-bone junction healing and regeneration.

Enthesis tissue engineering appoaches have been receiving increased attention as strategies to overcome some of the challenges encountered in the regeneration of tendon-to-bone interface. Enthesis is a multiphasic tissue that displays a complex gradient in composition, tissue organization and, therefore, mechanical properties, while it is maintained by a heterogeneous cell population. Therefore, different approaches, such as cell-based strategies, growth factors and gene therapy, scaffold-based strategies and mechanical stimulation, have been explored (Phillips et al. 2008; Bayrak and Yilgor Huri 2018; Bonnevie and Mauck 2018). However, up to now, the existing challenges faced in the clinics, when trying to restore musculoskeletal interfaces functionality, have led to a preference toward stem cell-mediated treatments, due to their regenerative and differentiation potential as nature tissue engineers. Hence, it is crucial to understand the cellular complexity observed at this junction toward generating adequate and straightforward cell-based approaches.

This chapter reviews the main characteristics of tendon-to-bone biology, including the cellular phenotypes and native niche properties, highlighting major challenges in enthesis tissue engineering.

2 Overview on Tendon-to-Bone Biology

2.1 Tendon Tissue

Tendons are well-organized and dense fibrous connective tissues that link muscle to bone. Due to their mechanosensitive nature, tendons have the ability to respond and adapt to the transmission of contraction-forces by muscles to the skeleton, allowing motion and maintenance of posture. Normally, tendons vary in structure, composition and mechanical properties,

depending on their anatomic location, which is coupled with their function while interacting within myotendinous (tendon-to-muscle) or tendon-to-bone junctions (Magnusson et al. 2003; Thomopoulos 2003; Kjaer 2004; Franchi et al. 2007).

Naturally, tendons have very limited and inefficient healing capacity. Intrinsic and extrinsic mechanisms are known to occur simultaneously during tendon healing, where resident cells and surrounding cells, respectively, are recruited (Fenwick et al. 2002). However, cell infiltration into the injury site and the creation of adhesions leads to the formation of fibrotic tissue (Docheva et al. 2015). Additionally, cells regulating fibrotic tendon healing have not yet been defined (Howell et al. 2017).

The limited knowledge on tendon biology relies on the fact that mature tendon tissue is relatively hypocellular, hypovascularized and mainly composed of extracellular matrix (ECM). Indeed, collagens account for up to 85% of its dry weight (Docheva et al. 2015). This tissue is a good example of a highly organized and multihierarchical structure composed of collagen fibrils densely packed into fiber bundles and these ones into fascicles (Fig. 1), interspersed at each level with different amounts of non-collagenous matrix (Thorpe et al. 2013). Although collagen type I is the most abundant element, other fribillar collagens can also be found, including collagen type III, V and IX. Additionally, although in smaller amounts, non-collagenous ECM elements, such as decorin, biglycan, fibromodulin, tenascin C, fibronectin, thrombospondin and elastin, also compose tendon ECM. Together with the collagenous and non-collagenous matrix, tendon resident cells coordinate the necessary cues to promote tissue repair and eventual regeneration (Gattazzo et al. 2014). Strikingly, tendons were considered to be only composed by terminally differentiated cells. But, despite its hypocellular nature, a mixed population of mature cells (tenocytes) and stem/progenitor cells (Bi et al. 2007) was found to compose the cellular environment, as addressed in detail below.

2.1.1 Tendon Cell Populations

2.1.1.1 Tenocytes

Tenocytes (TCs) or tendon cells are elongated fibroblast-like cells that lie arranged in long and parallel chains between collagen fibers and comprise about 90% of the cellular components of tendon. Therefore, they exhibit a stretched cytoplasm as a result of the stretching mechanical forces acting on tendons. Moreover, these cells present a prominent nucleus, a well-developed rough endoplasmic reticulum and are responsible for the synthesis, degradation and maintenance of the ECM (Patterson-Kane and Rich 2014). Tenocytes either reside between the collagen fibers within the fascicles or together in the interfascicular matrix between the fascicles, being denominated as intrafascicular or interfascicular TCs, respectively (Thorpe et al. 2015). Tenocytes communicate between each other by extending cytoplasmic processes into the three-dimensional (3D) tendon ECM network and establishing cell-cell contacts through adherens and gap junctions (Patterson-Kane and Rich 2014). Interestingly, differences have been highlighted regarding the influence of aging on the morphology, biochemistry and composition of tendon cells (Ippolito et al. 1980; Kannus 2000). Tendons from young donors have been referred as having spindle shaped cells, namely tenoblasts, while aged tendons tend to contain fewer and elongated cells, the tenocytes (Kannus 2000). Such morphological changes have been also shown to be accompanied by an evident decrease both in cell number and cell activity (Ippolito et al. 1980). Moreover, conversion of tenoblasts to tenocytes has been reported to occur under the influence of different stimulus, such as exercise or trauma (Chuen et al. 2004). Therefore, from developmental stage to adulthood, evidences confirm a gradual decrease in cell-to-matrix ratio; thus, the native cell-mediated reparative response seems to become very limited.

2.1.1.2 Tendon-Derived Stem Cells

A minor population of cells has been firstly identified in human and mouse tendons (Bi et al.

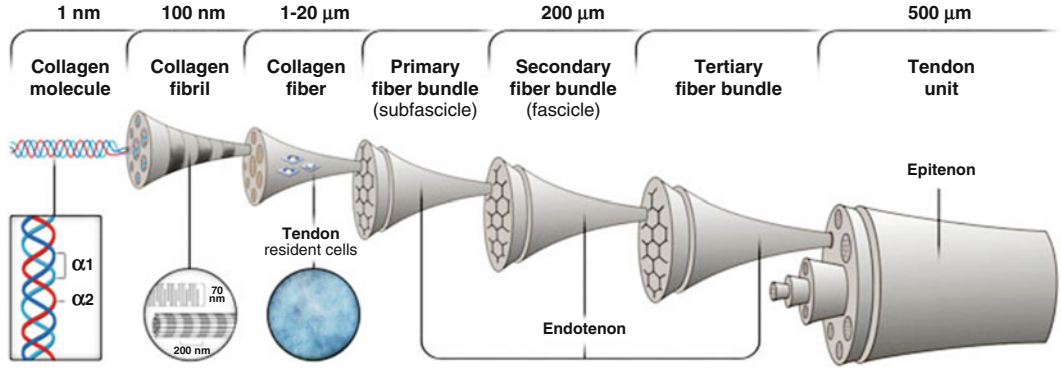

Fig. 1 Hierarchical structure of tendon. From Costa-Almeida et al. (2015). Copyright © Springer International Publishing Switzerland 2015

2007) and later in tendon samples of rabbit (Zhang and Wang 2010), rat (Yung-Feng et al. 2010; Tan et al. 2012; Rui et al. 2013), fetal bovine (Yang et al. 2016), horse (Lovati et al. 2011) and fetal human (Yin et al. 2010). These cells have been reported to fulfill universal criteria of stem cells, namely clonogenecity, self-renewal and multilineage differentiation capacities after *in vitro* expansion and *in vivo* transplantation (Bi et al. 2007). However, some heterogeneous properties have been reported, suggesting the existence of progenitor cells. Therefore, this cell population is usually termed as tendon-derived stem cells (TDSCs) or tendon stem/progenitor cells (TSPCs) (Bi et al. 2007).

Concerning the physiological niche, as cells residing within tendons, TDSCs share the same microenvironment with tenocytes and are expected to play an important role in tissue maintenance and repair. Mechanical loading, biological factors, matrix structure, resident cells, physiological factors (oxygen tension and metabolic products), topographical cues, among other possible signals, seem to influence the fate of TDSCs (Yin et al. 2010; Yu et al. 2017). *In vitro* induction studies have shown the ability of these cells to differentiate into tenocytes or towards the chondrogenic, osteogenic and adipogenic lineages and animal model studies have reported the formation of tendon-, cartilage-, bone- and tendon-bone junction-like tissues (Bi et al. 2007; Rui et al. 2010; Yin et al. 2010; Zhang and Wang 2010; Lovati et al. 2011; Yang et al. 2016).

Therefore, the application of this cell population in tendon and interfacial tissue engineering would be of great interest. However, its application still faces several limitations, including the lack of well-established enrichment and selection protocols for a straightforward isolation. A large uncertainty also remains around the markers that enable the differentiation between TDSCs, mesenchymal stem cells (MSCs) and TCs. Thus, the establishment of a set of criteria to differentiate the different cell types would be of great interest, enabling a better assessment of the tendon niche, influence of pathologies and, even, for interface tissue applications.

2.2 Bone Tissue

Bone is a highly mineralized connective tissue essential in important functions in the human body, such as locomotion, support of other organs, protection of soft tissues, harbor of bone marrow and blood production, as well as, storage and homeostasis of minerals (phosphate and calcium). Its structure and composition determine some unique mechanical properties that allow to have, not only, structural strength and lightweight design, but also stiffness, elasticity, resistance to deformation and capacity to absorb energy (Seeman and Delmas 2006). Macroscopically, two structural features can be distinguished: the cortical and trabecular bone (Fig. 2). Cortical

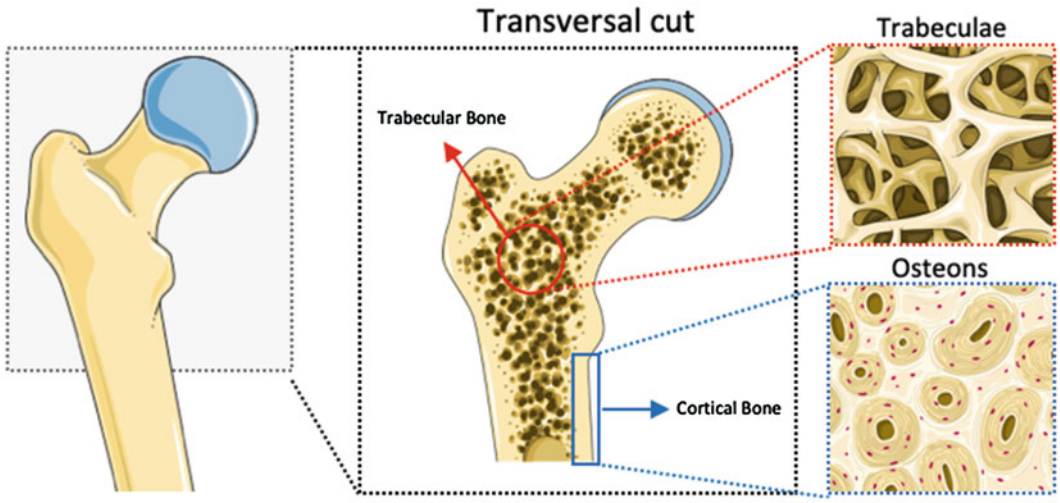

Fig. 2 Structural features of bone. The cortical and trabecular bone

bone is present around the bone, forming a dense and parallel shell of lamellar units – the osteons (Seeman and Delmas 2006). On the contrary, trabecular bone presents a less dense, spongy-like and homogeneous network of trabeculae, being always surrounded by the cortical bone (Seeman and Delmas 2006).

Bone, as a dynamic organ, undergoes a complex but continuous remodeling process in a cycle comprising three different phases: (1) bone resorption, (2) transition from resorption to new bone and (3) bone formation (Crockett et al. 2011). This process is only possible due to the balanced coordination of bone cells – osteoblasts, osteoclasts, osteocytes and bone lining cells – that altogether form a temporary anatomical structure, the so called basic multicellular unit (BMU) (Hattner et al. 1965). Herein, the Characteristics of the distinct cellular phenotypes will be highlighted, addressing their role in bone physiology.

2.2.1 Bone Cell Populations

2.2.1.1 Osteoblasts – Bone Forming Cells

Osteoblasts, the bone-forming cells, comprise up to 6% of the total bone resident cell population and are located along the bone surface. These cells derive from MSCs whose differentiation is controlled by the master transcription factor, run-related transcription factor 2 (*RUNX2*) (Jensen et al. 2010). Once this transcription factor is activated, osteoprogenitor cells undergo a proliferation process characterized by the expression of specific markers being afterwards denominated as pre-osteoblasts (Long 2011) (Fig. 3). These cells undergo morphological changes, becoming larger and with a cuboidal shape. The activity of alkaline phosphatase (ALP) increases and the cells start to secrete more bone matrix-related proteins (Capulli et al. 2014). Mature osteoblasts express higher levels of bone matrix proteins such as osteocalcin (OCN), bone sialoprotein (BSP) I/II and collagen type I, among others (Fig. 3). Eventually, these cells will be trapped within the bone matrix and start the deposition of organic matrix and its subsequent mineralization (Crockett et al. 2011). After osteoblast maturation, these cells can undergo one of three different fates: (1) become osteocytes as they are embedded in the bone, (2) transform into inactivated surface osteoblasts called bone-lining cells or (3) undergo programmed apoptosis (Rochefort et al. 2010).

2.2.1.2 Osteocytes – Bone Remodeling Cells

Osteocytes comprise up to 95% of bone cellular niche, being the most abundant and long-living cells with a lifespan of decades (Capulli et al. 2014). These cells are known for their central

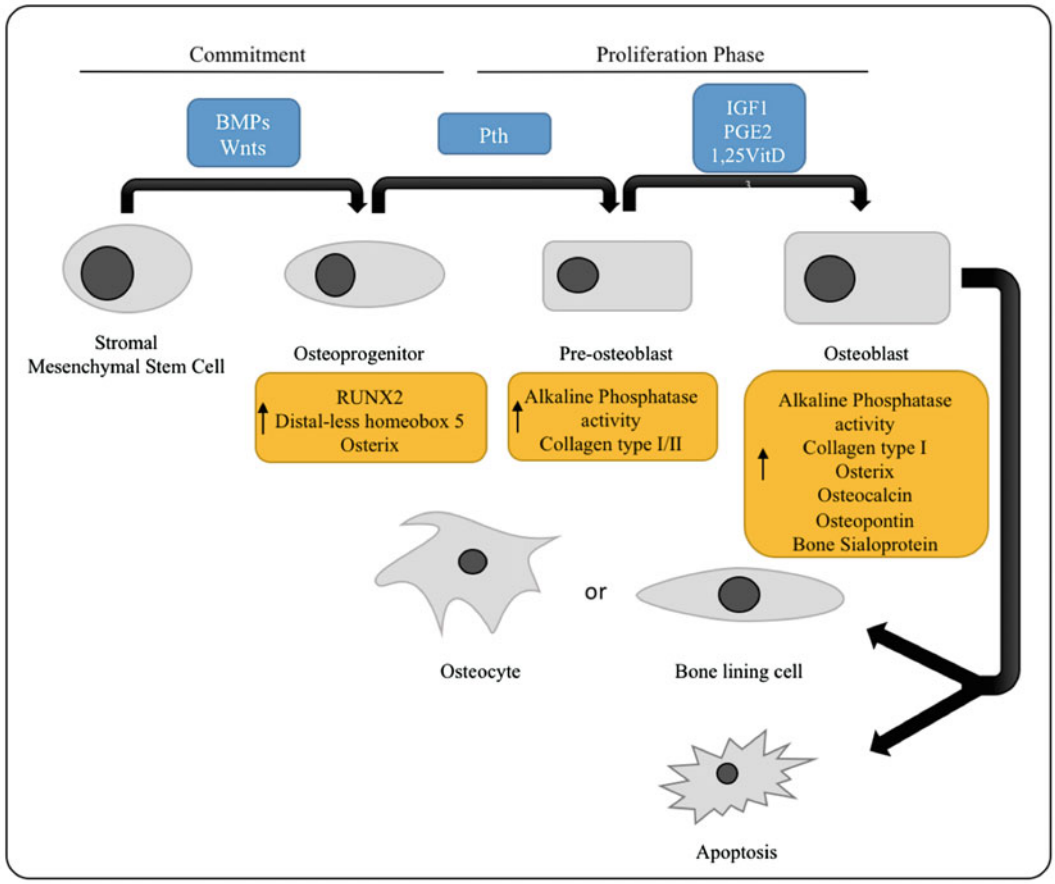

Fig. 3 Osteoblast differentiation process: from commitment to matrix synthesis. Schematic representation of the process of osteoblast differentiation and possible fate of mature osteoblast (*BMPs* bone morphogenic proteins, *Pth* Parathyroid hormone, *Wnts* Wingless, *IGF* insulin-like growth factor, *PGE* prostaglandin, *VitD3* vitamin D3)

role in bone formation and resorption. Contrary to osteoblasts, osteocytes are easily identified in a bone section because of their dendritic shape and localization within small lacuna spaces in the hard mineralized bone matrix (Noble 2008). Nevertheless, cell morphology varies between rounded and elongated shape depending on the bone type (Noble 2008).

As already mentioned, osteocytes are originated from old osteoblasts buried in the bone matrix. Cells at early stages of osteoblast to osteocyte differentiation have been extensively named as "preosteocytes", "young osteocytes" or "large osteocytes" (Rochefort et al. 2010). When mature, osteocytes are completely trapped in the mineralized matrix and previously expressed osteoblast markers are downregulated or switched off, while osteocyte markers start to be expressed (e.g. sclerostin) (Palumbo et al. 1990; Bonewald 2011). The majority of these markers are regulated, among other signals, by mechanical loading (Bonewald 2011). Once inside the lacunae, osteocyte cytoplasmic processes originate tinny tunnels, forming the so-called canaliculi, which will form the lacunocanalicular system, allowing not only the connection with neighboring osteocytes, but also osteoblasts and bone-lining cells at the bone surface (Civitelli 2008; Bonewald 2011). These communications are performed through the transport of small signaling molecules (such as prostaglandins and nitric oxide) and by interstitial fluid (Schaffler and

Kennedy 2012). Furthermore, the mechanosensitive function of osteocytes is possible due to the canalicular network and the spatial arrangement and shape of osteocytes (Bonewald 2011). However, the molecular mechanisms that trigger the convertion of mechanical stimuli to biochemical signals in osteocytes are not yet fully understood, even though some mechanisms have already been proposed (Xiao et al. 2006; Santos et al. 2010).

2.2.1.3 Bone Lining Cells

Bone lining cells are quiescent flat-shaped cells, originated from inactivated osteoblasts and are found at the bone surface, where neither bone resorption nor formation occurs (Miller et al. 1989; Rochefort et al. 2010). Some of these cells exhibit cytoplasmic processes and gap junctions to communicate with adjacent bone lining cells and osteocytes. Together with other bone cells, they are a component of the BMU, having a role in the bone remodeling cycle (Eriksen 1986). The function of bone lining cells is not fully understood but it is known that they have a secretory activity depending on the bone physiological status (Matic et al. 2016). Moreover, until now, there are no defined markers for the identification of bone lining cells or well-established and effective isolation protocols (Matic et al. 2016).

2.2.1.4 Osteoclasts – Bone Resorption Cells

Osteoclasts are large and terminally differentiated multinucleated cells originated from mononuclear precursors of the hematopoietic stem cell lineage. During osteoclastogenesis, or bone resorption, osteoclasts suffer a rearrangement of the cytoskeleton and become polarized; thus, different unique membrane domains can be distinguished: (1) the sealing zone and (2) ruffled border – that are in contact with the bone matrix – (3) basolateral and (4) functional secretory domains – which are not in contact with the bone matrix but with the extracellular fluid of other cells (Arana-Chavez and Bradaschia-Correa 2009). The ruffled border is responsible for the resorptive activity of osteoclasts, including the dissolution of hydroxyapatite due to the acidification in the resorption lacuna (Arana-Chavez and Bradaschia-Correa 2009). After mineral dissolution, the organic part is exposed to the action of proteolytic enzymes, leading to the degradation of both inorganic and organic matrix components (Arana-Chavez and Bradaschia-Correa 2009), resulting in bone matrix resorption.

2.3 Interfacing Tendon and Bone

Although enthesis bridges tendon and bone, cells within this interface reside within a unique microenvironment that is different from that found in tendon and bone niches, exhibiting geometrical and compositional dissimilarities (Rossetti et al. 2017). Two types of tendon-to-bone interface have been distinguished according to their location and structure: "fibrous" and "fibrocartilaginous" entheses (Benjamin et al. 2006). In fibrous attachments, tendon or ligaments attach directly or indirectly to long bones via the periosteum. In fibrocartilaginous attachments, typical of epiphyses and apophyses, there are locations where chondrogenesis has occurred. These insertions are the most common and with major clinical relevance, and include the bony attachments of rotator cuff and Achilles tendons, as well as of ACL.

The fibrocartilaginous insertion site has been classified into four stratified but continuous zones: zone 1, tendon/ligament; zone 2, non-mineralized fibrocartilage; zone 3, mineralized fibrocartilage; and zone 4, bone (Benjamin et al. 2002; Thomopoulos et al. 2002) (Fig.4). Tendon constitutes the first part of enthesis, where mechanical properties, as well as composition are similar to those found in the mid-substance tendon. This zone is characterized by its predominantly aligned collagen type I fibers and small amounts of decorin with interspaced spindle-shaped tendon resident cells (Waggett et al. 1998; Thomopoulos et al. 2002; Thomopoulos et al. 2003) (Fig.5a, c). Zone 2, also known as uncalcified fibrocartilage, comprises collagen type II and high amounts of pericellular collagen type III, together with small amounts of collagen type I, IX and X and proteoglycans (decorin and aggrecan) (Fukuta et al. 1998; Waggett et al. 1998; Thomopoulos et al. 2003;

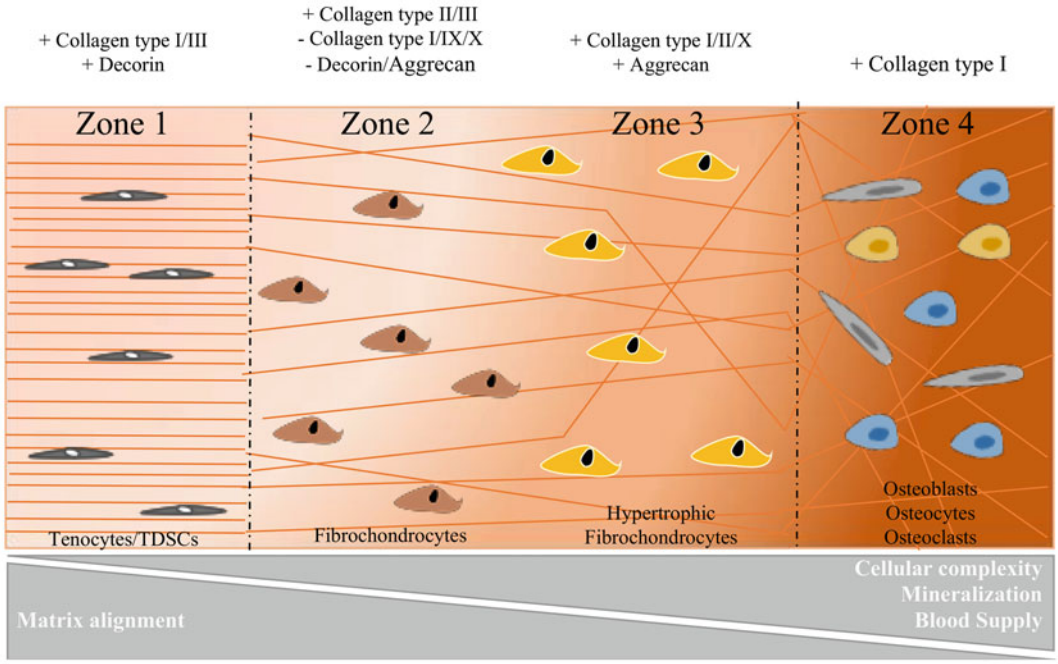

Fig. 4 Schematical image of tendon-to-bone structure and composition. Fibrocartilaginous insertion composed of four zones with the main matrix components and different cellular environment found in the interface. (Adapted from Tellado et al. (2015))

Lu and Thomopoulos 2013). This zone is also characterized by its low vascularization and the row-like arrangement of fibrochondrocytes (Fig. 4, Fig. 5a, b). The mineralized or calcified fibrocartilage (zone 3) is populated by hypertrophic fibrochondrocytes and is mainly constituted by collagen type II with significant amounts of collagen type I and X, as well as aggrecan (Thomopoulos et al. 2003; Thomopoulos 2011; Lu and Thomopoulos 2013). This zone represents the true transition between tendon and bone (Thomopoulos et al. 2010). Bone constitutes the last zone, thus containing osteoblasts, osteocytes and osteoclasts residing within a non-aligned collagen type I matrix together with high amounts of carbonated apatite minerals (Thomopoulos et al. 2002, 2010; Lu and Thomopoulos 2013).

Several studies have been performed to understand the composition and structure of enthesis. It is widely recognized that soft-to-hard tissue interfaces exhibit variations in viscoelastic and mechanical properties, architecture/structure, cellular and matrix composition along their length. Distinct regions along the supraspinatus tendon-to-bone insertion in rats and mice have been studied and gene expression, collagen organization, mineral content and biochemical properties evaluated (Thomopoulos et al. 2003; Wopenka et al. 2008; Schwartz et al. 2012). At gene level, the expression of decorin and biglycan was observed at the tendon end, while going to the bone end, only the expression of cartilage specific matrix genes was observed (such as aggrecan and collagen type II) (Thomopoulos et al. 2003). Polarized light and angular deviation have demonstrated a lower orientation of the collagen fibers at the bone insertion site compared to tendon (Thomopoulos 2003). Moreover, analysis using Raman microprobe demonstrated a linear increase in mineral content towards the interface (Wopenka et al. 2008; Schwartz et al. 2012). Interestingly, collagen type I is universally found along all the regions comprising tendon-to-bone interface; but the main striking difference relies on its organization. Genin et al. observed that a gradual increase in mineral composition

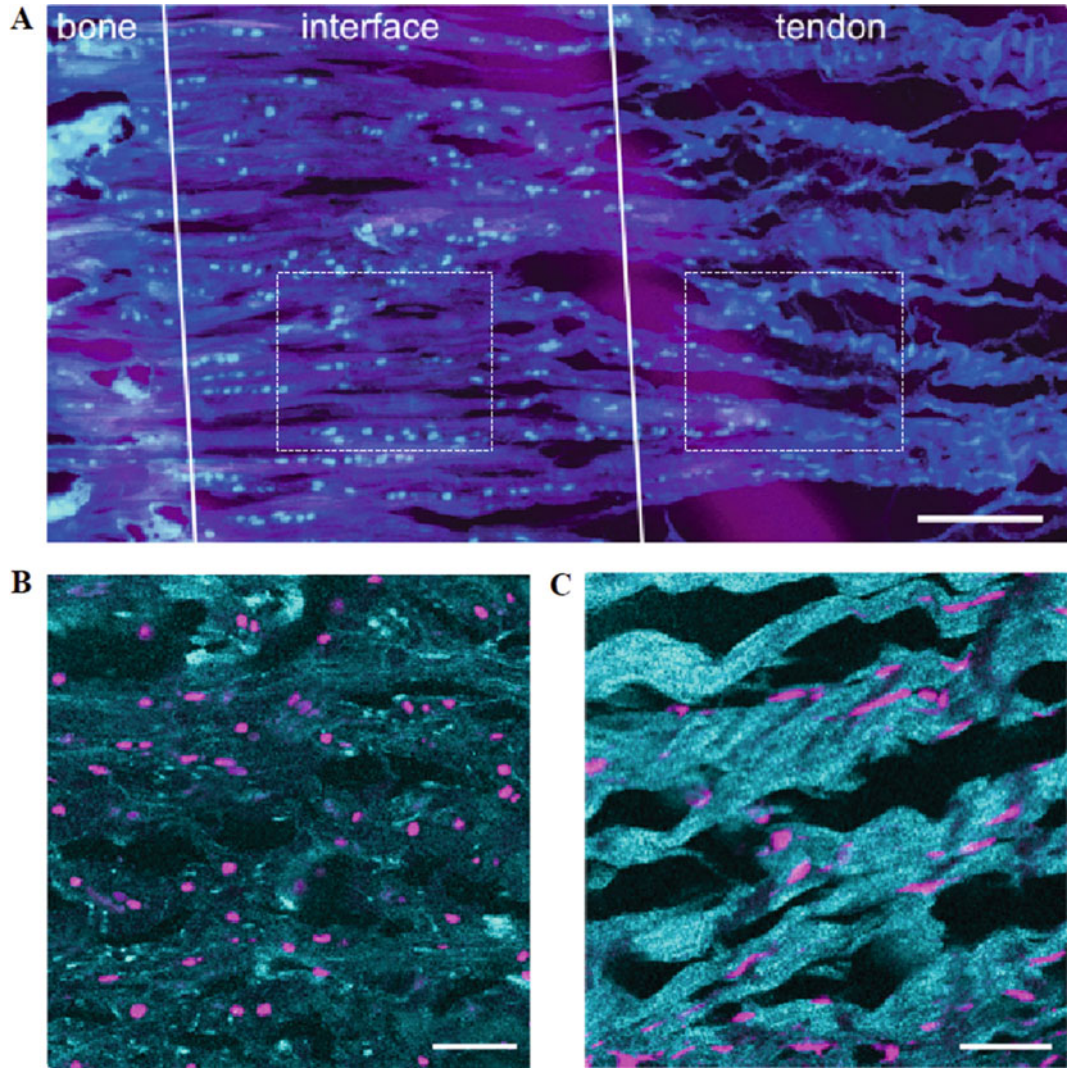

Fig. 5 Structure and cells morphology and localization in tendon-to-bone insertion site. (**a**) Localization of cells in tendon-to-bone interface. Confocal images staining cells with SYTO® 13. Scale bar 150 μm (**b**) Cells residing within interface showing a round-like shape. Scale bar 50 μm. (**c**) Tendon cells in arranged between tendon fibers. Scale bar 50 μm. (**a**–**c**) Copyright © 2018 Kuntz et al.

was accompanied by a decrease in collagen fiber organization while moving from tendon to bone (Genin et al. 2009). Based on these results, evidences support a continuous change in collagen fiber organization, but also in mineral content, ECM composition, geometry and, consequently, mechanical properties along the tendon-to-bone interface (Fig. 4). Altogether, these intrinsic properties are likely to effectively distribute the forces from a soft and flexible tissue like tendon, to a more rigid material as bone.

The structural and compositional complexity inherent to tendon-to-bone interface has led to the development of several tissue engineered strategies. However, a lack of understanding regarding molecular mechanisms and cellular interactions, makes enthesis regeneration a challenge. Thomopoulos et al. pointed out the importance of clearly understanding the

development and morphogenesis of enthesis before trying to address the healing process. The unique and transitional gradient present in tendon-to-bone interface is not recreated during the naturally occurring healing process. As already reported by Newsham-West et al., in a patella sheet model following surgical reattachment, the repaired enthesis resembled more a fibrous tissue than the original fibrocartilaginous interface, characterized by a gradient of morphological changes on the collagen fibrils between the two tissues and a hypercellular environment (Newsham-West et al. 2007). Moreover, studies in animal models of rotator cuff and ACL have clearly demonstrated a failed regeneration of tendon and bone with the formation of scar tissue and high probability of retear (Carpenter et al. 1998; Thomopoulos 2003; Galatz et al. 2010). Therefore, there is a fundamental need to better understand the relationship between structure, function and the healing process of the native tissue to be able to develop and regenerate tendon-to-bone interface.

2.4 Molecular Mechanisms Governing Enthesis Attachments

Currently, very little is known about the main mechanisms behind the embryonic development of this complex structure. It is believed that a synergistic effect between biological and biophysical cues triggers the generation of the highly multifaceted composition found at tendon-to-bone insertion sites. During fetal development, tendon and bone are formed almost at the same time, but the formation of the transition between them only occurs postnatally (Bland and Ashhurst 1997; Bland and Ashhurst 2001; Galatz et al. 2007). At this point, biological factors are likely to have a fundamental role in interfacial growth plate development. Tendon-to-bone developmental studies have demonstrated that the development of this tendon attachment occurs separately from bone development. This modular process provides a checkpoint to control musculoskeletal system assembly, without interfering with bone tissue morphogenesis (Zelzer et al. 2014).

Transcription factors associated with tenogenesis, scleraxis (Scx), and chondrogenesis, sex-determining region Y-box 9 (Sox9), have been highlighted as important elements for enthesis development (Blitz et al. 2009; Blitz et al. 2013; Killian and Thomopoulos 2016; Yoshimoto et al. 2017). Interestingly, deletion of *Scx* has been shown to prevent the formation of bony tuberosities where tendons attach, defective maturation of tendons and a decrease in the expression of *Sox9* in developing entheseal cartilage in mice (Killian and Thomopoulos 2016; Yoshimoto et al. 2017).

A common *Scx+/Sox9+* progenitor pool to tenocytes and chondrocytes has been proposed (Blitz et al. 2013; Sugimoto et al. 2013) but the regulation of the divergence process during lineage specification is still poorly understood. Recently, the interplay of these two factors on the development of bone eminence suggested different regulatory mechanisms to control the different pool of progenitor cells (Blitz et al. 2009, 2013). In a first study, the formation of the deltoid tuberosity was demonstrated to be initially regulated by tendon and, subsequently, muscle dependent (Blitz et al. 2009). Tendon *Scx*-expressing cells have been shown to drive the expression of bone morphogenetic protein 4 (*Bmp4*) and the deletion of either *Scx* or *Bmp4* in the limb arrested the development of cartilaginous bone eminences (Blitz et al. 2009, 2013). Therefore, the expression of *Bmp4* seems to be crucial for enthesis and associated bone ridges formation. In another study, a new regulatory mechanism for attachment formation was described. A distinct pool of *Scx*/*Sox*9-expressing progenitor cells has been shown to form bone eminences in a modular fashion, independent of primary cartilage chondrocytes descendance (Blitz et al. 2013) (Fig. 6). Moreover, a lack of eminences in limb mesenchyme of mouse embryos, where a loss of transforming growth factor beta (TGFß) signaling was induced by genetic ablation of transforming growth factor beta receptor II (TgfBrII), clearly demonstrated an earlier role of TGFß signaling pathway in skeletal/tendon progenitors (Pryce et al. 2009).

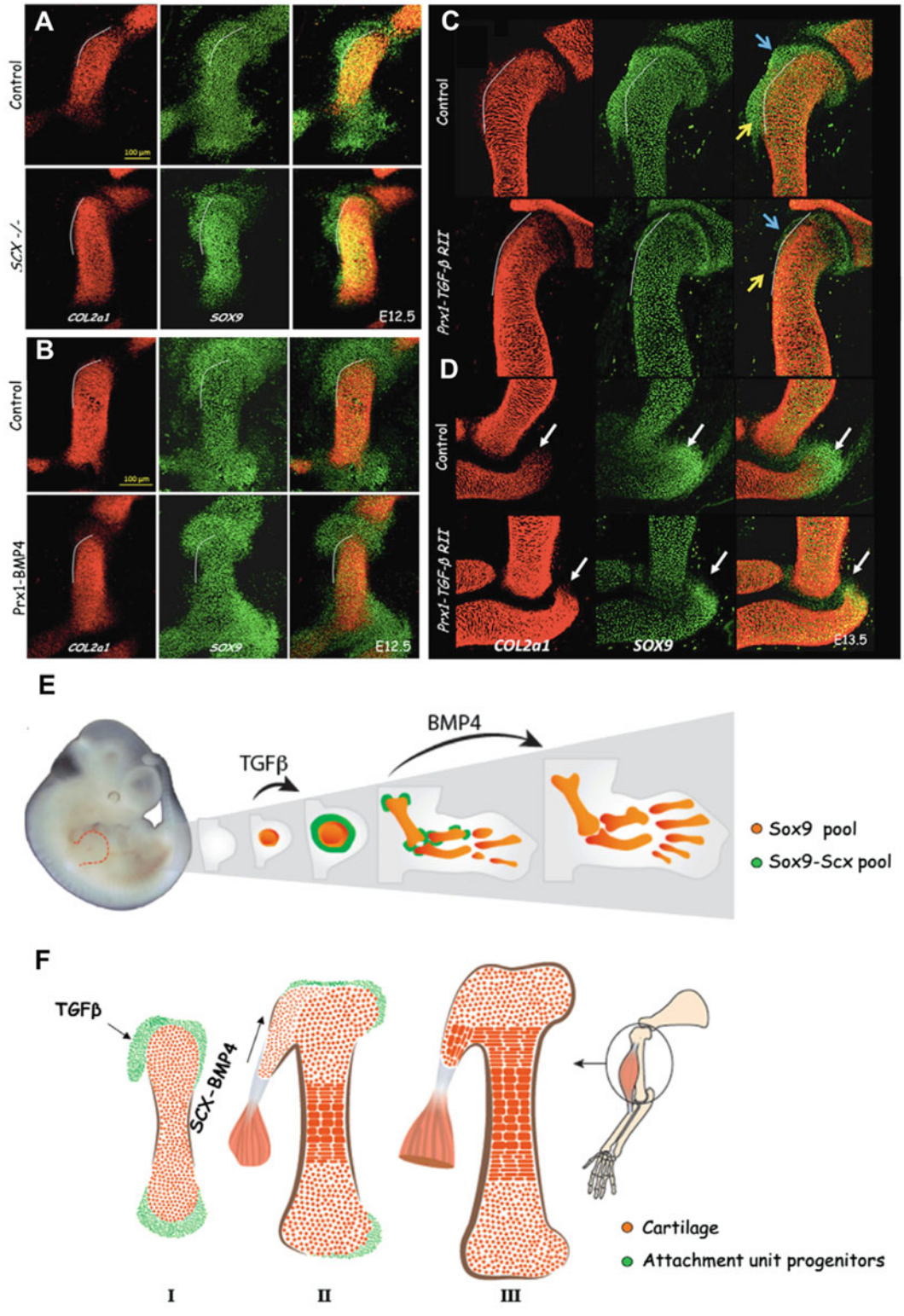

Fig. 6 SCX/BMP4 and TGFß signaling regulation of bone eminences. (**a–b**) Sections of *Scx–/–* and *Prx1-Bmp4* mutants showed the presence of S*ox9*-positive eminence progenitors. (**c–d**) Sections of *Prx1-Tgf-βRII* mutants demonstrating a lack in S*ox9*-positive progenitors. (**e–f**) Modular model of bone eminence development. Figure adapted from Blitz et al. (2013). Copyright © 2013. Published by The Company of Biologists Ltd

Interestingly, the loss of *Scx* has been shown to affect also *Sox9* expression in the developing deltoid tuberosity while decreasing the phosphorylation of Smad1/5 and Smad 3, intracellular downstream mediators of TGFß and BMP signaling pathways (Yoshimoto et al. 2017). Thus, it is tempting to assume that TGFß signaling plays an important role in tendon-to-bone formation trough the regulation of both tendon and bone eminence progenitors.

Other promising candidates have been pointed because of their involvement in the regulation of different stages of the attachment development (Ovchinnikov et al. 2006; Ho et al. 2008). Evidences have shown that the depletion of *Bmp4* in limbs did not lead to a complete loss of bone eminences. Moreover, the blockage of bone morphogenetic protein receptor 1a (*Bmpr1a*) in limb mesenchyme and a dominant-negative *Bmp5* mutation has led to alterations in bone eminences formation.

A more profound gene profiling of these candidates and other molecules playing a critical role in tendon-to-bone attachment and bone eminence formation would be of great interest. Nevertheless, exploring the role of growth factors implicated in skeletal morphogenesis and tendon or bone formation could light up some mechanisms important in the development and attachment of the tendon-to-bone junction.

2.5 Mineralization Process

The cellular and molecular mechanisms associated with matrix mineralization of tendon-to-bone interface follow a similar pathway to the mechanism observed in chondrocyte hypertrophy in the growth plate (Lu and Thomopoulos 2013; Zelzer et al. 2014).

A small population of cells pointed as responsible for entheseal mineralization has been identified in the fibrocartilage region between tendon and bone and denominated Gli1-positive cells (Breidenbach et al. 2015; Schwartz et al. 2015, 2017). These cells have been identified in neonatal enthesis but known to persist in the mature enthesis at the edge of mineralization and pointed as hedgehog (Hh)-responsive cells (Liu et al. 2013; Schwartz et al. 2015, 2017). The Indian hedgehog (Ihh) and parathyroid hormone-related protein (PTHrP) signaling pathways have been highlighted in a large number of mouse models due to their role in the regulation of chondrocyte differentiation towards hypertrophic chondrocytes and, therefore, preventing the mineralization at inappropriate zones of the growth plate (Chen et al. 2006; Liu et al. 2013; Schwartz et al. 2015). For example, deletion of *PTHrP* led to an arrestment of fibrous enthesis, meaning that a hypermineralized tuberosity was observed instead of a normal tibial crest (Wang et al. 2013, 2014). Similarly, when studying the role of Ihh in tendon-to-bone development, the deletion of smoothened (*Smo*), a transmembrane protein responsible for the Ihh signals transduction, in *Scx*-expressing cells led to an impairment in the formation of a fibrocartilaginous interface, evidenced by a downregulation of chondrogenic- and interface-related genes expression, along with a decrease in matrix mineralization (Liu et al. 2013).

Even though the molecular mechanisms are not yet fully understood, biomechanical cues, such as loading, have also been associated with the expression of PTHrP (Chen et al. 2006, 2007), and shown to modulate Ihh signaling and the size of Hh-responsive cells population (Schwartz et al. 2015). Unloading of the medial collateral ligament, semimembranosus and semitendinosus mouse enthesis led to a marked decrease in PTHrP expression. Nevertheless, cyclic mechanical stress has been also reported to influence Ihh expression in chondrocytes culture (Wu et al. 2001) and, more recently, Ihh expression has been correlated with the mechanical levels while studying mechanosensitive genes expression during embryonic bone formation (Nowlan et al. 2008).

Therefore, the expression of PTHrP and Ihh, along with the presence of Gli1-positive cells can be associated with the onset of mineralization in developing insertion sites of tendon-to-bone interface.

3 Main Challenges in Cellular Therapies for Enthesis Tissue Engineering

Cells are nature engineers that would ideally replicate tissue development when regeneration of a damaged enthesis is needed. Currently, various cell-based or culture-based strategies are used (Tellado et al. 2015). However, up to now, several issues remain unsolved regarding the application of cell-based therapies for enthesis tissue engineering and regeneration.

3.1 Cell Source Selection and Cell Differentiation Protocols

Given the cellular complexity described above, the identification of an ideal cell source poses a great challenge to the establishment of adequate cellular therapies. Co-culture systems have been demonstrated to be a useful tool while trying to mimic the heterotypic cellular environment found in tendon-to-bone junction. However, the attempt to replicate the native cell-cell communication and understand the molecular mechanisms behind tendon-to-bone healing and regeneration is far from resolution. Till now, culture-based strategies have focused on the use of multiple terminally differentiated cells (Wang et al. 2007; Cooper et al. 2014) or stem cells with terminally differentiated cells (He et al. 2009, 2012; Calejo et al. 2018). For instance, osteoblast-fibroblast interactions have been explored (Wang et al. 2007) under the rationale that the synergistic communication between distinct phenotypes could induce the formation of enthesis-like tissue. Although the oversimplicity of the system limited the formation of a true tendon-to-bone interface *in vitro*, it could support the deposition of fibrocartilage ECM components (e.g., collagen type II and aggrecan). Nevertheless, this type of strategy requires the isolation of cells from different tissues, often through invasive procedures, which limits clinical translation.

On the other hand, exploring the use of stem cells relies on the establishment of adequate differentiation protocols. So far diverse sources have been explored from, for example, not so well known ACL-derived stem cells (Mifune et al. 2012) to more established and readily available adipose-derived stem cells (ASCs) (Min et al. 2014; Kosaka et al. 2016; McGoldrick et al. 2017; Silva et al. 2017; Perikamana et al. 2018). Although there are well-established inductive protocols for the osteogenic and chondrogenic commitment of MSCs (Bunnell et al. 2008; Solchaga et al. 2011; Kwon et al. 2016), diverse attempts have been made to commit stem cells towards the tenogenic lineage. Normally, treatments with growth and differentiation factors (GDF) or genetic manipulation are applied. For example, ASCs cultured in the presence of different growth factors associated with tendon development and healing (endothelial growth factor, platelet-derived growth factor) showed an upregulation of tendon-related genes (Gonçalves et al. 2014), demonstrating the potential use of biochemical molecules to induce the cell commitment toward the tenogenic lineage. However, an ideal medium formulation is not yet available.

Furthermore, bone repair involves a progression from undifferentiated progenitors to actively mature cells with distinct phenotypes. Thus, the identification of an ideal skeletal progenitor is defying the fields of bone and tendon-to-bone tissue engineering.

3.2 Cell Isolation Protocols and Purity

Concerning the isolation methods of bone cells, osteoblast-like cells are often modeled through the use of immortalized cell lines derived from sarcomas. Alternatively, with interest for tissue engineering and clinical applications, primary cultures can be obtained by explant digestion or stem cell differentiation. These cell cultures are able to represent bone cells and to deposit mineralized ECM in response to osteoinductive stimuli (Jonsson et al. 1999), but their fundamental biology may not be comparable, limiting the comprehension of their effectiveness and

requiring extensive studies to understand their potential in tendon-to-bone therapies.

Furthermore, up to now, several problems remain unsolved regarding the use of tendon cell populations, and in particular of TDSCs for tendon-to-bone regeneration. The isolation of TDSCs still remains largely dependent on a well-established enrichment and selection approach. Several methods of tissue digestion have already been used (Yao et al. 2006; Ruzzini et al. 2014; Nagura et al. 2016; Vigano et al. 2017). Some authors reported the use of only collagenase type, collagenase type I with dispase (Bi et al. 2007; Zhang and Wang 2010), collagenase type II (Kohler et al. 2013) and collagenase type V (Utsunomiya et al. 2013) for tendon tissue digestion. Besides the lack of consensus between the described isolation methods, it is most likely that a mixed population of cells with heterogeneous characteristics in terms of clonogenecity, multidifferentiation potential, and self-renewal capability will be obtained, including also terminally differentiated cells, such as tenocytes (Lui 2015; Vigano et al. 2017). Isolation and characterization of these cells is of major importance because it will generate new insights on basic tendon biology and allow a correct use of the obtained cell population. The same problem is applied to the isolation of ACL-derived stem cells, which still lack standardized isolation methods (Huang et al. 2008; Steinert et al. 2011; Mifune et al. 2013).

3.3 Molecular Signatures

The lack of molecular markers to discriminate the tendon cell population at every discrete step of cell lineage differentiation, makes the expansion and purification of TDSCs and fully differentiated cells very difficult. Several additional factors have been reported to influence markers expression in TDSCs, such as cell source (age, donor variability, tendon type) and cell culture procedures (culture conditions and cell passaging). Indeed, some studies have reported depletion in the number of TDSCs when comparing cell populations isolated from young and aged individuals, as well as a variation in the number of obtained TDSCs according to the location of tissue collection, as mentioned above. Currently, there is no specific marker that is able to accurately distinguish between tendon cell populations and other cells from mesenchymal origin, like MSCs and fibroblasts. Although TDSCs share common surface markers, express identical genes and respond to different growth factors in a similar manner to that of other MSCs (such as bone-marrow MSCs), it was observed that expression patterns were closely related but not identical (Bi et al. 2007; Tan et al. 2012). Hence, some controversy may arise regarding the identity of these cell populations.

Furthermore, the limited knowledge regarding enthesis cells molecular biomarkers is challenging the interpretation of stem cell differentiation strategies for enthesis tissue engineering. Indeed, cell-based approaches relying on the transplantation of MSCs or on the seeding of MSCs (and other stem cells) onto bioengineered scaffolds require that the differentiated phenotype resembles the physiological phenotype of interface cells. Recently, several biomarkers have been proposed for tendon-to-bone interface. Despite morphological disparities, a transcriptomic analysis along with proteome data were used and the cellular patterns of enthesis cells were assessed and compared with tendon and cartilage cells. Kuntz and colleagues explored the transciptome of Achilles tendon-to-bone interface from porcine legs and have found 3,980 differentially regulated transcripts for enthesis and tendon and 395 for enthesis and cartilage, suggesting that cells at the interface are more chondrocyte-like than tenocyte-like given the strong overlap between cartilage and enthesis cells transcriptomes (Kuntz et al. 2018). Interestingly, symmetric differences were shown in the transcriptome of tendon in comparison with enthesis, where two genes showed enrichment in the tendon proteome but conversely in enthesis transcriptome (collagen type XIV alpha-1 chain and extracellular matrix protein 1), raising the hypothesis that differences resulted from the

influence of mechanical stimuli that cells undergo (Kuntz et al. 2018). Moreover, several markers of terminally differentiated hyperthophic chondrocytes were detected in cells within the interface (namely runt-related transcription factor 2 (RUNX2), integrin binding sialoprotein (IBSP) and matrix metallopeptidase 13 (MMP13)), among other cartilage-related biomarkers (aggrecan, chondroadherin, collagen type II and versican (Kuntz et al. 2018). Altogether, the integration of transcriptomics (Kuntz et al. 2018) and proteomics data (Rossetti et al. 2017) demonstrates that enthesis cells resemble chondrocyte-like cells, but in a different stage of chondrogenic differentiation, being less committed than cartilage cells.

The identification of several biomarkers is of major importance while trying to characterize the single cell unit or cells on tendon-to-bone interface. However, there is still a lack of knowledge about the cells at the interface along with a lack on molecular biomarkers for its identification. Even though advanced genetic tools have been developed, there is not a well established molecular signature for each cell type, being difficult to identify or clarify within the proper tissue or to clearly understand the differentiation process occurring in the tendon-to-bone interface.

4 Conclusions

Tendon-to-bone interface physiology and healing are still far from being completely understood. Insights from tendon and bone biology have been helping in the development of tissue engineering strategies for enthesis regeneration, but the fundamental differences within this interface need to uncovered. It is of major importance to understand the heterotypic cellular interactions occurring in the native tissues, such as the role of ECM-cell contact, cell-cell contact and production of paracrine factors, for the development of a fully functional tissue. Input signals from very dissimilar tissues, tendon (soft tissue) and bone (hard tissue) render the interface with a unique niche exhibiting specific structural and compositional properties. The identification of transcription factors and signaling pathways involved in enthesis development has been shedding light on putative mechanisms underlying interface repair and ultimately regeneration. The integration of transcriptomics and proteomics data has brought hope to a more precise identification of enthesis cells, which resemble a more chondrocyte-like than tenocyte-like phenotype. Nonetheless, the molecular signature (of both tendon cells and enthesis cells) is still to be unveiled, posing a strong challenge to the development of future cell-based therapies. Hence, further investigations are required to improve the knowledge on the molecular mechanisms regulating the physiology, as well as pathology and repair/regeneration of these musculoskeletal tissues.

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Induced Pluripotent Stem Cells in Disease Modelling and Regeneration

Burcu Talug and Zeynep Tokcaer-Keskin

Abstract

The reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) has raised extreme hope among both scientists and society by means of development of personalized and regenerative medicine. The field of stem cell research has been accelerating with a drastic speed afterwards and many iPSC lines has been produced for understanding the mechanisms of many debilitating diseases which arise in a variety of organ systems. In this review article we try to focus on the current research regarding the use of iPSCs in both disease modeling and regeneration.

Keywords

Disease modeling · Drug screening · Induced pluripotent stem cells · Regeneration

Abbreviations

CF	Cystic Fibrosis
CM	Cardiomyocyte
CRISPR/Cas9	Clustered Regularly Interspaced Palindromic Repeats/Cas9
FH	Familial Hypercholesterolemia
hiPSC	Human Induced Pluripotent Stem Cells
iPSCs	Induced Pluripotent Stem Cells
LQTS	Long QT Syndrome
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
WHO	World Health Organization
ZFN	Zinc Finger Nuclease

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1 Introduction

Reprogramming of somatic cells into a pluripotent state has always been a major concern of the scientists in a motive to understand developmental mechanisms, progression of certain diseases and cellular therapy. However the discovery of induced pluripotent stem cells (iPSCs) has been a major milestone in cellular reprogramming and opened the gates of the field of medicine and biological research. Thereafter, the research studies in the field of personalized and regenerative medicine has been accelerated. Takahashi and Yamanaka (2006) performed this cellular reprogramming first in mouse fibroblasts by investigating several transcription factors and ended up with 4 transcription factors delivered via retroviral vectors (Oct4, Sox2, Klf4 and c-Myc) that are able to convert somatic fibroblast cells into pluripotent stem cells. These factors, today also known as Yamanaka factors, then are

used to convert human fibroblasts to stem cells in 2007 (Takahashi et al. 2007). Moreover this scientific discovery brought Dr. Yamanaka the Nobel Prize in 2012. These creations of iPSCs raised the importance of generating stem cells for personal use, that are autologous and do not have a rejection possibility for cellular replacement therapies. In order to provide a better clinical use, many studies focused on the safer transdifferentiation protocols involving integration free delivery methods, use of less and different sets of transcription factors (Stadtfeld et al. 2008; Huangfu et al. 2008; Yu et al. 2009; Okita et al. 2011; Hou et al. 2013). Furthermore investigating the developmental stages of many diseases became available by generating 'disease in a dish' set ups by using iPSCs. In disease modeling, patient derived somatic cells are dedifferentiated to iPSCs. After careful examination and testing of the iPSC properties and characteristics (reviewed in Stadtfeld and Hochedlinger 2010) the developmental steps leading to the disease state can be investigated. Moreover if there is a known disease causing genomic alteration, this can be corrected in iPSCs by using different genome modification methods. The most important property of such modeling is the generation of isogenic controls. The use of present genome editing methods (CRISPR/Cas9, ZFN, TALEN) raises the chance that the disease causing mutation at the iPSCs generated from patient cells can be corrected so that the genetic background remains the same. This enables the investigation of the disease mechanism at the same genetic background eliminating the possible differences that can arouse due to genomic variety. Furthermore, the use of iPSC derived organoids- which are tiny 3D cell aggregates mimicking organs in a dish- to screen drugs or study disease mechanism, directed the researchers to another perspective. Presence of such organoid systems will most probably reduce the requirements and costs for clinical trials. Additionally, if multi organoid systems can be connected by the use of tissue chips then body on a chip mechanism would enable to investigate the effect of a disease or drug on multiple organs at a time. Accordingly, the aim of this review is to summarize the recent developments in regenerative and disease modeling studies conducted with iPSCs. Throughout this review, our focus will be on cardiac and gastrointestinal systems.

2 Regeneration

2.1 Cardiac Regeneration

Cardiovascular diseases are the main cause of death according to World Health Organization (WHO) 2018 report (Geneva: World Health Organization; 2018) therefore regeneration of cardiac cells especially by using iPSCs has been a great promise. However previous studies showed that the cells differentiated from iPSCs have to be maturated before use as they exhibit the characteristics of immature embryonic cardiomyocytes (reviewed in Yoshida and Yamanaka 2017). Furthermore the engraftment of the differentiated cardiomyocytes was varied regarding the differentiation stage of the cells (Funakoshi et al. 2016) and how they are engrafted. For this reason, the search of different strategies for better regeneration still continues to be a hot topic. Shiba et al. (2016) generated iPSCs from MHC class I and II haplotype cynomolgus monkey (*Macaca fascicularis*) fibroblasts. The iPSCs were derived integration free (Okita et al. 2013) and differentiated into cardiomyocytes by using the matrix sandwich model (Zhang et al. 2012) by sequential addition of growth factors. These iPSC derived cardiomyocytes regenerated myocardial infarction (MI) in monkeys after 4 weeks but at the same time increased the incidence of ventricular tachycardia. This study was important to show allogeneic transplantation and engraftment of the iPSC derived cardiomyocytes where autologous transplantation is not possible. In another study human iPSC derived cardiomyocytes were tested on an MI model generated by using nude rats (Li et al. 2017). The authors generated a biodegradable construct that has 1.5-12um thickness and seeded the hiPSC derived cardiomyocyte like cells on top

of this biomaterial and further cultured for 14 days. Then the cardiomyocyte seeded construct was engrafted to rats. As the construct provided a 3D organization rather than a 2D surface the coupling of the engrafted tissue with the rat heart and improvement at the functions of the MI area was successfully achieved however the authors also report an increase in ventricular arrhythmias. To overcome the low engraftment problem another study used genetically modified hiPSCs that overexpress cyclin D2 (CCND2). The cardiomyocytes derived from CCND2 over expressing hiPSCs showed better repair properties than the unmodified hiPSCs (Zhu et al. 2018). In conjunction with the studies trying to solve engraftment problem, research studies leading to the maturation of cardiomyocytes are still on going. It was reported that when mouse iPSCs were differentiated in the presence of neuregulin-1b, electrophysiologically mature murine cardiomyocytes were obtained (Iglesias-Garcia et al. 2015). Another study took advantage of a soft silicone surface as extracellular matrix and obtained electrophysiologically mature cardiomyocytes in 1 week period (Herron et al. 2016). Moreover for better response and engraftment of the iPSC derived cardiomyocytes, it has been shown that the ratio of the contracting cardiomyocytes to the non-cardiomyocyte cells determine the electrophysiological properties and maturation of the graft (Iseoka et al. 2018; Tiburcy et al. 2017).

2.2 Liver, Pancreas and Intestine Regeneration

Similar to cardiomyocyte differentiation the hepatocytes generated from iPSCs are considered immature (reviewed in Rashid et al. 2014) and many strategies include their maturation for their use in regenerative applications. In a recently published study Ong et al. (2018) reported the development of an algorithm to identify key factors in the maturation of hepatocytes by comparing human iPSC derived and primary hepatocytes. There have been further studies involving the use of liver organoids derived from iPSCs (Nie et al. 2018; Asai et al. 2017). One such study pointed to the successful improvement of the liver functions after implantation of 3D liver organoids that contain hiPSC derived hepatocyte like cells in a mouse liver failure model (Nie et al. 2018). Another strategy to provide hiPSCs a 3D differentiation environment could be the use of decellularized liver scaffold as reported in Lorvellec et al. (2017). These authors reported a better and faster differentiation of pluripotent stem cells into hepatocytes, when compared with 2D environments. However there are studies that also reported the importance of using supportive cells or extracellular signals to generate a niche for better differentiation and maturation of the hepatocytes as well (Nie et al. 2018; Asai et al. 2017; Koui et al. 2017; Takagi et al. 2017; Wang et al. 2016). Koui et al. (2017) developed strategies to differentiate both liver sinusoidal endothelial cells and hepatic stellate cells from hiPSCs to support again hiPSC derived liver progenitors. The authors achieved to maintain the proliferation of liver progenitors differentiated from hiPSCs for 14 days in 2D.

Pancreas is another organ of gastrointestinal system that has been subject for the research studies, especially for regeneration of insulin producing islet cells. In 2014 Pagliuca et al. reported the production of functional pancreatic beta cells from human embryonic and induced pluripotent stem cell lines; 2 years later in 2016 Vegas et al. achieved producing an alginate based, immunocompetent beta-cell encapsulation strategy by using above mentioned cells prolonging the activity of these cells as long as 174 days. Moreover production of pancreatic beta cells from patient derived iPSCs was reported by Millman et al. (2016) enabling the use of these cells for drug discovery and regeneration. On the other hand there is also need for research studies to better understand the biology of the differentiation process of beta cells from iPSCs. Transcriptome analysis was performed with mouse iPSCs undergoing beta cell differentiation at different time points to reveal the important genes for this

process (Huang et al. 2017). It has been reported that if human iPSC derived beta cells were obtained in spheroid cultures, the insulin secretion would be better than a monolayer differentiated counterpart (Yabe et al. 2017). There are similar studies reporting efficient hormone release from spheroid and 3D suspension culture differentiated iPSCs into beta-cells (Konagaya and Iwata 2016; Mihara et al. 2017) However there is still need for a durable and implantable device that won't form scars in the tissue and enable the testing of the produced cells and grafts in clinical trials.

Intestinal regeneration has also been performed by using iPSCs recently. Human pluripotent stem cells differentiated into functional intestinal organoids and engrafted into mouse intestine, where the enzyme secretions and contribution to tissue regeneration continued (Watson et al. 2014). Another approach to regenerate functional intestinal grafts was performed by decellularizing a part of porcine intestine and repopulating the scaffold by the help of a silicone tube with spheroids derived from human iPSC differentiated intestinal progenitors (Kitano et al. 2017).

3 Disease Modeling

iPSCs provide extreme importance for the modeling of debilitating diseases. The use of iPSCs enabled the investigation of such diseases that could not be possible to work at the bench otherwise. Furthermore, generation of disease models also facilitated discovery and investigation of many novel drugs and their efficacy in the laboratory environment. Many drugs can also be repositioned by testing them in 'disease in a dish' models. More importantly, testing of available drugs with iPSC-derived disease models can help patients to be treated with the best effective drug suitable for the patient's genetic background. In addition, generation of isogenic controls, by involving genome editing techniques available today, enables studying specific molecular mechanisms at the same genetic background.

3.1 Modeling of Cardiac Diseases

Human iPSC (hiPSC)-derived cardiac disease models, offer scientists the unique opportunity to work with diseased cardiomyocytes. Primary cardiomyocytes cannot be efficiently kept in the laboratory environment for long term studies. Although there are rodent models of cardiac diseases, the properties of the rodent cardiomyocytes are different from their human counterparts. For this reason, hiPSC derived cardiomyocytes are very valuable in that field of research.

Long QT syndrome (LQTS) is among the arrhythmic disorders of cardiomyocytes and can be lethal if left undiagnosed. There are many reported causative mutations related with LQTS (reviewed in Tester and Ackerman, 2014). Gelinas et al. (2017) reported a novel mutation (Gly52Val) at *KCNJ2* gene causing symptoms of LQTS by changing the I_{K1} currents. The authors used commercial hiPSC derived cardiomyocytes transduced with the vector carrying either the mutated cDNA or the normal cDNA. As a result of these transient transduction assays, the authors were able to recapitulate the disease profile observed at the patient. In another study, hiPSC lines were generated from 5 different patients carrying different mutations at *KCNH2* gene causing LQTS. The cardiomyocytes differentiated from these hiPSC lines were able to mimic the disease condition (Mehta et al. 2018). Furthermore regarding their drug screening data, the authors also suggest that a readily available drug Lumacaftor prescribed for cyctic fibrosis could be repositioned to treat LQTS. There are other studies that successfully report the use of hiPSC lines to screen different drugs in cardiac disease models (Kawatou et al. 2017; Miller et al. 2017; Lu et al. 2017; El-Battrawy et al. 2018; Kuramoto et al. 2018).

SCN5A-D1275N mutation was investigated by using a patient derived hiPSC line to reveal the real cause of lowered sodium currents associated with this mutation (Hayano et al. 2017). By using hiPSC disease model the authors were able to prove that this mutation was causing

the proteosomal degradation of the sodium channel. Another study (Veerman et al. 2017) regarding SCN5A-I230T mutation, where the expression levels of the protein is controlled by developmental maturation, was studied again by using 2 different hiPSC lines generated from the same family. One line was obtained from a homozygous patient and the other was from a heterozygous relative. This disease model recapitulated the clinical disease phenotype with increased expression of defected sodium channel levels by maturation after at least 66 days of culture period.

There are also research studies using hiPSCs to introduce mutations by CRISPR/Cas9 genome editing technology to eliminate the effects of genetic background. In such studies, the control group is called the isogenic control. One such study investigated the electrophysiological changes in hiPSC-CMs by introducing the I79N mutation to cardiac troponin T, which is linked to cardiac hypertrophy, with CRISPR/Cas9 technique. The authors compared the mutated line with its isogenic control and observed not only the signs of hypertrophy but also signs of pro-arrhythmia (Wang et al. 2018). Hinson et al. (2015) investigated mutations leading to the generation of truncated variants of titin, as a main cause of dilated cardiomyopathy. They generated hiPSCs from patients carrying titin mutations that cause truncated protein production. Furthermore the authors also generated similar mutations wih CRISPR/Cas9 gene editing technology in normal hiPSC lines to study the disease at the same genetic background. They found out that the mutations they investigated caused sarcomeric insufficiency and degenerated stress response in hiPSC-CM when compared with the isogenic controls. Their study enlightened the molecular background of dilated cardiomyopathy caused by truncated titin variants by using hiPSC disease models.

3.2 Modeling in Liver, Pancreas and Intestine

Primary hepatocytes cannot be kept at cell culture in 2D for a long time without losing their metabolic activities. For that reason the use of pluripotent stem cells provide an important tool to understand liver disease mechanism and evaluate the toxic effects of drugs on hepatocytes. Yusa et al. (2011) generated a hiPSC line derived from alpha 1 antitrypsin (A1AT) deficient patient and corrected the mutation causing the disease by using ZFN and piggyBac transposon systems generating the isogenic control of the disease hiPSC line. The authors proved that the corrected cells behaved very much like adult hepatocytes and were able to colonize and regenerate mice liver secreting human A1AT. Same group recently investigated and revealed novel mechanisms for the A1AT deficiency by using the above mentioned model with hiPSC derived hepatocytes (Segeritz et al. 2018). Discovery of such new mechanisms would lead to development of new drugs or repositioning of the readily available ones for the treatment of the disease. In another study hiPSCs were generated from hemophilia B patients and engineered with CRISPR/Cas9 technology (Ramaswamy et al. 2018). These genetically corrected cells were then differentiated into hepatocytes and transplanted to a hemophilic mouse model in order to investigate the effect of hepatocyte transplantation. This study is an important pioneer of using hiPSC derived hepatocytes for the cellular treatment of hemophilia. To study disease mechanism and drug testing in familial hypercholesterolemia (FH), both patient derived hiPSCs were generated and also iPSCs from an unaffected patient were engineered with ZFNs to introduce a similar knockout causing FH to generate isogenic knockouts. Then simvastatin and pro-protein convertase subtilisin/kexin type 9 antibodies were tested for the treatment of the disease both in vitro and in vivo mouse model of FH by transplanting iPSC derived hepatocytes (Yang et al. 2017).

Cystic fibrosis (CF) is one of the diseases that affect multiple organs like liver, pancreas and intestine. Hohwieler et al. (2017) generated hiPSCs from a CF patient to generate a CF disease model for pancreatic cells. They generated a pancreatic organoid system to investigate the effects of a mRNA mediated gene therapy and screen drugs for the treatment of CF. Again for

drug screening purposes in CF, Simsek et al. (2016) generated hiPSCs from a CF patient by differentiating the cells into pancreatic ductal epithelial cells.

Another interesting study generated hiPSC like cells from human pancreatic ductal adenocarcinoma cells (Kim et al. 2013). This model enables the investigation of the stages of pancreatic cancer and screening drugs against the disease. There are also many studies that report the use of iPSCs in diabetic models (reviewed in Kondo et al. 2018).

In a current study (Onozato et al. 2018a, b) a differentiation protocol was generated to optimize the metabolic activities of intestinal organoids derived from human iPSCs for use in drug screening. A similar approach was also optimized by Takahashi et al. (2018) to both screen drugs and pathogens of gut epithelium. Such methods would improve and fasten the discovery of new drugs by reducing the requirements for pre-clinical animal testing. Moreover the specific drugs could be prescribed according to the patient's own iPSC derived intestinal organoids response in cell culture.

4 Conclusion

Pluripotent stem cell use enabled the investigation of developmental stages of many different cell types and the mechanisms of many diseases. However the generation of iPSCs leveled up these studies towards a personal level. Moreover the improvements in genetic engineering methods also helped investigators to compare cells having the same genetic background, eliminating the noise that could arise from the differences about the genetic background. In addition to the advances in genetic engineering methods, the use of organoid cultures has been highlighted. iPSC derived organoid cultures are used to investigate disease mechanisms and drug responses. On the other hand the use of iPSCs for regenerative treatment still requires lots of research to ensure safety and follow up long term affects.

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Stem Cells for the Oromaxillofacial Area: Could they be a promising source for regeneration in dentistry?

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Abstract

Oromaxillofacial tissues (OMT) are composed of tooth and bone, together with nerves and blood vessels. Such a composite material is a huge source for mesenchymal stem cells (MSCs) that can be obtained with ease from extracted teeth, teeth structures and socket blood, flapped gingiva tissue, and mandibular/maxillar bone marrow. They offer a biological answer for restoring damaged dental tissues such as the regeneration of alveolar bone, prevention of pulp tissue defects, and dental structures. Dental tissue-derived mesenchymal stem cells share properties with bone marrow-derived mesenchymal stem cells and there is a considerable potential for these cells to be used in different stem cell-based therapies, such as bone and nerve regeneration. Dental pulp tissue might be a very good source for neurological disorders whereas gingiva-derived mesenchymal stem cells could be a good immune modulatory/suppressive mediators. OMT-MSCs is also promising candidates for regeneration of orofacial tissues from the perspective of developmental fate. Here, we review the fundamental biology and potential for future regeneration strategies of MSCs in oromaxillofacial research.

Keywords

Bone marrow mesenchymal stem cells · Dental pulp mesenchymal stem cells · Dental stem cells · Oromaxillofacial tissue · Tooth bank

Abbreviations

DP-MSCs	Dental pulp mesenchymal stem cells
iBM-MSCs	iliac bone marrow mesenchymal stem cells
MSCs	Mesenchymal stem cells
OMT	Oromaxillofacial stem cell
OMT-SCs	Oromaxillofacial stem cells
PDL-MSCs	Periodontal ligament mesenchymal stem cells
SHED	Exfoliated deciduous teeth mesenchymal stem cells

1 Introduction

Because human teeth and periodontium have a very limited capacity to regenerate, dental pulp and periodontium remain an immense clinical

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challenge (Mitsiadis et al. 2015). Throughout the life, expected healing in oromaxillofacial area is based on tissue homeostasis and regeneration without scar formation upon injury (Zhang et al. 2005; Pagella et al. 2015). Regeneration is identifed as replacement of a pathologic tissue, as well as the reconstruction of a missing or lost tissue, by a new one that can ensure its biologic function (Uccelli et al. 2008). To achieve regeneration on tooth structures and periodontium, the classical tissue engineering trials can be utilized through using stem cells, scaffold materials and relevant growth and differentiation factors.

To date, various stem cell (SC) types have been described, including embryonic SCs, adult somatic SCs (mesenchymal, hematopoietic and endothelial SCs), and induced pluripotent SCs (Herberts et al. 2011; Egusa et al. 2012). Lack of ethical concerns make adult SCs be preferred. Of these, mesenchymal stem cells (MSCs) are often of the promising cell types for regenerative medicine because they can be easily identified in adult tissues (Ghieh et al. 2015). MSCs were first identified as fibroblast-like cells in the bone marrow and, resemble appear colony forming unit-fibroblasts (CFU-Fs) at clonal density. Also MSCs, and have the capacity for differentiation into mesenchymal lineages, such as bone, cartilage, and fat. With the ongoing advances high progress in this field, introduced many alternative MSCs sources such as isolated from bone marrow, adipose tissue, synovium fluid, skeletal muscle (Dominici et al. 2006; An et al. 2015; Chen et al. 2017). Bone marrow and adipose tissue are conventional sources among MSCs. However, the highly invasive cell collection protocols, together with the considerable risk of donor site morbidity, have led to the search alternative tissues (Ducret et al. 2015; Kang et al. 2016). Dental pulp MSCs (DP-MSCs) with their easily surgical access, makes them a promising SC alternative. The non-invasive nature of obtaining DP-MSCs and easily isolation methods compared with other adult tissue sources makes these cells a valuable source of MSCs for tissue repair and regeneration.

After Gronthos et al. (2000) identified MSCs from dental pulp, oromaxillofacial tissue (OMT)-derived MSCs have been isolated and identified subsequently (Fukumoto et al. 2003;Tatullo et al. 2015a). Dental pulp is located in the central pulp cavity, called the pulp chamber, and contains a heterogeneous cell population involving fibroblasts, endothelial cells, neurons, odonto and osteoprogenitors, inflammatory and immune cells (Ledesma-Martínez et al. 2016; Nuti et al. 2016). DP-MSCs are ectodermal derived and originate during tooth development from ectodermal cells that migrate from the neural tube to the oral region and finally they differentiate into mesenchymal cells (Aurrekoetxea et al. 2015). This feature gives them special biological properties of neural crest cells. Dental pulp is closed into the dental cavity surrounded by mineralized dentin, generating a kind of sealed niche that preserves it from environmental differentiation stimuli and maintains SCs properties in the adult tissue.

This review brings together these advances and provides an overview of some of the key findings in the identification and heterogeneity of OMT-MSCs, especially dental pulp (DP)-MSCs. The difference between bone marrow MSCs and DP- MSCs is also described. We tried to explain how these cells can be used to treat several different dental disorders. Finally, the future aspects provide a perspective on how to improve this field forward in order to realise the potential that these cells hold for oromaxillofacial regenerative medicine.

2 Top View of the Problems in Oral and Maxillofacial Tissue

Oral and maxillofacial tissue (OMT) consists of hard and soft tissue. OMT affects not only the functions of breathing, chewing, speech and smell etc. but also esthetics and have much influence on the patients psychologically especially after accident injuries or tumour section (Mao and Prockop 2012).

The defects occurred in OMT can be divided into three main groups: (i) dental defects, (ii) maxillofacial defects, and (iii) mucosal defects (Fig. 1).

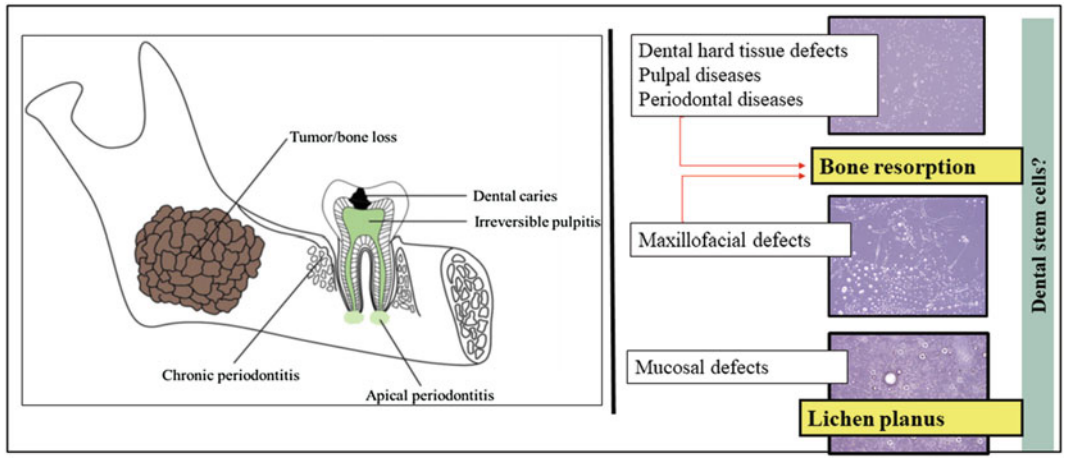

Fig. 1 OMT defects, their results and promising therapeutics as mesenchymal stem cells. The Left panel is modified from Yang et al. (2017)

2.1 Dental Defects

Dental defects could be subclassfied as dental hard tissue defects, pulpal diseases and periodontal diseases. One of the most common reasons to cause dental hard tissue defects is dental caries. It is a multifactorial disease caused by the demineralization of tooth enamel surface by an oral microorganism, *Streptococcus mutans*, which keads to weaken enamel structure (Soares et al. 2016). As a result of normal, abnormal, or pathological wear, non-carious lesions could cause abfraction, abrasion, and erosion or chemical degradation of dental tissues (Mjor 2001). Up to now, the direct filling was the main treatment method of dental hard tissues defects, where as either amalgam or zinc oxide composite resins would be used to restore the dental defects (Sequeira-Byron et al. 2015). However, there still exists problems like removing healthy tissues and/or side effects of zinc oxide composites (Mendi et al. 2017).

2.1.1 Pulpitis

Pulpitis is an inflammatory state of the dental pulp and clinically could be described as reversible or irreversible. Root canal therapy involves the extraction of inflammatory tissues, and mechanical preparation of the root canal, after that filling materials were used to stop the bacteria from stepping into the root canal system and periodontal tissue (Hargreaves et al. 2016). However, clearing up all the pulpal tissues leave the teeth intensive, losing nutrients and changing properties of enamel and dentin.

2.1.2 Periodontitis and Gingivitis

The most common disease which is an inflammatory response of the periodontium to the bacteria is perodontitis and gingivitis. Periodontitis cause lesions of gingiva, attachment, alveolar bone and eventually tooth loss (Caton et al. 2011). The methods to treat the periodontitis are subgingival scaling and root planing. However, the tissue loss can't be a reversal and these methods would only stabilize the situations.

2.2 Maxillofacial Defects

The irreversible process of three dimensional (3D) alveolar bone resorption begins as early as 6 months following tooth loss or extraction that may pose a challenge for predictable implant placement (Shabestari et al. 2010; Khojasteh et al. 2012). Since inadequate bone volume may jeopardize long-term prognosis of dental implants, reconstruction of resorbed alveolar ridges has been a goal and a challenge for clinicians to optimize outcomes of oral implant placement (Shayesteh et al. 2013; Kolerman et al. 2014).

A variety of surgical approaches have been proposed to enhance the alveolar bone volume including ridge splitting, distraction osteogenesis, J-bone iliac, anterior ileum bone grafts onlay or particulate bone grafts with or without membranes (von Arx and Buser 2006; Hämmerle et al. 2008; Boronat et al. 2010; Khojasteh et al. 2012; Morad and Khojasteh 2013; Ataç and Kılınç 2014). Autogenous bone, harvested from extraoral and intraoral donor sites, has been extensively used because of its osteoinductive, osteoconductive, and osteogenic properties (Urban et al. 2013). On the other hand, high resorption rate could compromise the clinical outcomes of autogenous bone grafts (Meijndert et al. 2005; Hassani et al. 2005; Beitlitum et al. 2010; Khojasteh et al. 2013). In animal and human studies autologous cortical bone graft resorption up to 56% is reported (Berglundh and Lindhe 1997; Ozaki and Buchman 1998; Merkx et al. 1999). In addition, these grafts are associated with morbidity depending on the harvest site (Kolerman et al. 2014). Bone grafting techniques have several disadvantages, including postoperative pain, infection risk, and immunologic problems, underscoring the need for appropriate bone substitutes that mimic the osteogenic potential of autologous bone (Polo-Corrales et al. 2014).

Recent advances brought by tissue engineering suggest that significant changes in "traditional clinical dentistry" and may circumvent many of the limitations of the available traditional dentistry techniques and its power has been explored to minimize the need of surgery to obtain bone graft or teeth extraction. Achievement of tissue engineering strategies depends on identifying and understanding the biology and behaviour of oromaxillofacial stem cells.

2.3 Mucosal Defects

Oral lichen planus is a chronic inflammatory disease that affects the skin and the mucus membrane. The disease affects 1–2% of the population. Clinically, it is seen as reticular, papular, plaque-like, erosive, atrophic or bullous types. Intraorally, the buccal mucosa, tongue and the gingiva are commonly affected. Although calcineurin inhibitors, retinoids, dapsone, hydroxychloroquine, mycophenolate mofetil and enoxaparin have subscribed to treatment of the disease, corticosteroids have been the primary management of OLP. Analysis of current data on pathogenesis of the disease suggests that blocking IL-12, IFN-γ, TNF-α, RANTES, or MMP-9 activity or upregulating TGF-β1 activity in OLP may be of therapeutic value in the future (Bouquot and Gorlin 1986; Scully et al. 1998; Sugerman et al. 2002; Ismail et al. 2007; Roopashree et al. 2010).

3 Oral and Maxillofacial Tissue (OMT) Stem Cells: Isolation, Identification, and Biology

OMT-SCs have been found in several tissues and can be divided into epithelial and oral mucosal stem cells (EOM-SCs), cranial bone MSCs, mandibular bone marrow MSCs, and dental MSCs (dental ulp MSCs (third molar and exfoliated deciduous teeth), dental follicle MSCs, periodontal ligament MSCs, apical papilla MSCs) (Fig. 2a).

In 2000, adult human dental stem cells were first reported by Gronthos et al. (2000), they applied a methodology that had been previously developed for the isolation and characterization of bone marrow MSCs. By using a colony forming efficiency assay that determines the CFU-F number in bone marrow cell suspensions, they demonstrated a minor population within adult human dental pulp is clonogenic. Subsequently, different types of dental MSC-like populations were isolated and characterized. Similarly, dental pulp isolation, cranial bone MSCs, epithelial and oral mucosal stem cells and dental MSCs were obtained by explant culture method preferably with enzymatic digestion (Figs. 2b and 3).

Of these, dental pulp, as a heterogeneous cell reservoir, consists of odontoblasts that reside on mineralized dentin surface, in addition to abundant interstitial fibroblasts that are located among a web of blood vessels and nerve endings. On the

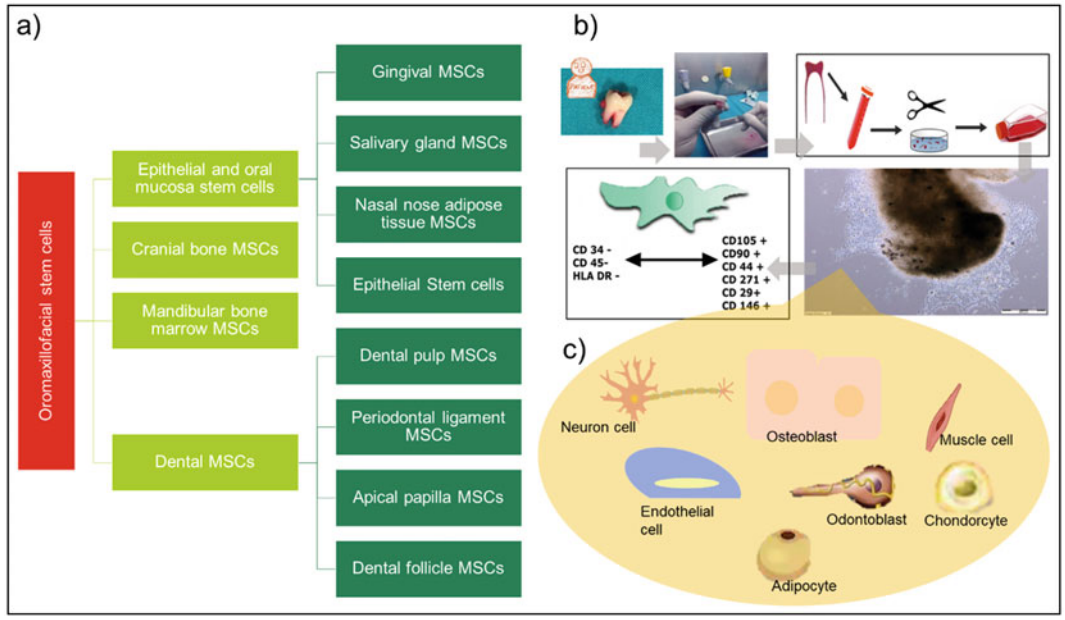

Fig. 2 (**a**) Classification of OMT stem cells, (**b**) Solid tissues or blood samples from mandibular bone marrow are taken and cultured by explant culture method. Occurred colonies then sorted by flow cytometer for positive and negative surface markers of MSCs. (**c**) Differentiation potential of OMT-SCs

other hand, the iliac bone marrow niches are formed by stromal mesenchymal stem cells, osteoblasts and, hematopoietic stem cells (Sacchetti et al. 2007; Mendez-Ferrer et al. 2010; Bianco 2011). This heterogeneity may give rise to isolate stem cells with various differentiation stage and cells from different origin shows different biological functions.

According to the literature search and our findings, we suggest that donor age is a significant factor for isolation of MSC from dental pulp, tooth extraction socket and mandibular/maxillar bone marrow (Kim et al. 2012; Bressan et al. 2012; Kellner et al. 2014). A study which compared DP-MSCs cultured from children, adolescents, adults and elderly donors showed that association among isolation, efficacy, increase in duplication time and the greater number of apoptotic cells with the propagation have been described from aged donors.

As for the culture method, different culture medium formulations have been tested including several basal media and growth supplements and also chemically defined media (Bonnamain et al. 2013). Although several concerns related to safety issues and batch variation have lead to the exploration of alternatives to meet regulatory criteria for clinic usage, 10% fetal bovine serum is still the gold standard (Brindley et al. 2012; Ducret et al. 2015). As a result, many studies have investigated the possibility of isolating, differentiating OMT-SCs in serum-free culture media.

Iliac bone marrow-derived mesenchymal stem cells (iBM-MSCs) frequently serve as a reference for the characterization of stem cells that reside in OMT, given that both are mesenchymal origins. (Sacchetti et al. 2007; Mendez-Ferrer et al. 2010; Bianco 2011) The studies of isolation of OMT stem cells has been profoundly influenced by earlier studies of iBM-MSCs. OMT-SCs, especially dental pulp-MSCs are positive for cell surface markers similar to those of iBM-MSCs, including CD44, CD73, CD105, STRO-1, and CD146, but are negative for CD45, CD34, CD14, CD11b, CD79, CD19 and HLA-DR (Huang et al. 2009). Different techniques, such as fluorescence activated cell sorting (FACS) and

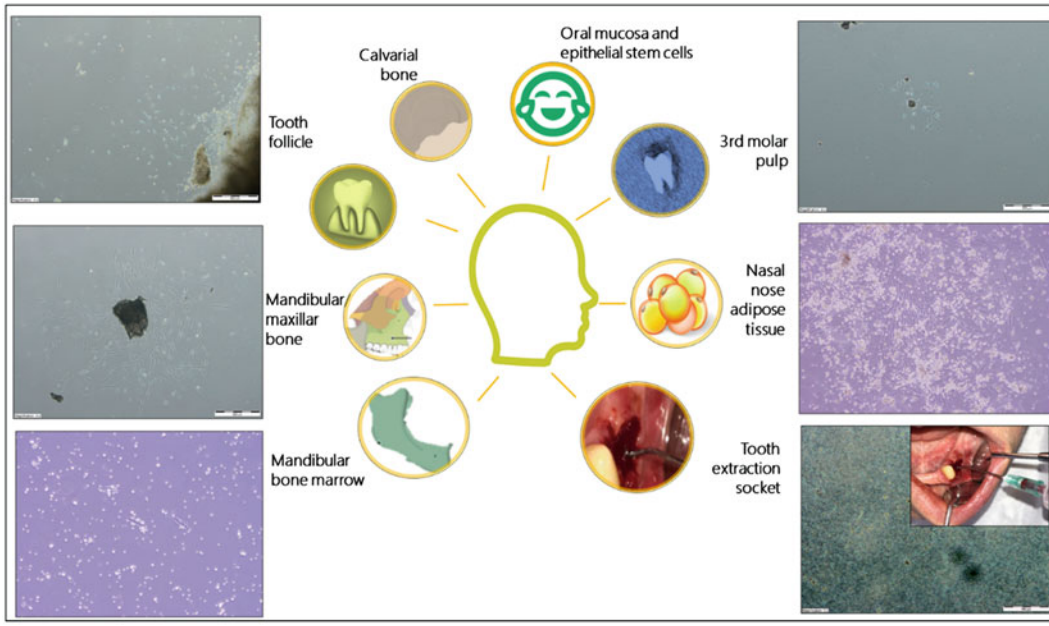

Fig. 3 SCs from OMT. Our group isolated MSCs from 3rd molar pulp, nasal adipose tissue, tooth extraction socket, mandibular bone marrow, cranial bone, and tooth follicle (Olympos CKX 41, Japan)

magnetic-activated cell sorting (MACS). have been tested to isolate and purify clonal subsets of stem cells from dental pulp. However there is a remarkable need to enrich pure MSCs because since MSCs isolated from bone marrow, adipose or OMT tissues are each highly heterogeneous cell populations. Numerous other surface markers have been studied in the characterization of OMT-SCs (Table 1) (Gronthos et al. 2002; Guilak et al. 2006; Keating 2012; Eleuterio et al. 2013; Liu et al. 2015; Ledesma-Martínez et al. 2016; Aghajani et al. 2016; Niehage et al. 2016; Werle et al. 2016; Mendi et al. 2018).

The classical application for stem cells is based on their long term self renewal and the ability to differentiate into new mature spacialized cells to facilitate replacement and regeneration of tissues. In basic, the ISCT propose as a criterion to define human MSCs that must differentiate in vitro into ostoeblasts, adipocytes, and chondroblasts (Dominici et al. 2006). Our group showed that proliferation studies using xCELLigence Analysis System (Roche) of dental pulp MSCs exhibited higher rates of proliferation when compared to the iliac bone marrow MSCs (Mendi et al. 2017; Mendi et al. 2018). Colonies of dental MSCs, cranial bone MSCs, and epithelial and oral mucosa MSCs occur at an apparently higher frequency than mandibular bone marrow MSCs and iliac bone marrow MSCs (Gronthos et al. 2000; Mendi et al. 2017; Mendi et al. 2018). Higher proliferation rate is exhibited compared with iliac bone marrow MSCs. This may be clarified by the developmental state of the respective tissues.

The osteogenic potential of OMT-SCs has been well documented in several studies. Osteogenic differentiation is well- known to be induced by dexamethasone, L-ascorbic acid and β-glycerophosphate supplementation (Pittenger et al. 1999; Riccio et al. 2010; Atari et al. 2012; Teti et al. 2015; Goto et al. 2016; Bhuptani and Patravale 2016; Mendi et al. 2017; Mendi et al. 2018). Alizarin Red S staining and von Kossa dying allow confirmation of the matrix mineralization and calcium deposition after induction. However, we have to speculate that there is an handicap for DP-MSCs that differentiated cells should be considered as osteoblast or odontoblast. To verify the differentiation, the expression of

Table 1 Summary of surface markers expressed on OMT-MSCs

ISCT	Mesenchymal	Stemness	Neural	Others
CD73	CD 13	OCT ¾	Nestin	CD40
CD 90	CD 29	SSEA4	B-III tubulin	CD 120a
CD 105	CD 44	NANOG	S100	CD261
	CD 146		Notch 1	CD262
	CD 166		CD 271	CD264
			Synaptophysin	CD266
				Integrin alpha-4
				Integrin alpha-6
				Integrin alpha-10
				CD121a
				CD130
				CD213a1
				CD217
				CDw210b

bone-specific and dentin specific proteins such as alkaline phosphatase, and dentin sialoprotein respectively should be done. Also, early and late osteogenesis markers including osteonectin, osteocalcin, osteopontin, osterix, and runt-related transcription factor 2 (RUNX2) could be examined (Grottkau et al. 2010; Ferro et al. 2012; Riccio et al. 2010; Cha et al. 2015; Ajlan et al. 2015; Goto et al. 2016; Alraies et al. 2017).

Adipogenic differentiation has been reported for DP-MSCs and other OMT-SCs. Insulin, dexamethasone, ɪndomethacin and 3-isobutyl-1-methylxanthine (IBMX) are using to induce adipogenic differentiation and adipocyte droplets are stained by Oil Red O (Pittenger et al. 1999). Several markers, such as peroxisome proliferator-activated receptor γ, glucose transporter type 4, fatty acid binding protein 4 and lipoprotein lipase are shown for OMT-SCs especially DP-MSCs. However, the power of adipogenic differentiation is not as strong as iliac BM-MSCs (Gronthos et al. 2000; Mendi et al. 2017; Mendi et al. 2018).

As distinct from adipogenic differentiation, chondrogenic differentiation potential is higher in DP-MSCs. A chondrogenic lineage phenotype is mediated by several compounds, including ITS (insulin, transferrin and selenium), dexamethasone, L-ascorbic acid, L-proline and sodium pyruvate (Pittenger et al. 1999; Nemeth et al. 2014; Jang et al. 2016).

Hereunder, studies revealed that OMT-SCs have functional and phenotype differences compared to the iliac BM-MSCs Akintoye et al. (2006) reported specific site properties of the mandibular BM-MSCs and iliac crest of the same individual, where a greater proliferation and osteogenic differentiation ability was observed from the mandibular BM-MSCs compared to the ones from the iliac crest. Indeed, orofacial derived MSCs 'adipogenic potential is lower than those of the iliac (Mendi et al. 2017; Mendi et al. 2018) which can lower the production of fat during bone tissue regeneration. In contrast with iliac BMSCs, OMT-SCs are more committed to being differentiated to odontogenic tissues rather than osteogenic tissues.

The detailed relationship among OMT stem cell populations is still unclear. Initially, it was hypothesized that because dental and orofacial tissues are specialized tissues that do not undergo continuous remodelling as do osteogenic tissues, MSCs derived from OMT tissues may be more committed or restricted in their differentiation potency than human iliac BM-MSCs. There are several studies comparing the the properties of dental, oral mucosa derived MSCs with those of BM-MSCs and datas have confirmed the multidifferentiation capacity of OMT –SCs (Zhang et al. 2009; Carinci et al. 2006; Wang et al. 2010; Moshaverinia et al. 2012; Moshaverinia et al. 2013; Moshaverinia et al. 2014).

4 OMT-SCs in Clinical Trials and in Vivo Regeneration Studies

Stem cell therapy has become a promising alternative in dentistry and oromaxillofacial rehabilitation (Rada et al. 2002; D'Aquino et al. 2009; Caton et al. 2011; Tatullo et al. 2015b). Regenerative dentistry aims to regenerate the damaged dental tissues and to regain the tooth morphology and functions (Fig. 1). There are a total of 110 clinical trials correlated with oral diseases and stem cells (Fig. 4, and Table 2). The stem cells used in the clinical trials include iBM-MSCs, DP-MSCs, periodontal ligament, buccal fat, cord blood, oral mucosal epithelial MSCs. As outlined in Table 2, there are proposed to treat periodontal disease, primary Sjogren's Syndrome, mandibular fractures and cleft lip and palate.

To achieve successful regeneration with stem cells in dentistry accurately designed scaffolds may improve the oral and maxillofacial regeneration (Mitsiadis et al. 2012; Hayashi et al. 2015). Among total 44 clinical trials, we found, 12 cases those conducted by scaffolds, correlated with oral diseases and oral stem cells. Eight of these cases are for the treatment of bone diseases including craniofacial abnormality, mandibular fractures, bone atrophy, cleft palate, maxillar cyst, and edentulous alveolar bone loss. BioOss scaffolds and commercially available collagen scaffolds are used to hold periodontal ligament MSCs and iliac BM-MSCs (Baba and Yamada 2016).

Among the clinical trials, it is important to report the follow-up results. Manimaran et al. (2016), were able to demonstrate bone regeneration using this technique with no recurrence of ameloblastoma tumour. Ameloblastoma is a benign odontogenic tumour, which is locally aggressive in behaviour. A 14-year-old male with ameloblastoma was treated with autologous DP-MSCs and stromal vascular fraction (SVF) and evidence of bone regeneration was observed. On the day of surgery, SVF was processed from buccal pad of fat, and platelet-rich fibrin (PRF) was prepared from patient's peripheral blood. Enhanced bone formation was seen in post-operative OPG and CT Scan after 10(th) month. The study showed an innovative approach to manage these cases by using a combination of autologous DP-MSCs and buccal pad of fat SVF to regenerate a mandibular defect left by the resection of an ameloblastoma with 1.5 -year follow-up.

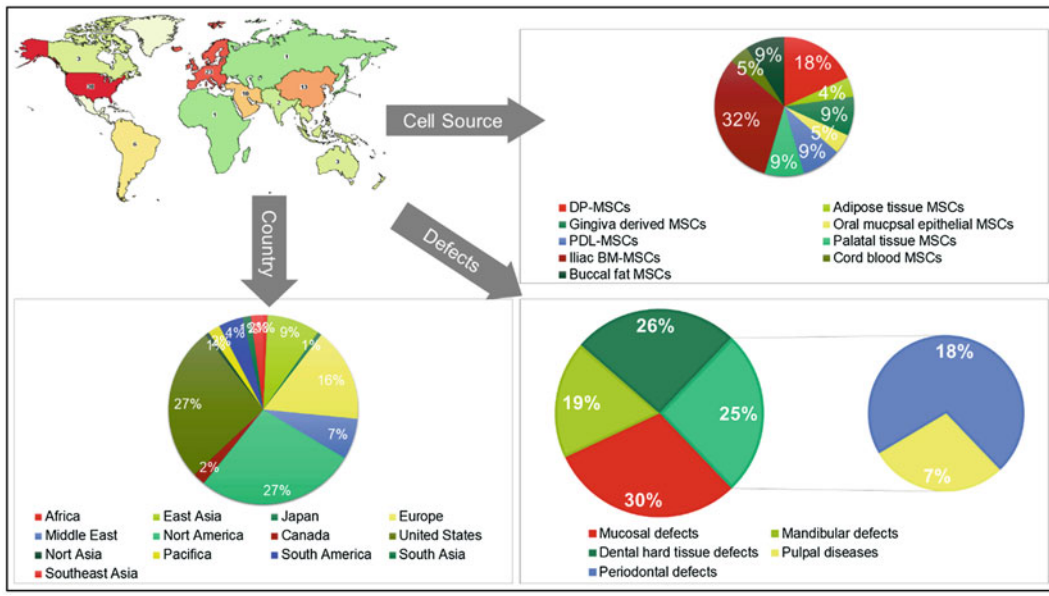

Fig. 4 Clinical trials searched for 2018 with oral disease and mesenchymal stem cell keywords

Table 2 Stem cells used in the clinical trials correlated with different oral disease conditions and mesenchymal stem cells (clinicaltrials.gov)

Code	Conditions	Procedure	Cell source	Status	Region
NCT03137979	Periodontitis	GMSCs + collagen scaffold	Gingiva-MSCs	Recruiting	China
NCT02513238	Xerostomia (radiation induced)	MSCs in NaCl	Autologous adipose tissue derived MSCs	Completed	Denmark
NCT02449005	Chronic periodontitis	Fibrin glue + collagen fleece + alveolar BM-MSCs	Autologous alveolar bone marrow derived MSCs	Active, not recruiting	Greece
NCT01932164	Celft lip and palate	Maxillary alveolar graft technique mesenchymal stem cells transplantation	Decidious tooth pulp derived MSCs	Completed	Brazil
NCT01309061	Rombergs disease	Cell transplantation	Autolog adipose tissue derived MSCs	Completed	Korea
NCT02751125	Bone Atrophy	The stem cells mixed with Bi Calcium Phosphate (BCP)	BCP with autologous bone marrow MSCs.	Completed	Norway
NCT02731586	Edentulous Alveolar Ridge	Application of MSCs	Dental pulp derived allogenic MSCs	enrolling by invitation	India
NCT00221130	Adult Periodontitis	Injectable gel is the mixture of ex-vivo cultured mesenchymal stem stem cells, ex-vivo cultured osteoblast-like cells differentiated from mesenchymal stem cells and scaffold (include, platelet rich plasma, human thrombin and calcium chloride	MSCs and osteoblst cells	completed	Japan
NCT00953485	Primary Sjögren's Syndrome (pSS)	Transplantation	Allogeneic mesenchymal stem cells transplantation	unknown	China
NCT02055625	Oral Graft vs Host Disease	Allogenous mesenchymal stromal cells will be injected directly underneath the mucosal lesions	Mesenchymal stromal cells	suspended	Sweeden,
NCT02755922	Mandibular Fractures	Application of autologous mesenchymal stem cells	Autolog MSCs	Completed	Mexico
NCT03102879	Regenerative Endodontic Procedure Procedure: Conventional Root Canal Treatment	Umbilical cord-derived mesenchymal stem cells encapsulated in a plasma-derived biomaterial and	Umbilical cord-derived mesenchymal stem cells	Completed	Chile
NCT03070275	Implant therapy	aBM-MSCs/fibrin glue/collagen fleece	Autologous alveolar bone marrow mesenchymal stem cells	Completed	Aristotle University of Thessaloniki
NCT03563495	Cleft lip and palate	Tissue engineered group autogenous bone graft group	Autologous bone marrow derived	Completed	Cairo University

(continued)

Table 2 (continued)

Code	Conditions	Procedure	Cell source	Status	Region
			mesenchymal stem cells		
NCT02149732	Limbal Stem Cell Deficiency	Cultivated oral mucosal epithelial sheet transplantation	Autologous cultivated oral mucosal epithelial cell sheet	Available	Seoul
	Stevens-johnson Syndrome				
	Ocular Cicatricial Pemphigoid				
	Chemical Burn				
NCT02745366	Alveolar Bone Loss	BFPSC+FDBA+PRF	Buccal fat pad derived mesenchymal stem cells (BFDPMSC)	Recruiiting	Iran
	Atrophy	FDBA+PRF			

Successful reconstruction of a bony defect is a difficult task in craniofacial surgery. The gold standard treatment consists of the autogenous bone utilization alone or in combination with other autologous biomaterials (Anitua 1999; Anitua et al. 2004). Yamada et al. (2011) attempted to regenerate bone in a significant osseous defect with various stem cells. Cells were grafted into a parent canine mandible as an allograft, using platelet-rich plasma. Initially, teeth were extracted from a child and parent hybrid canine mandible region and bone marrow (canine mesenchymal stem cells; cMSCs), and parent teeth (canine dental pulp stem cells; cDPSCs), and stem cells were extracted from deciduous teeth (puppy deciduous teeth stem cells; pDTSCs). After 4 weeks, bone defects were prepared on both sides of the mandible with a trephine bar and graft materials were implanted into these defects. Their results demonstrate that stem cells from deciduous teeth, dental pulp, and bone marrow with PRP have the ability to form bone, and bone formation with DTSCs might have the potential to generate a graft between a child and parent. This preclinical study could give the way for stem cell therapy in orthopaedics and oral maxillofacial reconstruction for clinical application.

Cleft lip and palate is the most common congenital anomaly in the orofacial region. Autogenous iliac bone graft, in general, has been employed for closing the bone defect at the alveolar cleft as well. However, such iliac bone graft provides patients with substantial surgical and psychological invasions. Nakajima et al. (2018) elucidated the nature of bone regeneration by exfoliated deciduous teeth (SHED) as compared to that of human DP-MSCs and iBM-MSCs. The stems cells derived from pulp tissues and bone marrow were transplanted with a polylactic-co-glycolic acid barrier membrane as a scaffold, for use in bone regeneration in an artificial bone defect of 4 mm in diameter in the calvaria of immunodeficient mice. They found that degree of bone regeneration with SHED relative to the bone defect was almost equivalent to that with hDPSCs and hBMSCs 12 weeks after transplantation according to the micro CT analysis. SHED was found one of the best candidates as a cell source for the reconstruction of alveolar cleft due to the bone regeneration ability with less surgical invasion (Nakajima et al. 2018).

Sjögren's syndrome (SS) is characterized by autoimmune activation and loss of function in the salivary glands. Recent studies reported that bone morphogenetic protein 6 (BMP6), which is a

member of transforming growth factor beta (TGF-β) superfamily, was highly expressed in SS patients. To investigate the role of BMP6 in SS, Xu et al. (2018) treated the salivary gland-derived mesenchymal stem cells (SGMSCs) with BMP6. They found that BMP6 could impair immunomodulatory properties of normal SGMSCs by downregulating the Prostaglandin E2 synthase through DNA-binding protein inhibitor-1. Neutralizing the BMP6 could significantly restore the SGMSC's immunoregulatory function *in vitro* and delay the SS disease activity in vivo. They saw that BMP6 not only affect the secreting function of epithelial cells in the salivary gland but also influence the immunomodulatory properties of SGMSCs, which may trigger or enhance the autoimmune reflection in SS.

5 The Potential for Non-oral Tissue Engineering

As mentioned, OMT-SCs are able to differentiate into odontoblast, cementoblast, osteoblast, chondrocyte, melanocyte, endothelial cell, hepatocyte a deven myoblast and neural cell. Hence besides the area of oral diseases, OMT-SCs have the potential to be applied in brain, eye, liver, bone, skin, and muscle diseases as well (Liu et al. 2015) (Fig. 5).

5.1 Neuronal Diseases

In terms of animal models, Mita et al. (2015), reported that intranasal administration of SHED in a mouse model of Alzheimer's disease could result in a substantially improved cognitive function. Also, regarding the Parkinson's disease, a chronic neurodegenerative disease caused by the loss of dopaminergic neurons in the substantia nigra, the potential of DP-MSCs to differentiate into dopaminergic neurons under appropriate conditions has been evaluated (Chang et al. 2014; Mead et al. 2014). In 2014, Chang et al. (2014) reported an arrested proliferation and the acquisition of a phenotype resembling mature neurons of a greater proportion of DP-MSCs after neuronal differentiation, with high expression of nestin and other mature neuron cells markers such as βIII-tubulin.

On the other hand, Schwann cells play a major role in the regeneration axon of the peripheral nervous system. In a rat model, DP-MSCs and conditioned medium examined and reported that DP-MSCs promoted axon regeneration through trophic factors on Schwann cells and promote angiogenesis (Yamamoto et al. 2016).

5.2 Spinal Cord Injuries

DP-MSCs stand out as the most prominent source for in vitro neuronal differentiation. To evaluate the *in vivo* peripheral nerve regeneration potential of DP-MSCs and differentiated neuronal cells from DP-MSCs (DF-DPSCs), a total of 1×10^6 hDPSCs or DF-hDPSCs labelled with PKH26 tracking dye and supplemented with fibrin glue scaffold and collagen tubulization were transplanted into the sciatic nerve resection (5-mm gap) of rat models. At 12 weeks after cell transplantation, both groups showed notably increased behavioural activities and higher muscle contraction forces compared with those in the non-cell transplanted control group. Pretransplanted labelled PKH26 were also distinctly detected in the regenerated nerve tissues, indicating that transplanted cells were well-preserved and differentiated into nerve cells. Furthermore, no difference was observed in the nerve regeneration potential between the hDPSC and DF-hDPSC transplanted groups. (Ullah et al. 2017).

The transplantation of SHED has been studied as a possible treatment strategy for spinal cord injuries due to its potential for promoting tissue protection and functional recovery in a Wistar rat model (Nicola et al. 2018). The SHED transplantation promoted functional recovery, measured as from the first week after spinal cord contusion by Basso, Beattie, and Bresnahan scale. Twenty-four and 48 h after lesion, flow cytometry revealed a spinal cord vimentin+ cells increment in the SHED group. The results supported that SHED act as a neuroprotector agent after transplantation,

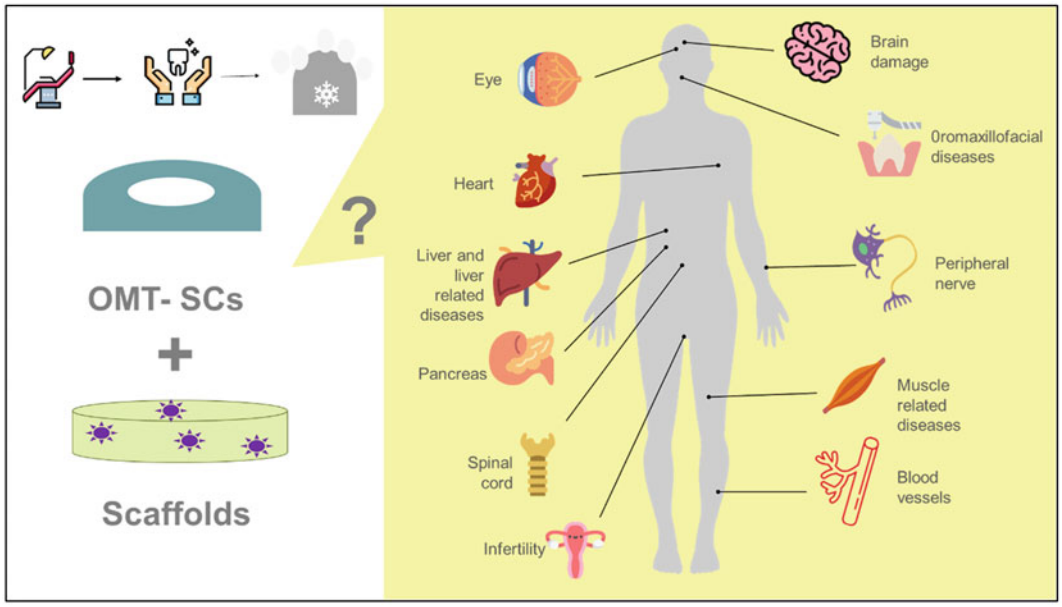

Fig. 5 Different OMT-SCs could be transplanted alone or with appropriate scaffolds for non-oral tissues

probably through paracrine signalling to reduce glial scar formation, inducing tissue plasticity and functional recovery. Asadi-Golshan et al. (2018) showed that intraspinal administration of SHED-CM loaded in collagen hydrogel leads to improved functional recovery and proposes a cell-free therapeutic approach for spinal cord injury.

Also, numerous experimental studies have shown that cellular therapy, including DP-MSCs, is an attractive strategy for ischemic brain injury systemic delivery of human. In this context, Nito et al. (2018) DP-MSCs was shown to reduce ischemic damage, and improved functional recovery in a rodent ischemia model, with a clinically relevant therapeutic window.

5.3 Angiogenesis

Insufficient vessel growth associated with ischemia remains an unresolved issue in vascular medicine. MSCs have been shown to promote angiogenesis through a mechanism that is potentiated by hypoxia. Overexpression of hypoxia-inducible factor (HIF)-1α in MSCs improves their therapeutic potential by inducing angiogenesis in transplanted tissues. Gonzalez-King et al. (2017) studied the contribution of exosomes released by HIF-1α-overexpressing donor MSCs (HIF-MSC) to angiogenesis by endothelial cells. Exosome secretion was found enhanced in HIF-MSC. Their results indicate that exosomes derived from MSCs stably overexpressing HIF-1α have an increased angiogenic capacity in part via an increase in the packaging of Jagged1, which could have potential applications for the treatment of ischemia-related disease (Gonzalez-King et al. 2017).

The perivascular characteristics of DP-MSCs were described as the potential application of DP-MSCs. Nam et al. (2017) investigated whether DP-MSCs had angiogenic capacity by co-injection with human umbilical vein endothelial cells (HUVECs) *in vivo*. They saw DP-MSCs expressed perivascular markers such as NG2, α-smooth muscle actin (α-SMA), platelet-derived growth factor receptor β (PDGFRβ), and CD146. On the other hand, Paino et al. (2017) also found that human DP-MSCs could express high levels of angiogenic genes, such as vascular endothelial growth factor and platelet-derived growth factor

A. Human DP-MSCs, after 40 days of culture, give rise to a 3D structure resembling a woven fibrous bone. When these woven bone (WB) samples were analysed using classic histology and synchrotron-based, X-ray phase-contrast microtomography and holotomography, it was found that histological and attractive physical qualities of bone with few areas of mineralization and neovessels. Such WB, when transplanted into rats, was remodelled into vascularized bone tissue.

5.4 Corneal Regeneration

Glaucoma is a common cause of irreversible blindness and is characterised by a degenerative loss of retinal ganglion cells (RGC) and their axons, leading to optic disc cupping and reduced visual acuity (Quigley 1996). Hence, enhancing viability and function of these cells is a major goal of basic SNF translational researches. As mentioned, OMT-SCs, especially DP-MSCs, have the potential to differentiate into neuronal cells. Mead et al. (2015), compare the efficacy of adipose-derived stem cells, iBM-MSCs and DP-MSC for preventing the loss of RGC and visual function when transplanted into the vitreous of glaucomatous rodent eyes. The study encouraged the use of DP-MSCs as a neuroprotective cellular therapy in retinal degenerative disease such as glaucoma.

DP-MSCs therapeutic potential on on axotomized adult rat retinal ganglion cells (RGCs) was investigated through in vivov and in vitro model by Mead et al. (2013). Apart from other studies they used conditioned media collected from cultured rat DP-MSCs and iBM-MSCs to examine nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) secretion. DP-MSCs or iBM-MSCs were cocultured with retinal cells, with or without Fc-TrK inhibitors, in a Transwell system, and the number of surviving βIII-tubulin$^+$ retinal cells and length/number of βIII-tubulin$^+$ neurites were quantified. For the *in vivo* study, DP-MSCs or iBM-MSCs were transplanted into the vitreous body of the eye after a surgically induced optic nerve crush injury. At 7, 14, and 21 days postlesion (dpl), optical coherence tomography (OCT) was used to measure the retinal nerve fiber layer thickness as a measure of axonal atrophy. At 21 dpl, numbers of Brn-3a$^+$ RGCs in parasagittal retinal sections and growth-associated protein-43$^+$ axons in longitudinal optic nerve sections were quantified as measures of RGC survival and axon regeneration, respectively. It was seen that both DP-MSCs and iBM-MSCs secreted NGF, BDNF, and NT-3, with DPSCs secreting significantly higher titers of NGF and BDNF than BM-MSCs. DP-MSCs, and to a lesser extent iBM-MSCs, promoted statistically significant survival and neuritogenesis/axogenesis of βIII-tubulin$^+$ retinal cells in vitro and in vivo where the effects were abolished after TrK receptor blockade. Intravitreal transplants of DP-MSCs promoted significant neurotrophin-mediated RGC survival and axon regeneration after optic nerve injury (Mead et al. 2013).

To determine the outcome of the use of a tissue-engineered cell sheet composed of human undifferentiated immature DP-MSCs for ocular surface reconstruction in an animal model of total limbal stem cell deficiency (LSCD), a tissue-engineered hIDPSC sheet was transplanted onto the corneal bed. Subsequently,covered with deepithelialized human amniotic membrane (AM). After 3 months, a detailed analysis of the rabbit eyes was performed with regard to clinical aspect, histology, electron microscopy, and immunohistochemistry. Corneal transparency of the rabbit eyes that underwent DP-MSC transplantation was found to be improved throughout the follow-up, while the control corneas developed total conjunctivalization and opacification. Rabbits from the MCB group showed clearer corneas with less neovascularization (Gomez et al. 2010).

5.5 Treatment of Diabetes

Despite advances in the treatment of diabetic patients, diabetes mellitus remains one of the most serious health care problems in the world.

Using transplants of Langerhans islets in cell-based therapy of type 1 diabetes mellitus has shown major limitations because of the side effects associated with immunosuppressive therapy and insufficient supply of islets (Moshtagh et al. 2013). Besides, DP-MSCs could differentiate i*n vitro* toward therapeutically functional insülin producing cells (IPCs), their immunomodulatory functions via secretion of active molecules and/or direct interaction with immune cells, make them an option for cell therapy. DP-MSCs were differentiated into IPCs that were morphologically similar to pancreatic islet cells and were shown to express insulin and C-peptide, as confirmed by immunofluorescence staining (Suchanek et al. 2017). Moreover, IPCs demonstrated higher expression of PDX-1 and HES1 in comparison to the cells cultivated in standard cultivation media, and newly occurring mRNA for Glut2, which was not detectable in the negative control, confirming the differentiation of DP-MSCs into IPCs. This study was found is in line with Huang et al. (2009) that has shown that SCs from other dental origin were capable of secreting insulin. Also, periodontal ligament (PDL) cells, can differentiate into pancreatic cells capable of insulin secretion (Suchanek et al. 2017).

The effects of factors secreted by SHED on β-cell function and survival was investigated also. Izumoto-Akita et al. (2015) suggested that conditioned medium of SHED provides direct protection and encourages the propagation of β-cells, and has potential as a novel strategy for treatment of diabetes also.

5.6 Liver Diseases

Liver transplantation is a gold standard treatment for intractable liver diseases. Because of the shortage of donor organs, alternative therapies have been required. It is a good candidate for regenerative medicine, where stem cell-based therapies play a central role. Due to their potential to differentiate into a variety of mature cells, OMT-SCs are considered feasible cell sources for liver regeneration.

Ishkitiev et al. (2015) were the first to report that DP-MSCs differentiated into hepatocyte-like cells. They cultured SHED in the presence of hepatocyte growth factor (HGF), dexamethasone, and oncostatin, and found that they transformed into a hepatocyte-like shape and produced IGF-1 and albumin. They also identified the presence of urea in the culture medium, which suggested the possibility that the urea cycle was functioning in these cells.

In 2015, Yamaza and coworkers investigated *in vivo* capability of SHED homing and hepatocyte differentiation and therapeutic efficacy for liver disorders in carbon tetrachloride (CCl4)-induced liver fibrosis model mice. SHED were transplanted into CCl4-induced liver fibrosis model mice through the spleen and analyzed the *in vivo* homing and therapeutic effects by optical, biochemical, histological, immunological and molecular biological assays. It was seen that transplanted SHED homed to recipient livers, and expressed HLA-ABC, human hepatocyte specific antigen hepatocyte paraffin 1 and human albumin.

6 Dental Stem Cell Banking: Is It Obligation or Choice?

The process of obtaining, handling, and storing stem cells obtained from patients' deciduous teeth and wisdom teeth or OMT-SCs in a facility operated according to good manufacturing proactices (GMP) is called dental stem cell banking (Arora et al. 2009; Kaku et al. 2010; Abedini et al. 2011). To implement dental stem cell banking for regenerative therapy extensively, it is important to obtain confidential data from in vitro/in vivo studies and clinical trials. Recently, dental cell/tissue banks have been planned and placed into practice in several countries, such as Advanced Center for Tissue Engineering Ltd., Tokyo, Japan (http://www.acte-group.com/); Teeth Bank Co., Ltd., Hiroshima, Japan (http://www.teethbank.jp/); Store-A-ToothTM, Lexington, USA (http://www.store-a-tooth.com/); BioEDEN, Austin, USA (http://www.bioeden.com/) and Stemade Biotech Pvt. Ltd., Mumbai, India (http://www.stemade.com/).

Once OMT, such as PDL, pulp tissues, apical papilla, or the tooth itself, are obtained from the patient, they can be cryopreserved for approximately 10–12 years to retain their regenerative potential (Seo et al. 2005; Tirino et al. 2011). Dental stem cells can be isolated from the cryopreserved tissue/tooth whenever required for future regenerative therapies (Oh et al. 2005).

Although successful autologous transplantation of banked teeth has been achieved in the clinic (http://www.teethbank.jp/), stem-cell-based tissue engineering therapies using stem cell banking have not yet been reported. Therefore, the utility of stem cell banking in dentistry should be carefully evaluated. In addition, legislation for the banking system is necessary because it provides bio-insurance for a future use that is highly unlikely. Checks and audits must be conducted to determine whether the banking company can operate well into the future and whether the cryopreserved cells and tissues are maintained in good quality for future use in transplantation.

Thus, appropriate cryopreservation of these dental cells, tissues and teeth are imperative to realize the opportunities of these OMT-SCs for medical applications, particularly for autotransplantation (Mazur 1984). However, the optimal methods for tissue cryopreservation and OMT obtaining remain largely unknown. Kaku et al. (2010) described long-term tooth cryopreservation using a programmed freezer with a magnetic field, the so called Cell Alive System (CAS).6 Using the CAS method, the PDL showed good cell viability and differentiation capability after cryopreservation. Ducret et al. (2015) proposed a protocol for DP-MSCs to achieve more good manufacturing practices (GMP) compliant approach. According to the protocol, there is no rule for tooth selection, cell stress, microbial contamination. Since cell stress modificate cell fate, cell differentiation and cell phenotype the procedure applied to tooth-extraction and post-operation rules should be standardized. Of course, a variability of serum production, and the oral flora of the donor should be discussed.

7 Conclusion

Growing evidence has demonstrated that the oral and maxillofacial region is a rich source of adult stem cells. Many intra-oral tissues, such as deciduous teeth, wisdom teeth and the gingiva, are not only easily accessible from the oral cavity but can also often be obtained as a discarded biological sample. Therefore, dental professionals should recognize the promise of the emerging field of regenerative dentistry and the possibility of obtaining stem cells during conventional dental treatments that can be banked for autologous therapeutic use in the future.

It should be noted that these tissues are often discarded in the clinic as medical waste and therefore present a particularly attractive source for stem cells because of their availability. Many research groups have therefore used dental stem cells to elucidate various biological phenomena and to establish potential clinical applications. However, these cells are heterogeneous with various differentiation states, as they include true "stem" cells, progenitor cells and possibly fibroblasts (Miura et al. 2003; Seo et al. 2008; Sonoyama et al. 2008). Therefore, it is necessary to effectively classify and purify these cells to prevent unexpected clinical results.

To achieve more scientific evidence, more studies, such as clinical randomized controlled trials with long follow-ups, must be carried out. There must also be a complete understanding of biological processes on both donor and recipient sides during bone regeneration which is extremely important to be able to structure more effective clinical strategies for stem cell-based bone regeneration.

Immunomodulatory function of OMT-SCs' is important in suppressing the local immune response during transplantation and in achieving optimal tissue regeneration. Prosthodontists are being motivated to get involved in stem cell biology by the increased requirement for new technologies for implant dentistry, mucosal defects, and may be organ transplantation.

Even though DSC banking may constitute a potential solution by cryopreserving them for future use, such a possibility is not only time-consuming and costly but limits their use in clinical applications. Obtaining the the teeth, or OMT, culture conditions, dose of cell infusion, and route of cell delivery should be determined.

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Conflict of Interest No competing financial interests exist.

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Stem Cells Derived from Dental Tissues

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Abstract

Stem cells are undifferentiated cells located in different parts of the body. The major role of stem cells is to restore of injured tissues. Since the discover of stem cells, they gained a big attention due to their differentiation and regeneration capacity. The main source of stem cells was known as bone marrow. However, different sources for obtaining stem cells were discovered. Dental tissues, a new source for stem cells, provide cells having mesenchymal stem cell characteristics such as fibroblast-like structure, expression of surface antigens specific for mesenchymal stem cells, regeneration ability, multilineage differentiation capacity and immunomodulatory features. Dental pulp stem cells (DPSCs), dental follicle progenitor cells (DFPCs), stem cells from apical papilla (SCAP), tooth germ stem cells (TGSCs) and periodontal ligament stem cells (PDLSCs) are stem cells derived from dental tissues as well as stem cells from exfoliated deciduous teeth (SHED). Dental stem cells express mesenchymal stem cell markers like Stro-1, CD146, CD106, CD90, CD73 CD29 and CD13. However, they do not express hematopoietic stem cell markers such as CD11b, CD45 and CD34. Dental stem cells are able to undergo myogenic, chondrogenic, adipogenic, neurogenic, osteogenic and odontogenic differentiation. Thanks to these differentiation ability of dental stem cells, they can easily be manipulated in regenerative medicine. Dental stem cells, that can effortlessly be transfected, can also be used in cell therapy application. Immunomodulatory features of dental stem cells make them suitable candidates for the therapy of immune-related disorders. Dental stem cells with high potentials such as ability of self-renewal, mesenchymal stem cell characteristics, multilineage differentiation and immunomodulation are promising tool for *in vitro* and *in vivo* differentiation studies as well as the therapy of immune-related diseases.

Keywords

Dental tissues · Stem cells · Dental stem cells · Mesenchymal stem cells

Abbreviations

ADSCs	Stem cells derived from adipose tissue
ASCs	Adult stem cells
BMP	Bone morphogenic protein
BMSCs	Bone marrow stem cells
CFU-F	Colony forming unit fibroblast assay
Col-I	Collagen-I
DFPCs	Dental follicle progenitor cells
DPSCs	Dental pulp stem cells

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ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
HGF	Hepatocyte growth factor
HLA-G	Human leukocyte antigen G
ICA	Islet cell aggregates
IDO	Indole amine 2,3-dioxygenase
IDPSCs	Immature DPSCs
IL-6	Interleukin-6
iPSCs	Induced pluripotent stem cells
MI	Myocardial infarction
MSCs	Mesenchymal stem cells
NO	Nitric oxide
PBMCs	Peripheral Blood Mononuclear Cells
PDLSCs	Periodontal ligament stem cells
PGE2	Prostaglandin E2
SCAP	Stem cells from apical papilla
SHED	Stem cells from exfoliated deciduous teeth
SLE	Systemic lupus erythematosus
SSEA	Stage-specific embryonic antigens
TGF-β	Transforming growth factor beta
TGSCs	Tooth germ stem cells
Th17	T helper 17
VEGF	Vascular endothelial growth factor

1 Introduction

Unspecialized cells having regeneration and multilineage differentiation potential under certain situations are identified as stem cells (Blau et al. 2001). When stem cells are exposed to an appropriate signal, they start to transform into a specialized cell type. The main role of stem cells is to obtain restoration of injured cells, tissue augmentation and general homeostatis (Suchánek et al. 2007). In recent years, stem cells are mainly seperated into 3 categories: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (ASCs) (Nuti et al. 2016). ESCs, which are totipotent, are collected from embryo and potentially can generate all embryonic cell types. Cells in the inner mass of the blastocyte are called pluripotent and can develop hundreds of cell types. ASCs are multipotent and limited in terms of differentiation potential (Yalvaç 2008).

Hematopoietic, adipose, neural, hepatic, limbal and mesenchymal stem cells (MSCs) are included in the adult stem cell group (Nuti et al. 2016). Mesenchymal stem cells have clonogenic differentiation capacity (Pittenger et al. 1999). The primary source of MSCs is known as bone marrow. However, it is not the only source of MSCs. Distinct sources are available for the isolation of MSCs, for instance; umbilical cord blood, fetal liver, adipose tissue, dermis, skeletal muscle, lung and dental tissues (da Silva Meirelles et al. 2006). Forms of MSCs are very similar to fibroblasts that is spindle-like (Pittenger et al. 1999). In 1970, Friedenstein firstly identified MSCs in the bone marrow from guinea pig (Friedenstein et al. 1970). The maintenance of mesenchymal stem cells is very easy in cell culture flasks. They are negative for CD45, CD34 and CD14 which are hematopoietic stem cell markers while positive for mesenchymal stem cell markers like CD166, CD105, CD90, CD73 and CD29 (Chamberlain et al. 2007; Friedenstein et al. 1970). Fluorescence activated cell sorting is widely used to characterize MSCs as well as Colony forming unit fibroblast assay (CFU-F) (Friedenstein et al. 1970). Differentiation ability of MSCs is quite high. Under specific inducers, MSCs can undergo differentiation into adipocytes, osteoblasts and chondroblasts *in vitro* and in *vivo* conditions (Takahashi et al. 2007). Also, it was shown that environment of the cell is also effective on the differentiation of MSCs (Khan et al. 1991). Ascorbic acid, dexamethasone and β-glycerol phosphate induce osteogenic differentiation of MSCs, while TGF-β3 and dexamethasone stimulate chondrogenic differentiation. Moreover, glucocorticoids also have a pivotal role in the differentiation of MSCs into chondrogenic cells. They boost collagen type II upregulation which is TGF-β-mediated and trigger dermatopontin, collagen type XI and aggrecan which are the matrix componenets (Derfoul et al. 2006; Jaiswal et al. 1997; Mackay et al. 1998; Pereira et al. 1995; Pittenger et al. 1999).

There are many studies in which MSCs are used in gene therapy applications. For instance, MSCs infected with vascular endothelial growth

factor (VEGF) were transplanted into rats after myocardial infarction (MI) to ameliorate heart function (Matsumoto et al. 2005; Yang et al. 2007a). In another study, MSCs were used as vehicles to deliver interferon-β into mice's tumor. Furthermore, the ability of bone formation of MSCs were increased by transfecting them with bone morphogenic proteins (BMPs) (Hasharoni et al. 2005; Tang et al. 2005; Zhang et al. 2004).

2 Dental Stem Cells

Third molar teeth, deciduous teeth and other teeth obtained from various dental applications can be counted some sources of dental tissues. Since dental stem cells were discovered as a derivation of adult stem cells, they have gained importance (Miura et al. 2003). Dental pulp found in the center of tooth is a soft tissue and comprises blood vessels, neural fiber and connective tissue. Dental pulp contains both mesenchymal and ectodermal contents as well as neural crest cells (Kerkis et al. 2006).

The formation of tooth begins approximately at the sixth of the embryogenesis and it is the combination of ectomesenchyme coming from the ectoderm and neural crest. Enamel organ is formed by the ectoderm which interacts with enamel and the ectomesenchyme. Thanks to this interaction, periodontal ligament and cementum are created (Chatterjee 2006). After the synergy of the ectoderm and the mesoderm, the tooth germs start to develop. Meantime, dental follicle, dental organ and dental papilla, which give rise to the main part of the periodontal and dental structures, are formed from the neural crest cells (Bosshardt 2005). Subsequently, mineralization around the teeth occurs. Thanks to this mineralization, dental pulp tooth germs stay in a hard structure protecting them from environmental factors including differentiation stimuli (Erickson and Reedy 1998). The development of the third molar starts at age six meaning that tissues having highly differentiation capacity in the dental lamina stay undifferentiated and dormant (d'Aquino et al. 2008) (Fig. 1).

Stem cells having mesenchymal stem cell properties from the dental pulp of the third molar firstly obtained and identified by Gronthos (Gronthos et al. 2000). Dental pulp stem cells (DPSCs), dental follicle progenitor cells (DFPCs), stem cells from apical papilla (SCAP), tooth germ stem cells (TGSCs) and periodontal ligament stem cells (PDLSCs) are stem cells derived from dental tissues as well as stem cells from exfoliated deciduous teeth (SHED) (Miura et al. 2003; Morsczeck et al. 2005; Seo et al. 2005; Sonoyama et al. 2006). MSCs isolated from dental tissues have an important differentiation potential *in vitro*. Chondrocytes, adipocytes, osteoblasts, odontoblast and myoblasts can be obtained by differentiation of dental stem cells (Liu et al. 2015).

Markers spesific for mesenchymal stem cells like Stro-1, CD146, CD106 and CD44 are expressed in dental stem cells. Nonetheless, hematopoietic stem cell markers such as CD11b, CD45 and CD34 are not expressed (Liu et al. 2015; Shi et al. 2005). It was revealed that the expression of Stro-1 in stem cells derived from dental tissues indicates odontogenic and multilineage differentiation potential of these cells (Yang et al. 2007b).

Differentiation profile of DSCs and has been shown in common with bone. Differentiation ability of dental stem cells are utilized in tissue engineering. It was demonstrated that odontoblasts can be obtained from the differentiation of DPSCs (Gronthos et al. 2000). DSCs also have the ability of differentiation into chondrogenic, myogenic and osteogenic cells (Zhang et al. 2006a). Thanks to osteogenic differentiation ability of DSCs, they are great candidate for osteogenesis and scaffold studies (Liu et al. 2007; Zhang et al. 2006b). Chondrogenic, osteogenic and adipogenic differentiation of DSCs were succesfully achieved and differentiation capacity of DSCs was stimulated by amphiphilic pluronic block copolymers (Doğan et al. 2012). Although MSCs and ADSCs (stem cells derived from adipose tissue) have neurogenic differentiation potential (Deng et al. 2006; Huang et al. 2007; Wislet-Gendebien et al. 2005), it was revealed that DSCs have also neurogenic

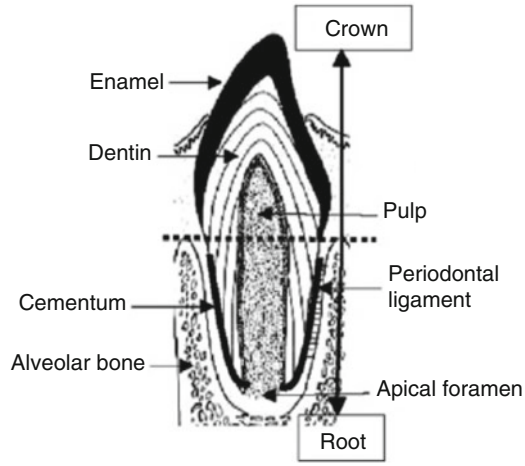

Fig. 1 The structure of tooth. (Liu et al. 2005)

differentiation capacity in certain situations (Arthur et al. 2008). It was announced that dental stem cells express Nanog, Oct-4, TRA-1-60 and SSEA-4 which indicate pluripotency (Kim et al. 2012). There are many studies showing the neurogenic differentiation potencial of DSCs *in vitro* and *in vivo*. Studies in the literature indicate that DSCs could be applied in neural tissue engineering and may be used in neural regeneration (Kim et al. 2012).

Dental stem cells also have immunomodulation features as well as differentiation capacity. This function of DSCs make them suitable candidates for transplantation applications and immunosuppressive cure against autoimmune and inflammatory illness.

3 Dental Pulp Stem Cells (DPSCs)

Dental pulp stem cells (DPSCs) are firstly derived from dental pulp in 2000 by Gronthos (Gronthos et al. 2000). DPSCs, which are adherent cells, possess morphology like fibroblasts and have multilineage differentiation capacity (Martens et al. 2013). Cryopreservation of DPSCs is quite safe. Also, DPSCs have a high proliferation capacity. They express surface antigens such as CD146, CD105, CD90, CD73, CD59, CD44, CD29, CD13, and STRO-1, which are spesific for mesenchymal stem cells. However, they do not express surface antigens such as CD45, CD34, CD24, CD19, and CD14 which are hematopoietic stem cell markers (Huang et al. 2009; Karaöz et al. 2010; Lindroos et al. 2008). Differentiation capacity of DPSCs is very high. There are numerous studies showing the differentiation of DPSCs. They can undergo odontogenic, myogenic, adipogenic, osteogenic and neurogenic differentiation (Armiñán et al. 2009; Carinci et al. 2008; d'Aquino et al. 2007; Nör 2006; Zhang et al. 2006a). In addition, islet cell aggregates (ICA) like pancreatic islet cells can be obtained from differentiation of DPSCs. It was shown that ICA derived from DPSCs express pancreatic islet cell markers such as pdx1, pdx4, pdx6, C-peptide, Isl-1 and ngn3. These cells also secrete insulin by a glucose stimuli. DPSCs may be a promising candidate for the treatmet of diabetes for the future (Govindasamy et al. 2011). Moreover, DPSCs can secrete anti-apoptotic and proangiogenic agents. This feature of DPSCs may be useful in the therapy of myocardial infarction. Cultured DPSCs were injected into nude rats and an increase in myocardial function was achieved (Gandia et al. 2008). Embryonic stem cell markers such as TRA1-60, nanog, Oct-4 and TRA-1-80-1 are expressed in DPSCs (d'Aquino et al. 2009). These expressions of embryonic markers in

DPSCs suggest that pluripotent stem cells may be left in the tooth structure (Huang et al. 2008).

Apart from differentiation abilities of DPSCs, they have immunomodulatory features. It has been reported that immunogenicity of DPSCs is quite low. The expression of histocompatibility complex class II antigen does not exist on DPSCs (Lee and Song 2018; Lin and Du 2018). Hepatocyte growth factor (HGF), Human leukocyte antigen G (HLA-G), transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2) and interleukin-6 (IL-6) which are anti-inflammatory cytokines, are released from DPSCs. In addition, indole amine 2,3-dioxygenase (IDO) and nitric oxide (NO) having critical positions in the immune response are produced by these stem cells (Anitua et al. 2018; Johnston et al. 2016; Liu et al. 2015). Increasing the number of regulatory T cells can be succeeded by DPSCs (Özdemir et al. 2016; Yildirim et al. 2016).

4 Dental Follicle Progenitor Cells (DFPCs)

Ectomesenchymal ancestor cells create the dental follicle which is a detached and veined tissue. Dental follicle contains a number of precursor cell types for odontoblasts, cementoblasts and periodontal ligament cells. Pregenitor cells are obtained from the dental follicle of human third molars (Morsczeck et al. 2005). They exhibit mesenchymal stem cell features with surface antigens and proliferation ability. DFPCs express mesenchymal stem cell markers like CD90, CD59, CD29 and CD13. Nevertheless, the expression of hematopoietic stem cell markers is not observed in DFPCs (Estrela et al. 2011). If they receive appropriate stimuli, DFPCs can undergo osteogenic, adipogenic and chondrogenic differentiation (Yao et al. 2008). Because DFPCs are derived from developing tissue, their plasticity is much better than other dental stem cells (Volponi et al. 2010). DFPCs also have immunosuppressive properties. It was reported that they can secrete TGF-β and IL-6. Proliferation of Peripheral Blood Mononuclear Cells (PBMCs) is frustrated by DFPCs. It is considered that DFPCs are convenient candidates for the treatment of chronic inflammatory disease treatment (Liu et al. 2015).

5 Stem Cells from Exfoliated Deciduous Teeth (SHED)

The pulp of human exfoliated deciduous teeth is another source of dental stem cells. Miura et al are firstly obtained SHED cells from the pulp of exfoliated deciduous teeth (Miura et al. 2003). Due to immature cell population in exfoliated deciduous teeth, they are also named as "immature DPSCs" (IDPSCs) (Kerkis et al. 2006). The morphology of SHED cells are like fibroblasts which is similiar to DPSCs and DFPSCs. Proliferation of SHED cells are very high (even than bone marrow stem cells) and they can generate sphere-like clusters (Estrela et al. 2011). SHED cells are positive for CD166, CD146, CD90, CD73, CD29, which are mesenchymal stem cell markers, as well as Oct4, whereas they are negative for CD45, CD34 and CD14 (Huang et al. 2009; Pivoriūnas et al. 2009). Differentiation ability of SHED cells is very similiar to MSCs. It was reported that SHED cells are differentiated into chondrocytes, myocytes, adipocytes, osteoblasts and even neuron-like cells (Kerkis et al. 2006; Miura et al. 2003; Wang et al. 2010). Gene expression of SHED cells are distinct from DPSCs. SHED cells express genes relevant to extracellular matrix formation, for instance transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), and collagen-I (Col-I) (Nakamura et al. 2009). In addition, stage-specific embryonic antigens SSEA-3 and 4; nanog, nestin and oct-4, which are the markers of pluripotency, are expressed by SHED cells (Esmaeilzadeh et al. 2016; Huang et al. 2009). SHED cells also demonstrate immunomodulatory functions. It was displayed that SHED cells successfully repressed the function of T helper 17 (Th17) playing a crucial role in the defense against microorganisms. It is suggested that SHED cells may be used in the therapy of systemic lupus erythematosus (SLE) (Yamaza et al. 2010).

6 Stem Cells from Apical Papilla (SCAP)

Apical papilla is a tissue softly connected to the top of developing tooth. Apical papilla of human immature permanent tooth is another source where dental stem cells are obtained from (Sonoyama et al. 2006, 2008). Cells from apical papilla are attained by primary culture techniques and these cells are a community of stem cells. SCAP have mesenchymal stem cell characteristics. Cell surface antigens specific for mesenchymal stem cells for such as CD146, CD90, CD44, CD24 and STRO-1 are expressed on SCAP. However, the expression of cell surface antigens specific for hematopoietic stem cells is not detected in SCAP. It is thought that CD24, not perceptible in bone marrow stem cells (BMSCs) and DPSCs, is a certain marker for SCAP (Esmaeilzadeh et al. 2016; Rodríguez-Lozano et al. 2011). Differentiation of SCAP into osteo-, neuro-, and adipogenic cells *in vitro* is reported in the literature. SCAP also exhibit immunosuppressive features. It was shown that SCAP impede the proliferation of T cells which is apoptosis-independent *in vitro* and display minimal immunogenicity. Cryopreservation does not effect immunosuppressive properties of SCAP (Taşlı et al. 2016a).

7 Tooth Germ Stem Cells (TGSCS)

Tooth germ is an accumulation of precursor cells that generate a tooth and tissues in it (Liu et al. 2005). Tooth germ of the third molars in humans generally start to form at age 6 (Kerkis et al. 2006). Up to this time, remaining tissues from the embryonic period are undifferentiated and developmentally fresh. For this reason, proliferation capacity of these cells is very high (d'Aquino et al. 2008). Human tooth germs are able to procure from wisdom teeth (third molar) of young adults. Cells attained from the tooth germ of the third molar represent mesenchymal stem cell features. It was revealed that human tooth germ cells express CD166, CD105, CD90, CD73 and CD29 which are spesific surface antigens for mesencymal stem cell. Nevertheless, the expression of hematopoietic stem cell markers is not observed in tgscs (Taşlı et al. 2013). These cells exhibit multipotency. It was reported that human tooth germ stem cells are able to differentiate into osteo-, odonto-, and adipogenic cells which originate from the endoderm, mesoderm and ectoderm (Gronthos et al. 2002; Ikeda et al. 2008). Differentiation ability of tooth germ stem cells (tgscs) can be increased by using external substances. Doğan et al. indicated that osteogenic and chondrogenic differentiation of tgscs is enhanced by F68 which is a pluronic block copolymer (Doğan et al. 2012). Tgscs are alsp able to differentiate into neurogenic cells due to they come from neural creast and include neuroectodermal and mesenchymal constituents (Yalvaç et al. 2011). Moreover, myogenic cells can be obtained by differentiation of tgscs (Taşlı et al. 2016b). The use of tgscs has some other advantages as well as differentiation ability. Differentiation capacity of tgscs is rather high. These cells are easily accesible because they are obtained from removed teeth for a regular dental therapy. Isolation procedure of tgscs is not painful and does not pose ethical issues (Yalvac et al. 2010). Tgscs are also used for gene therapy applications. Odontogenic differentiation capacity of tgscs can be increased by different transfection techniques. It was demonstrated that bone morphogenic protein 7 (BMP-7) and bone morphogenic protein 2 (BMP-2) which are enhancer of odonto- and osteogenesis, are transferred into tgscs by electroporation. Increasing the odonto- and osteogenic differentiation abilities of tgscs may be a possible treatment techniques used in bone repair (Taşlı et al. 2014). Electroporation and chemical transfection efficiency of tgscs was also boosted by extraneous materials (Aydin et al. 2016). Tgscs derived from human have immunomodulatory features. It was demonstrated that tgscs attained from human were used as an immunosuppressive agent in rats (Guzmán-Uribe et al. 2012).

8 Periodontal Ligament Stem Cells (PDLSCS)

Periodontal ligament (PDL) is a neural crest-originated tissue. Cells in the periodontal ligament provide the connection between bone and cementum. Tooth located in dental alveolus is also supported by PDL cells. In addition, biologic equilibrium of the tooth and rehabilitation of harmed tissue are provided by these cells (Bartold et al. 2000; Lee et al. 2011; Mao et al. 2006; Pitaru et al. 2002). Enzymatic digestion can be used to obtain the cells from periodontal ligament as well as explant culture techniques. It was demonstrated that cells obtained from periodontal ligament display mesenchymal stem cell characteristics. Markers spesific for MSCs like CD105, CD73, CD44, CD29 and CD10 are expressed in PDL cells. However the expression of surface antigens spesific for hematopoietic stem cells like CD14, CD34 and CD45 is not observed in PDLSCs (Feng et al. 2010; Seo et al. 2004; Shi et al. 2005). Differentiation capacity of periodontal ligament stem cells (PDLSCs) is similiar to other stem cells attained from dental tissues. Chondrogenic, osteogenic and adipogenic cells can be obtained by differentiation of PDLSCs *in vitro* (Gay et al. 2007). It was reported that neuronal differentiation ability of PDLSCs was revealed in the literature. Also, PDLSCs are capable of differentiating in vitro and in vivo environments to form cementoblasts (Techawattanawisal et al. 2007). There are studies showing the transplantation of PDLSCs into tissues with tooth damage to generate cementoblasts (Seo et al. 2004; Sonoyama et al. 2006). PDLSCs have the ability of immunosuppression. It was shown that these cells can release Indoleamine 2, 3-Dioxygenase (IDO), HGF and TGF-β. The doubling time of PBMCs is hindered by these substances released from PDLSCs (Kim et al. 2010). Immunosuppression of PDLSCs is succeeded via the anergy of prostaglandin E2 (PGE2)-induced T cells. In addition, induction of Treg is reduced by PDLSCs (Liu et al. 2015).

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A Novel Virtue in Stem Cell Research: Exosomes and Their Role in Differentiation

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Abstract

In the past decade a number of different stem cell types have entered the clinical applications increasingly as a therapeutic option, due to their tissue maintenance capacity at the site where they localize. Although it was initially thought that conferral of resilience to damaged tissue largely depends on the stem cells themselves through orchestration of signaling among the local epithelial and immune systems at the injury site, recent findings point out that the remarkable regenerative capacity of stem cells is rather due to their nanovesicular products that emerge as the new active players of tissue repair processes. Among these extracellular vesicles exosomes generated particularly by stem cells have been receiving a substantial interest both in the fields of stem cell biology and extracellular vesicles. In this chapter fundamental facts about stem cell biology, biogenesis of extracellular vesicles and exosomes, their structure, and function are summarized. Moreover, properties of both tumor-derived exosomes as well as those derived from stem cells are discussed relatively in-depth in terms of their influence on proximal and distal tissue physiology. Last but not the least, among countless studies in an exploding field, we summarize those that attempt to unravel the complex signaling networks through which stem cell-derived exosomes alter the fate of differentiating stem cells as well as the molecular make-up of exosomes released from differentiating stem cells by conducting thorough proteomic and genomic analyses with the ultimate goal of identifying effector gene products mediating exosomal cues in stem cell biology.

Keywords

Differentiation · Exosomes · Extracellular vesicles · Stem cells

Abbreviations

Alix	Apoptosis-linked gene 2–interacting protein X
ASCs	Adult Stem Cells
BMSCs	Bone marrow stem cells
DCs	Dendritic cells
EGF	Epidermal growth factor
ESCs	Embryonic stem cells

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EVs	Extracellular vesicles
HMSCs	Human marrow stromal cells
HSCs	Hematopoietic Stem Cells
HSP	Heat-shock proteins
ILVs	Intraluminal vesicles
iPSC	Induced pluripotent stem cells
MHC	Major histocompatibility complex
miRNA	MicroRNA
MSCs	Mesenchymal stem cells
MVB	Multi-vesicular bodies
Runx2	Runt-related transcription factor 2
TEX	Tumor-derived exosomes
TLR	Toll-like receptors

1 Basic Characteristics of Stem Cells

Stem cells are located in various parts of body and have a differentiation potential into functional cell types. They have self-renewal capacity because when a stem cell divides, two different cells generate; one of them retains all parent stem cell characteristics and the other so-called "progenitor cell" acquires a specialized function (Yamashita et al. 2003). According to their differentiation capacities, stem cells are grouped into four different classes; Totipotent Stem cells can create all parts of body and derived from morula stage (Karin and Mintz 1981), Pluripotent Stem Cells (PSCs) include Embryonic Stem Cells (ESCs) and Induced Pluripotent Stem Cells (iPSCs) and are able to differentiate into 200 various cell types (Takahashi et al. 2007; Takahashi and Yamanaka 2006; Thomson et al. 1998; Yu et al. 2007), Multipotent Stem Cells obtained from different tissue types are able to differentiate into fewer cell types than PSCs (Henningson Jr et al. 2003). Unipotent Stem Cells are also called as progenitor cells and have differentiation capacity into one crucial cell type. It is challenging to work with PSCs -especially those isolated from human- in the sense that they cannot be easily induced to differentiate into a desired cell lineage due to their unstable nature. Therefore, several factors including cues from the microenvironment, cell-to-cell interactions, physical and chemical factors are required to be tightly controlled in order for a proper guidance of their differentiation into a desired cell type (Higuchi et al. 2011). Applications involving ESCs have been hindered due to their tumorigenicity and ethical as well as social concerns regarding their use (Witkowska-Zimny and Walenko 2011).

Multipotent stem cells, which are also known as Adult Stem Cells (ASCs), are abundant in the body and they are divided into two main sub-classes such as Hematopoietic Stem Cells (HSCs) and Mesenchymal Stem Cells (MSCs). Although ASCs isolated from various tissues have limited differentiation capacity under *ex vivo* conditions, *in vivo* they execute major roles in maintaining blood homeostasis, tissue, and skin turnover. While differentiation of HSCs into some cell types have been demonstrated, they can mainly produce all types of blood cells (Wilson and Trumpp 2006). On the other hand, MSCs have fibroblast cell-like morphology, self-renewal capacity, and are isolated from a number of tissue types including bone marrow, adipose, muscle connective tissue, amniotic fluid, placenta, dental tissue, and placenta (De Coppi et al. 2007; Friedenstein et al. 1970; Gronthos et al. 2000; Mareschi et al. 2001; Miao et al. 2006; Morsczeck et al. 2005; Young et al. 2001; Zuk et al. 2001). MSCs also have some characteristic features including, adherence to plastic under standard culture conditions, possession of CD105, CD73 and CD90, but lack of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR expression as surface markers. Besides, MSCs are easily differentiated into osteo-, chondro- and adipogenic cell lineages under appropriate culture conditions in *in vitro* (Dominici et al. 2006). It has been proven that MSCs can also differentiate into additional cell types such as neuronal cells (Yalvaç et al. 2011). Due to biological functions of MSCs including differentiation into various cell types, tissue maintenance and repair, immunosuppression and neuroprotection, they have been commonly used

in clinical applications (Pfister et al. 2014). MSCs maintain tissue homeostasis by controlling cell turnover, inducing tissue repair and regeneration, and regulating the immune system overall coordination of which enable restoration of damaged tissues. In repair of the damaged tissue MSCs may operate in two different ways; (i) they can either differentiate directly into required cell types, or (ii) they release vital factors that are required for the repair process (Ratajczak et al. 2017). In this respect, MSCs are crucial for bone tissue integrity and regeneration due to their osteogenic potential. They are also privileged -not only- to have the ability to escape an unwanted immune response (DelaRosa and Lombardo 2010), but also to release immunomodulatory agents and therapeutic factors (da Silva Meirelles et al. 2009; Ghannam et al. 2010). According to previous studies very few of the transplanted MSCs to the injured or damaged organ/tissue could localize to the target site (about <1%) (Phinney and Prockop 2007). More recently it has been discovered that rather the secretion of extracellular vesicles by MSCs is what drives their therapeutic effect (Chin et al. 2011). Subsequently, researchers were able to demonstrate precisely that purified exosomes from conditioned medium can exert the therapeutic efficacy of MSCs during tissue regeneration, maintenance, and repair (Akyurekli et al. 2015; Yu et al. 2014). Hence, the term "exosomes" has been proposed to collectively describe these extracellular vesicles that can reduce injury or repair damage in the tissue. In terms of assessing the clinical benefit, exosomes appear to be more advantageous than cell-based therapy due to lower rate of immune rejection after allogenic application (Yu et al. 2014). In this chapter we summarize the current status of our knowledge on extracellular vesicles in general while focusing on function, biogenesis, biological properties of exosomes, and -in particular- their impact on stem cell differentiation in detail. In this respect, we highlight comprehensive studies that shed light on the molecular circuitry downstream to exosome-mediated differentiation of MSCs into specific cell types taking proteomic or genomic approaches.

2 Extracellular Vesicles

The release of extracellular vesicles (EVs) was firstly discovered during maturation of sheep reticulocytes in 1983 (Pan and Johnstone 1983). EVs -subcellular structures encapsulated in lipid bilayer membrane- are produced by various cell types such as immune cells, cancer cells, and stem cells, and they can be found in physiological fluids including, blood, urine, saliva, milk, amniotic fluid, and malignant ascites (Raposo and Stoorvogel 2013). EVs play a crucial role in immune response, cancer development, tissue homeostasis, inflammation, and angiogenesis due to their function as major mediators of cell-to-cell communication (Konala et al. 2016; Raposo and Stoorvogel 2013). According to recent studies, the size of EVs range between 3 to 2000 nm in diameter and they can transfer nucleic acids and proteins from one cell to another in complex organisms (Keshtkar et al. 2018; Rani et al. 2015).

EVs are divided into three main groups; apoptotic bodies, microvesicles, and exosomes that are distinguished from one another based on their size, content, and origin (Hao et al. 2017). Apoptotic bodies constitute the largest members of EVs with their size ranging between 1–5000 nm and they are derived from cells undergoing apoptosis. Microvesicles are second largest group of EVs with 50–1000 nm in diameter that are derived from almost all cell types following cell membrane activation during various cellular transport events or apoptosis. The smallest type of EVs are exosomes having a diameter between 40–100 nm and their biogenesis takes place via special mechanisms (Bakhshandeh et al. 2017; Whiteside 2018). (Table 1).

3 Biogenesis of Exosomes

Pan and Johnstone were first to report presence of exosomes as couriers for removing undesirable transferrin from maturating sheep reticulocytes in 1983. Later, the term "exosomes" was coined

Table 1 Basic characteristics of different extracellular vesicles

Vesicle types	Size (diameter)	Origin	Contents
Exosomes	40–100 nm	Luminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	mRNA, miRNA, non-coding RNAs; cytoplasmic and membrane proteins (receptors and MHC molecules)
Microvesicles	50–1000 nm	Outward budding of cell membrane	mRNA, miRNA, non-coding RNAs, cytoplasmic and membrane proteins
Apoptotic bodies	1–5000 nm	Outward blebbing of apoptotic cell membrane	Nuclear fractions, cell organelles

by Johnstone in 1987 (Johnstone et al. 1987). Unlike the case for the other extracellular vesicles, exosomes stem from an endosomal biogenesis process. During exosome biogenesis, the membrane of endosomes buds inwardly to form what is called an "intraluminal vesicles (ILVs)" within a membrane vesicle that ultimately results in a multivesicular bodies (MVBs). Next, when the endosomal MVBs form into a complex with the cell membrane to release their vesicular contents (mainly exosomes) into the extracellular environment exosomes become generated (Xu et al. 2018). Based on electron microscopic images of isolated exosomes a unique cup-like structure is evident of their morphology (Choi and Mun 2017).

Both components as well as contents of exosomes are very different from each other depending on the cell type they originate from. For example, many studies have shown that several cell types such as cancer cells, stem cells, dendritic cells (DCs), epithelial cells, or different types of immune cells can produce exosomes. So far 9769 proteins, 2838 miRNAs, and 1116 lipids have been discovered as biologically active components of exosomes produced by living cells (Xu et al. 2018).

For example, the tumor-sensitive gene product (Tsg101), tetraspanins (CD9, CD63, CD81 and CD82), annexins, Rab, apoptosis-linked gene 2–interacting protein X (Alix), glycosylphosphatidylinositol (GPI)-anchored proteins, heat-shock proteins (HSP), and flotillin are commonly encountered signature proteins in an exosome that are not found in other types of EVs and, therefore, they are typically used to characterize the exosomes (Raposo and Stoorvogel 2013; Clayton et al. 2004; Ge et al. 2015; Simpson et al. 2008; Skog et al. 2008). In cellular physiology these proteins such as tetraspannins, Hsp70, Hsp90, and Tsg101 are found either in the cytoplasm or surface of the donor cells and they operate in the endosomal pathway (Bobrie et al. 2011; Van der Pol et al. 2012). Tetraspanins have several interaction partners such as major histocompatibility complex (MHC) molecules and integrins, indicating their key roles in forming assemblies of large molecular complexes and membrane subdomains. One key role of heat-shock proteins Hsp70 and Hsp90 is to bind antigenic peptides and assist their loading onto MHC class I and MHC class II (Schorey and Bhatnagar 2008). Molecules in MHC I class can be found in exosomes derived from numerous cell types. Likewise, molecules in MHC II class are typically present in exosomes of those cells that express MHC class II. Similarly, exosomes from DCs contain CD86 -an important co-stimulatory molecule for T cells- and those derived from T cells are enriched in T-cell receptors. The growing list of exosomal proteins continue with a series of cell-specific transmembrane proteins including α- and β-chains of integrins (e.g., αMon DCs, β2 on DCs and T cells, and α4β1 on reticulocytes), immunoglobulin-family members (e.g., intercellular adhesion molecule 1 (ICAM1)/CD54 on B cells, A33 antigen on enterocytes and P-selectin on platelets) or cell surface peptidases (e.g., dipeptidylpeptidase IV/CD26 on enterocytes and aminopeptidase N/CD13 on mastocytes) (Théry et al. 2002). Epidermal growth factor (EGF) VIII (MFGE8)/lactadherin26 is a milk-fat-globule protein and is abundantly found in the exosomes of the cells where they are expressed such as DCs and

some tumor-cell lines (Théry et al. 1999). These proteins are thought to operate in "addressing" of the exosomes to the recipient cells by anchoring them onto the targeted cells.

Even though exosomes vary from one another according to their origin, proteins pertained to cytoskeleton structure (ß-actin, myosin, and tubulin), cell metabolism (glyceraldehydes-3-phosphate dehydrogenase, (GADPH)), intracellular signaling pathway (Wnt, ß-catenin, notch ligand) and those that maintain cell-to-cell communication (interleukin-1ß and tumor necrosis factor alpha (TNF-α)) can be commonly found in all exosomes (Derkus et al. 2017). Intriguingly, only a subset of the endosomal/lysosomal proteins can be found in exosomes while lysosomal proteases or other soluble endocytic residents, or any subunits of the v-ATPase are not present (Théry et al. 2002).

Regarding the rest of the exosomal architecture, reticulocyte exosomes have a similar composition to that of the reticulocyte plasma membrane. Similarly, B-cell-derived exosomes are composed of lyso-bis-phosphatidic acid, a lipid that is abundant in the late endocytic compartments of these cells (Denzer et al. 2000). Exosomes derived from platelets and DCs contain Phosphatidylserine (PS), a lipid that is found at the cytosolic side of the plasma membrane at relatively low levels (Théry et al. 2002). Exosomes predominantly become released from structurally and functionally unique microdomains in the plasma membrane which are termed as "lipid rafts", hosting important membrane activities such as endocytosis, cell signalling, cell adhesion, and membrane trafficking (Laude and Prior 2004), and endocytosis (Rajendran and Simons 2005). Hence, exosomes are enriched in certain raft-associated lipids such as cholesterol, ceramide, sphingolipids and phospoglycerides with long and saturated fatty-acyl chains which are preponderant components of the exosomal membrane and they facilitate the budding of inward ILVs into MVBs (Rani et al. 2015). In Epstein-Barr virus (EBV)-transformed B cells both internal vesicles of late endosomes and exosomes are enriched in cholesterol, as well as plasma-membrane microdomains (Möbius et al. 2002). In mast cells and DCs, one study detected an increase in phosphatidylethanolamines and flip-flop rate between the two leaflets of the bilayer of exosome membranes (Laulagnier et al. 2004).

Several different types of biological cargo -including mRNA, micro RNA (miRNA), pre-miRNA, other noncoding RNA species and proteins- may be released into the recipient cells by exosomes via three different mechanisms; (i) endocytic uptake, (ii) direct fusion with the cell membrane or (iii) adhesion that depends on ligand-receptor interaction, underscoring the physiological importance of these microvesicles in maintaining intercellular communication and immune regulation (Camussi et al. 2010; Konala et al. 2016; Raposo and Stoorvogel 2013; Van der Pol et al. 2012).

Valadi et al. (Valadi et al. 2007) reported that, although the RNA extracted from exosomes contains mostly short RNA fragments (less than 200 nt), the fact that identifiable full-length mouse proteins can be translated from exosomal RNA pool by human cells suggests presence of full length mRNAs in these nanovesicles. Several studies indicate that the RNA "cargo" of exosomes can be significantly at different levels than the endogenous levels of these messages in the parental cell (Skog et al. 2008; Mittelbrunn et al. 2011; Zomer et al. 2010). On the other hand, in the case of cancer-cell-associated exosomes, miRNA content of circulating exosomes is reported to be similar to that found in their originating cancer cells which can be releasing exosomes at a higher rate than normal cells, a feature proposed to be exploited to develop novel diagnostic markers (Rabinowits et al. 2009; Taylor and Gercel-Taylor 2008; Kalluri 2016). Current procedure-of-preference to verify the identity of exosomes involves EM-based imaging, which serves as an ultimate proof, yet it is a costly and time-consuming technique. Hence, there is clearly an urgent need to develop more practical and cost-effective molecule-based tools and protocols to confirm the presence of exosomes. In that respect, a characteristic miRNA may serve as an exosomal biomarker, but this area of research needs more comprehensive studies (Vlassov et al. 2012).

4 Biological Function of Exosomes

As mentioned above increasing number of studies point to participation of exosomes in diverse cellular processes such as cell-to-cell communication, proliferation, angiogenesis, migration, and differentiation (Fig. 1). The modulation of protein synthesis in target cells via the action of their cargo mRNA (Skog et al. 2008) and miRNA is the most common mechanism exosomes use to alter gene expression in the recipient cells (Rabinowits et al. 2009; Skog et al. 2008; Taylor and Gercel-Taylor 2008).

Elegant studies by Valadi et al. demonstrated that the so-called shuttle RNA is transferred from a subset to the rest of a mast cell population by exosomes harvested from a human (HMC-1) and mouse (MC/9) mast cell lines. The authors further showed that upon uptake by the recipient cells the transferred RNA was translated into a protein product, suggesting biological activity (Valadi et al. 2007). Remarkably, rather than mRNAs that are abundantly present in secreting cells a selected subset of transcripts were packaged into exosomes, pointing to the involvement of a selective "*handling&shipping*" machinery which is yet to be uncovered (Schorey and Bhatnagar 2008). Meanwhile, Pegtel et al. showed that miRNA containing exosomes derived from EBV-infected B-cells transported viral miRNAs to uninfected cells where expression of host genes targeted by miRNA was altered (Pegtel et al. 2010). Whether similar mechanisms for propagation of infection exist under *in vivo* conditions remains to be determined (Bang and Thum 2012). In another study, Alvarez-Erviti and colleagues demonstrated that DCs engineered to express a fusion gene (comprised of neuron-specific rabies virus glycoprotein (RVG) and exosomal membrane protein LAMP2B sequences) produced exosomes with RVG peptide on their periphery. These authors were able to further show that exosomes derived from engineered DCs can be loaded with siRNA that then could cross the blood-brain barrier to achieve a more than 60% gene silencing in the targeted genes of neurons, microglia, oligodendrocytes, and their precursors (Alvarez-Erviti et al. 2011).

This feature of these nanovesicles proposes exosomes as a promising tool for novel drug delivery strategies. For a number of reasons

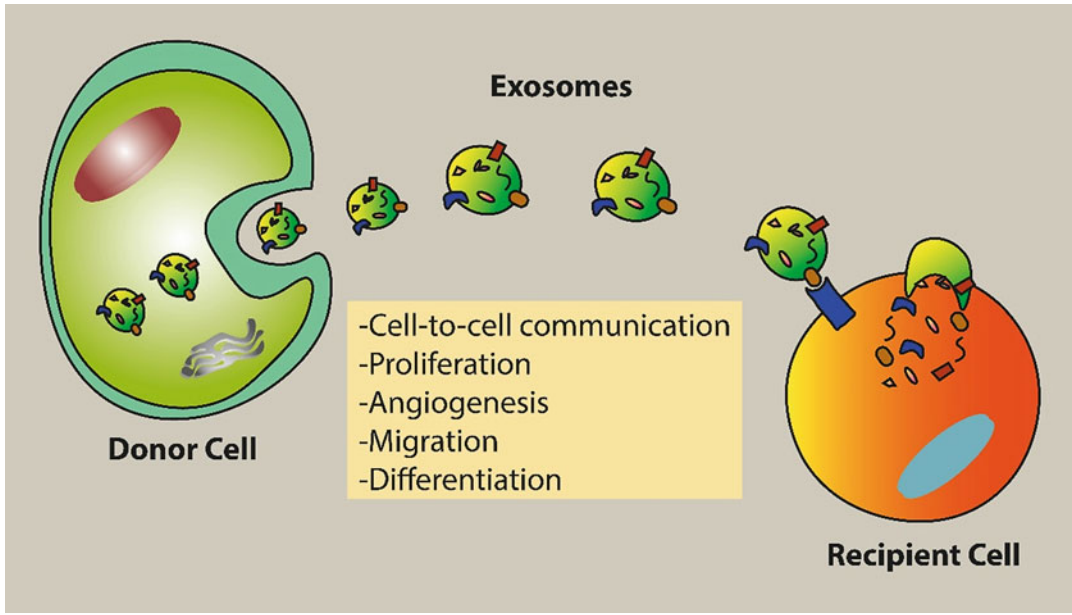

Fig. 1 Biological functions of exosomes

exosomes are eminently suitable to be utilized as couriers of therapeutic agents (Lai et al. 2013a): (i) As routine cargo of exosomes subcellular materials such as protein and nucleic acids can be easily loaded into exosomes. (ii) Exosomes are readily detected in body fluids such as blood (Caby et al. 2005), urine (Zhou et al. 2006) and breast milk (Admyre et al. 2007), suggesting that as drug delivery vehicles with demonstrated longer circulating half-life and improved efficacy they can be well tolerated by human body (Kamerkar et al. 2017). (iii) Materials packaged in exosomes can be easily taken up by the target cells upon fusion with their plasma membrane (Valadi et al. 2007). (iv) Although the existent evidence is to be strengthened further, exosomes can be directed to aim for a tissue-of-interest depending on the their sourcing cell type to deliver their cargos (Hood et al. 2011). (v) Notably, exosomes are compatible with membrane modifications allowing enhancement of precision for cell-type-targeting (Alvarez-Erviti et al. 2011).

Another important known function of exosomes is to remove unwanted metabolites or misfolded proteins to outside of the cells, a function *heavily* relied upon by diverse cell types, including neurons, astrocytes, and glial cells for the disposal of unwanted stress proteins to prevent amyloid fibril formation, that is a hallmark subcellular pathology seen in neurodegenerative disorders such as Alzheimers Disease (Lai et al. 2011).

5 Properties of Exosomes Derived from Tumor

Exosomes derived from tumor cells, abbreviated as TEX, are heavily found in tumor microenvironment (TME) and body fluids of all cancer patients (Ludwig et al. 2017; Szczepanski et al. 2011). TEX plays a major role in transferring messages from a donor cancer cell to other malignant cells or normal cells such as MSCs that may be found in the microenvironment to induce phenotypic changes in the latter (Boyiadzis and Whiteside 2015). TEX can promote survival of cancer cells by mediating autocrine, juxtacrine, and paracrine signaling (Whiteside 2017). TEX-dependent paracrine signaling affect -not only- the tumor site, but also distal tissues and cells from the tumor via transfer of the intercellular messages (Whiteside 2018). For example, human brain tumor cells utilize exosomes for transferring oncogenic receptor EGFRvIII to other cells, besides exosomes derived from a highly aggressive form of melanoma cells can convert less metastatic tumor cells to a more aggressive phenotype (Hao et al. 2006; Dominici et al. 2006). According to another study, exosomes produced by tumor were able to evade the immune system by enhancing immunosuppressive myeloid cells (Lai et al. 2015).

Communication between tumor cells and endothelial cells takes place by the transfer of specific miRNA enclosed in exosomes that elicit a stimulatory effect on migration, metastasis, angiogenesis, tumor proliferation, and malignancy (Boon and Vickers 2013). For example, Toll-like receptors (TLR) -activated by exosome-shuttled miRNAs- can contribute to progression of malignancy either by inducing relevant phenotypic changes in host cells or by igniting a pro-metastatic inflammatory response that ultimately fuels the tumor growth at distal sites. In an elegant study, Fabbri et al. (Fabbri et al. 2012) showed that miR-21 and miR-29a secreted by the tumor, can bind to TLR as ligands resulting in stimulation of tumor growth. Furthermore, these two miRNAs can bind to murine TLR7 and human TLR8 in immune cells and stimulate TLR-mediated pro-metastatic inflammatory response contributing tumor growth and metastasis. In another study Zhuang et al. showed that exosome-mediated secretion miR-9 by tumor cells stimulates endothelial cell migration and suppression of SOCS5 levels resulting in promotion of tumor angiogenesis (Zhuang et al. 2012).

6 Properties of Exosomes Derived from MSCs

MSC-secreted exosomes have been firstly discovered in a mouse model of myocardial ischemia/

reperfusion injury in 2010 (Lai et al. 2010). Then, MSC-derived exosomes were tested in different disease models. Interestingly, MSCs were found to secrete higher amount of exosomes than other cell types (Tsao et al. 2014). MSC-derived exosomes are originated from lipid-raft microdomains in the plasma membrane and their biogenesis operates via the endocytic-exocytic pathway. MSC-derived exosomes are implicated in stem cell differentiation due to their expression of stem cell markers, such as prominin-1/CD133 (Fatima and Nawaz 2015). In addition to the widely expressed surface markers of exosomes, including CD9 and CD81, exosomes of MSC origin express CD29, CD44, and CD73 (Yu et al. 2014).

Contents of MSC-derived exosomes can also be nucleic acids, structural proteins and enzymes, and lipids like in the case of exosomes derived from other cell types. Two studies identified 857 specific protein (Lai et al. 2012) and more than 150 miRNAs in MSC-derived exosomes (Chen et al. 2009) most of which were reported to play critical roles in intercellular communication, immune response, biogenesis of exosomes, tissue repair and regeneration, metabolism, and differentiation. MSC-derived exosomes enable MSCs to execute their essential role in maintenance of tissue homeostasis in cases of diseases and injury. Furthermore, they allow MSCs to remain responsive to the cues of their microenvironment within the tissue where they reside. (Lai et al. 2013b). To restore normal tissue function a number of critical enzymatic activities enclosed in MSC-derived exosomes provide tissue regeneration. Several of these enzymes stored in MSC-derived exosomes are regulated by feedback mechanisms governed by the tissue microenvironment (Lai et al. 2015). Increasing number of evidence both from *in vitro* and *in vivo* studies underscore the stunning regenerative capacity of MSC-derived exosomes that stimulate epithelial and immune cells at the site of injury and promote angiogenesis (Hao et al. 2017).

Upon fusion with target cells MSC-derived exosomes release their contents and ultimately alter target cell behavior. For example, exosomes collected from MSCs that ectopically express TNF-related apoptosis-inducing ligand (TRAIL) induced apoptosis in cancer cells in a dose-dependent manner (Yuan et al. 2017). Moreover, analysis of MSC-derived exosomes suggest presence of proangiogenic factors such as platelet-derived growth factor (PDGF), EGF, fibroblast growth factor (FGF), and nuclear factor-kappaB (NF-κB) signaling pathway proteins as candidate paracrine effectors (Anderson et al. 2016; Zheng et al. 2018). In contrast, Lee and colleagues reported anti-angiogenic effects of MSC-derived exosomes, through downregulation of vascular endothelial growth factor (VEGF) level in tumor cells (Behbahani et al. 2016).

7 Role of Exosomes in Stem Cell Differentiation

In 1990's, taking advantage of the remarkable differentiation capacities of stem cells into diverse cell types, intense research efforts advanced our knowledge of stem cell biology using various protein-based products (Schuldiner et al. 2000; Jiang et al. 2010). At the turn of the century, with the development of iPSC technology, researchers changed their perspective and directed them towards the use of vector-transported gene fragments (Okita et al. 2007; Suzuki et al. 2012). Consequently, a decade later studies on miRNA-supported iPSC production as well as stem differentiation became popular in the field with the aim of transferring mi-RNAs to the nucleus through the use of viral vectors or chemicals (Giobbe et al. 2015; Li et al. 2015). In fact, it has been also demonstrated that exosomes can trigger differentiation of stem cells without requiring any growth factors or stimulants such as chemokine or cytokine.

More recently, it has been demonstrated that tumor-derived exosomes can also induce differentiation of stem cells into target cells. One such example involves impact of exosomes derived from prostate cancer cells on differentiation of bone-marrow MSCs (BM-MSCs) (Chowdhury et al. 2015).

Stem cells are becoming increasingly therapy-of-choice in bone tissue engineering or regenerative

medicine. However, outcomes these studies are not yet mature enough for human clinical applications. Therefore, the impact of exosomes on stem cell differentiation should be investigated in all aspects in order to expedite use of stem cells as therapeutic agents (Hao et al. 2017).

Raghuvaran Narayanan studied differentiation of human marrow stromal cells (HMSCs) that were incubated with exosomes isolated either from undifferentiated HMSC population or those undergoing osteogenesis to compare the osteogenic potential of stem cell-derived exosomes from both conditions. Regardless of the differentiation status, the expression levels of several genes associated with growth factors such as bone morphogenetic protein 9 (BMP9), transforming growth factor β1 (TGFβ1), transcription factors, and ECM molecules were increased in stem cell-derived exosomes. In addition to *in vitro* studies, researchers conducted *in vivo* research by implanting clinical-grade collagen membranes with or without exosomes together with HMSCs in the back of athymic nude mice (for 4 weeks). The results showed that scaffolds containing exosomes displayed substantial vascularization and calcium phosphate nucleation compared to the control scaffolds lacking exosomes. Accordingly, expression levels of specific proteins related to matrix mineralization, vascularization, and osteogenic differentiation were increased consistently with the physiological differences. Thus, results of both *in vitro* and *in vivo* studies underscore the pivotal role of exosomes in stimulating osteogenic differentiation of HMSCs (Narayanan et al. 2016).

Some transcription factors such as runt-related transcription factor 2 (Runx2) is a positive regulator for the osteogenic differentiation of stem cells into bone cells. Activity of this key transcription factor of osteogenesis can be enhanced by the action of elongating binding protein (C/EBPß) and Smad5, while its activity can be attenuated by the action of another transcription factor such as Twist. Hence, changes in the levels of transcription factors Runx2, Osterix or ß-catenin can have either a negative or positive outcome on osteogenic differentiation of stem cells (Komori 2006; Franceschi et al. 2007). In a comprehensive study Xu et al. were first to report the genetic and proteomic analyses of exosomes originating from BM-MSCs during their differentiation into bone cells. Consistent with the findings of prior studies, expression levels of let-7a, miR-199b, miR-218, miR-148a, miR-135b, miR-203, miR-219, miR-299-5p, and miR-302b were increased, while expression levels of miR-221, miR-155, miR-885-5p, miR-181a, and miR-320c were decreased in exosomes which were isolated on days 5–7 post to the induction of differentiation compared to the profiles of exosomes which were isolated day 0 in this study. Based on the miRNA and mRNA analysis of exosomes harvested at different time points during osteogenesis of bone marrow stem cells (BMSCs) a vital regulatory function of these nanovesicles in differentiation process is evident. By taking advantage of a new web-based computational tool, so called DNA Intelligent Analysis (DIANA) DIANA-miRPath, a number of signature pathways -under miRNA-dependent control- came forth including those related to RNA degradation, the mRNA surveillance, Wnt signalling, and RNA transport which were enriched in differential exosomal miRNA patterns associated with osteogenic differentiation. In the same study, the anti-osteogenic activity of miR-885-5p due to inhibitory impact of its high levels on bone morphogenetic protein 2 (BMP2) via suppression of Runx2 was also detected during osteogenic differentiation of BMSCs. Furthermore, suppressive effect of miR-885-5p on osteogenic differentiation involves Wnt pathway through downregulation of Wnt5a mRNA (Xu et al. 2014). Meanwhile, let7a acts as a positive regulator of osteogenesis by blocking the adipogenic differentiation of human stromal/mesenchymal cells through its influence on high mobility group AT-hook 2 (HMGA2). Likewise, positive regulation of osteogenic differentiation exerted by miR-218 involves Wnt/ß-catenin pathway which has an important role in osteogenesis of adipose-derived stem cells (Zhang et al. 2014).

In another study, Zhang et al. demonstrated that certain signaling pathways play key roles in mediating the pro-osteogenic effects of exosomes

on BMSCs based on the microarray and bioinformatics analyses. One such signaling pathway was phosphatidylinositol 3-kinase (PI3K)-Akt which was involved in MSC proliferation, migration, and osteogenic differentiation. Pharmacological intervention of PI3K/Akt signaling by specific inhibitors resulted in suppression of various early osteogenesis-related marker protein levels, ALP production, and calcium mineral deposition in BMSCs, providing further evidence for the key role PI3K/Akt signaling has in osteogenic differentiation of these cells (Zhang et al. 2016).

Recent attempts to employ iPSC based technology in bone tissue engineering utilized exosomes harvested from human induced pluripotent stem cells that were originally derived from mesenchymal stem cells (hiPS-MSCs-Exos) and β-TCP scaffolds by Zhang et al. These results supported the notion that exosomes play an active role in proliferation, migration, and osteogenic differentiation of BMSCs (Zhang et al. 2016). In a different study, Xin Qi et al. demonstrated that combination of hiPS-MSC with Exos stimulated bone regeneration through improved angiogenesis in addition to osteogenesis in an ovariectomized rat model (Qi et al. 2016).

Takeda and Xu investigated impact of exosomes harvested from differentiating neural cells on differentiation of stem cells. They were able to demonstrate that these exosomes trigger differentiation of MSCs toward neuronal cells (Takeda and Xu 2015). MSCs which are exposed to PC12-derived exosomes, exhibited high expression of neural markers, such as microtubule associated protein 2 (MAP 2), 160 kDa neurofilament, and neuron-specific enolase (NSE), indicating that PC12-derived exosomes could trigger neurogenic differentiation of MSCs. According to the microarray analysis, PC12-derived exosomes have high abundance of miR-125b that regulates some neuronal genes, including diacyl glycerol o-acyl transferase 1 (DGAT1), sphyngosin-1-phosphate-lyase 1 (SGPL1), and TBC1D1 all of which impact neural differentiation (Le et al. 2009). In addition, miR-221 and miR-222 are other examples that are abundantly found in PC-12-derived exosomes. Both play roles in proliferation and migration of Schwann cells as well as in the outgrowth of neurites (Yu et al. 2012).

8 Conclusion

Increased incorporation of stem cell-based therapies in the clinic represents a pivotal change in the regenerative medicine where tissue repair means cure for several disorders involving ischemia/injury, including stroke, cardiovascular diseases, and others. Although stem cells were initially proposed as a safe therapeutic strategy during the booming years of the field, there are still potential unresolved risks and complications encountered in their clinical applications such as culture-induced senescence, immune rejection, genetic instability, failure to home in the site-of-interest, loss of their functional properties, and induction of malignant growths. In this perspective, the remarkable regenerative properties of exosomes offer to become a promising and rescuing alternative for these limitations of stem cells in the clinical applications. Despite their promise as novel and safe cell-free therapeutic opportunities, entry of exosomes in the clinic has to await further investigations that uncover the details of their biology.

Nonetheless, exosomes emerge as invaluable products of MSCs in the execution of reparative functions of these types of stem cells that can be easily harvested from adipose tissue, bone marrow, muscle connective tissue, amniotic fluid, placenta, dental tissue, and placenta. Moreover, several aspects of their biology including longer retainment in the circulation, specific targeting of recipient cells via expression of unique surface marker, and their compatibility with loading of drugs offer a potential to engineer novel approaches. Hence, there is no doubt understanding the precise molecular architecture of exosomes and the details of their crosstalk with epithelial as well as local immune cells will mark the beginning rather than the end-point of designing further alternative exosome-based strategies.

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Mesenchymal Stem Cells as Regulators of Carcinogenesis

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Abstract

Mesenchymal Stem Cells (MSCs) are adult stem cells; isolated from various body parts including bone marrow, adipose tissue and dental tissue, have been characterized well and used in regenerative medicine applications. The promising potential of MSCs makes them great candidates in many disorders. It has been well known in the literature that MSCs interact with cancer cells and regulate the carcinogenesis process at different stages. The dual role of MSCs in cancer progression should be clearly identified at the physiological and molecular level to identify clinical potential in cancer treatment. The promoting or suppressive role of MSCs in cancer is controlled by various growth factors, cytokines and chemokines which affect the cell proliferation, angiogenesis and metastasis. Although many studies have been conducted to explore MSC-cancer cell interactions, it is still unclear how MSCs communicate with cancer cells and tumor microenvironment. Further studies are required to investigate secreted factors and paracrine effects, tumor stroma environment, molecular regulators and downstream pathways that are involved in MSC-cancer interaction loop. MSC type, cancer type and stage specific phenotypic and transcriptomic profile changes should be identified in detail to improve clinical use of MSCs in cancer either as a target or as a tool.

In the current book chapter, we review the literature to summarize current information about the MSC-cancer cell interactions in terms of soluble factors, angiogenesis, metastasis and drug resistance. The role of MSCs in tumor progression or suppression was discussed based on the current literature.

Keywords

Cancer therapy · Carcinogenesis · Mesenchymal stem cells · Metastasis · Paracrine interactions

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Abbreviations

ALL	Acute Lymphoblastic Leukemia
Ang-1	Angiopoietin
ASNS	Asparagine Synthetase
CAFs	Cancer Associated Fibroblasts
CCL2	CC Chemokine Ligand 2
CCL5 or RANTES	Chemokine ligand 5
CCR5	CC Chemokine Receptor 5
CML	Chronic Myeloid Leukemia
CSCs	Cancer Stem Cells

CXCL12	Chemokine Ligand 12
CXCL16	CXC Chemokine Ligand 16
CXCR4	CXC Chemokine Receptor 4
CXCR6	CXC Chemokine Receptor 6
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
ERK	Extracellular Signal-Regulated Kinase
ERK1/2	Extracellular Signal Regulated Kinase 1/2
ET-1	Endothelin-1
EVs	Extracellular Vesicles
HGF	Hepatocyte Growth Factor
IGF-2	Insulin-like Growth Factor-2
IL-6	Interleukin 6
IL-8	Interleukin 8
LOX	Lysyl Oxidase
MAPK	Mitogen-Activated Protein Kinases
MMP-1	Matrix Metalloproteinase-1
MMP9	Matrix Metalloproteinase 9
MMPs	Matrix Metalloproteinases
MSCs	Mesenchymal Stem Cells
NK	Natural Killer
PDGF	Platelet- Derived Growth Factors
Tregs	Regulatory T Cell
VEGF	Vascular Endothelial Growth Factor
α-SMA	α-Smooth Muscle Actin

1 Introduction

Tumors consist of not only malignant cells but also surrounding components including stroma tissue, other regulatory cells, immune components and blood vessels. Interaction of those components can either drive an active tumor state leading to cancer progression and metastasis or repress the tumor growth. One of the important cellular components of the tumor environment is adult mesenchymal stem cells (MSCs) which have a dual role in tumor survival and create a complex interaction network in multiple cancers (Zhang 2008). MSCs as a heterogeneous cell population was first discovered in bone marrow and then identified in multiple tissues in the adult body (Uccelli et al. 2008).

MSCs, also known as mesenchymal stromal cells, are multipotent stem cells dwelling in diverse tissues, including bone marrow, adipose tissue, umbilical cord blood mainly, but also placenta and amniotic fluid (De Ugarte et al. 2003; Erices et al. 2000; Igura et al. 2004; Tsai et al. 2004). Their multi-lineage differentiation potential into osteocyte, adipocyte, chondrocyte, endothelial cell and myocyte, and immunomodulatory capacity make them active players of wound healing (Pittenger et al. 1999; Oswald et al. 2004; Gang et al. 2004; Lee et al. 2016). The similarities between wound healing and tumorigenesis made a reputation for tumors as "wounds that don't heal" (Dvorak 1986) and point out possible contributions of MSCs to tumor progression. Thus, the MSC-related events taking place during regeneration might also happen during tumorigenesis. In this context activation, migration, homing to tumor microenvironment, secretion of chemokines and cytokines and differentiation account some of them.

Besides their role in tissue regeneration, homeostasis and differentiation, MSCs contribute to the tumor microenvironment to modulate cancer cell proliferation and death. Understanding the cross-talk between MSCs and cancer cells has been the aim of interest for the past decade. MSCs contributes to several processes including cancer progression, epithelial to mesenchymal transition (EMT), drug resistance mechanism, angiogenesis and metastasis to other tissues (Papaccio et al. 2017). MSCs residing in the different organs in the adult body recruit to the tumor stroma to maintain direct and indirect interactions, therefore controlling the cancer cells, tumor environment, immune system and

other cellular components (Melzer et al. 2016) All those interactions are regulated by complex molecular cascades that take place in either cancer cells or MSCs. Understanding the possible molecular mechanisms that drive tumor stimulation or suppression is crucial for identification of new therapeutic pathways and controlling cancer. In this book chapter, we will focus on the MSCs-cancer cell interactions by explaining the identified molecular partners published in the literature.

2 Growth Factors and MSC Interactions

Growth factors (GFs) are defined as low molecular polypeptide constructs that are active as regulators of embryonic development, cellular differentiation and proliferation (Goustin et al. 1986). GFs are mediators of interaction between various cells such as cancer cells, myofibroblasts, macrophages and endothelial cells (Witsch et al. 2010). GFs have important roles as promoters of tumor growth through various signal transduction pathways**.** The role of GFs in the cancer progression has gained a lot of importance in recent years in clinical oncology including disease stratification, prognosis, and cancer therapy (Halper 2010). Due to close cross talk between GFs, cancer cells and adult stem cells, evaluation of MSCs and their migration to tumors in terms of GFs and MSCs interaction processes is important. The role of MSCs in carcinogenesis is an interesting concept for the identification of new treatment modalities (Yagi and Kitagawa 2013). The collaborative activity of GFs and MSCs needs to be investigated in detail to understand regulatory mechanisms which control cancer cell proliferation leading to tumor growth and metastasis.

Cancer cell interactions with the surrounding stroma are regulated by the release of pro-inflammatory cytokines and GFs. Tumor structure consists of complex molecules and cells including GFs, immune cells, fibroblasts, extracellular matrix and MSCs (Farahmand et al. 2018). GFs secreted to the tumor environment not only controls cancer cell proliferation but also trigger MSC migration to the tumor area which then initiate proliferation of cancer cells. Following accumulation in tumor areas, MSCs are believed to differentiate into tumor-associated fibroblasts by the microenvironment that secretes trophic factors such as vascular endothelial growth factor (VEGF), interleukin 8 (IL-8), transforming growth factor β (TGF-β), epidermal growth factor (EGF) and platelet-derived growth factors (PDGF) (Nwabo Kamdje et al. 2017).

Bone marrow MSCs have been found to stimulate *in vitro* proliferation, migration and invasion of prostate cancer cell line PC3 through TGF-β pathway. Blocking TGF-β from human bone marrow derived MSC hinders pro-angiogenic function indicating the oncogenic activity of MSC derived TGF β (Ye et al. 2012). A similar study showed that TGF-β immunodepletion in MSC-derived condition media, obtained from human adipose MSCs treated with oncostatin M, reduced adhesion of PC3 cells *in vitro* (Lee et al. 2013b). Under physiological conditions, TGF-β prevents cell cycle progression and induces apoptosis or differentiation. Genetic and epigenetic events during tumor formation may transform TGF-β into a tumor promoter (Witsch et al. 2010). TGF-β as a dual player in cancer may have an inhibitory effect at early stages of carcinogenesis by inhibiting cell proliferation of the tumor progenitors that normally lead to epithelialization in the mesenchymal transition supporting the development of the disease at later stages (Costanza et al. 2017; Ridge et al. 2017).

VEGF is one of the most important growth factor that enhances and directs stem cell motility to the tumor site. VEGF regulates both vasculogenesis and angiogenesis in the tumor environment which is required for cancer cell proliferation and metastasis. Chang et al. reported that VEGF, secreted by transplanted umbilical cord derived MSCs, might be an important paracrine factor that has a critical role in alveolarization, angiogenesis, cell death and inflammatory response (Chang et al. 2014). Bone marrow MSCs exhibit a migratory behavior at high VEGF concentrations and in response to glioma condition medium towards glioma

spheroids (Schichor et al. 2006). Ritter et al. reported that VEGF which has its receptors on MSC surface, induces MSCs migration followed by their secretion from breast cancer cells (Ritter et al. 2008). MSCs have EGF and PDGF receptors on their surface, and blocking PDGF or EGF with antibodies reduces the migration capacity of MSCs (Yagi and Kitagawa 2013).

MSCs produce and secrete a number of paracrine factors such as chemokines, cytokines, and growth factors, thus regulate tumor progression and metastasis in many tumors. Recent studies have shown that MSCs typically facilitate angiogenesis by paracrine secretion of angiogenic growth factors (Lee and Hong 2017). Hypoxic conditions in the tumor area trigger the secretion of many growth factors such as PDGF and VEGF, which are important chemotactic and mitogenic factors for MSCs. Studies in recent years show that MSCs secrete VEGF autonomously and migrate to the tumor area to induce vasculogenesis (Papaccio et al. 2017). In a melanoma model of growth factor induced angiogenesis; adipose MSCs were isolated from mice with melanoma and formed capillary like structures followed by VEGF and bFGF secretion from MSCs (Vartanian et al. 2016).

In addition to growth factor secretion or response to growth factors by cell membrane receptors, MSCs could also contribute to cancer progression via Extracellular vesicles (EVs). Vallabhaneni et al. demonstrated that bone marrow MSCs-derived EVs, contain large amounts of tumor-promoting small RNAs (miRNA-21 and 34a) and approximately 150 different factors, most of which are tumor-bearing proteins such as PDGFR-b TIMP-1 and TIMP-2 (Vallabhaneni et al. 2015). MSCs derived EVs promote tumor growth *in vivo* by increasing VEGF expression and extracellular signal regulated kinase 1/2 (ERK1/2) signaling activity in gastric and colon cancer xenograft models (Zhu et al. 2012). Lee et al. observed that MSCs-EV downregulated VEGF expression in tumor cells leading to inhibition of angiogenesis *in vitro* and *in vivo* in breast cancer (Lee et al. 2013a).

In general, MSCs may modulate the sensitivity of cancer cells to chemotherapeutic agents through the production of certain GFs. Understanding the interactions of GFs and MSCs will be valuable to understand how MSCs might control the different phases of carcinogenesis. It is important to understand molecular mechanisms that are controlled by either GFs secretion from cancerous tissue or MSCs during carcinogenesis.

3 Chemokines, Cytokines and MSC Interactions

Chemokines and cytokines are communication agents regulating cellular responses and behaviors. MSCs are recruited to tumor site and involved in tumorigenesis through autocrine/paracrine signaling and cell-to-cell contact. MSCs show tropism towards the tumor site in response to chemokines, cytokines and other soluble factors secreted from tumor cells (McGrail et al. 2012). The interaction between cancer cells and the stroma (Pietras and Ostman 2010) leads to the release of various chemokines: CXC Chemokine Ligand 8 (Kim et al. 2009), CC Chemokine Ligand 2 (CCL2) (Klopp et al. 2007), CC Chemokine Ligand 15 (Lejmi et al. 2015), CC Chemokine Ligand 20 (Lejmi et al. 2015) and CC Chemokine Ligand 25 (Xu et al. 2012), cytokines: TGFβ (Klopp et al. 2007), Hepatocyte Growth Factor (HGF) (Lin et al. 2008), VEGF (Klopp et al. 2007), neurotrophin-3 (Birnbaum et al. 2007) and platelet-derived growth factor BB (Klopp et al. 2007), and some soluble factors: Cyclophilin B (Lin et al. 2008), urokinase plasminogen activator (Gutova et al. 2008) and leucine-leucine 37 (Coffelt et al. 2009). Those factors are released into the extracellular environment or packed in extracellular vesicles (EVs), such as exosomes, for delivery to MSCs, bearing the corresponding surface receptors, to attract MSCs to the tumor stroma (Hall et al. 2007; Hogan et al. 2012).

One of the recent research has proposed that CXC Chemokine Ligand 16 (CXCL16) secreted by cancer cells is the main reason for tumor tropism of MSCs (Jung et al. 2013). CXCL16, which is a chemoattractant chemokine found in soluble form or as a transmembrane receptor on

the cell surface (Deng et al. 2010), is over-expressed in prostate cancer cells compared to healthy prostate epithelial cells in response to tumor necrosis factor α and interferon ɣ (Darash-Yahana et al. 2009; Wang et al. 2008a; Lu et al. 2008). According to the report, CXCL16, expressed by prostate cancer cells, not only recruited MSCs to tumor microenvironment but also promoted the conversion of MSCs to their activated state, known as Cancer Associated Fibroblasts (CAFs). Additionally, the downstream of CXCL16/CXCR6 signaling was also reported to regulate invasive and metastatic characteristics of cancer. CXCL16 was found to enhance expression of CXC Chemokine Ligand 12 (CXCL12) in CAFs and thus lead to EMT conversion of cancer cells, eventually supporting tumor growth and metastasis to secondary sites. Another process affected by CXCL16/CXCR6 signaling is tumor vascularization which is controlled by the expression of some pro-angiogenic factors such as VEGF and IL-8. Although CXCL16/CXCR6 signaling primarily promotes the recruitment of MSCs to tumor stroma, the downstream regulatory pathways manintain the activation of MSCs, invasion, metastasis and angiogenesis processes (Jung et al. 2013; Papaccio et al. 2017).

Another mechanism claimed to control migration of MSCs to tumor stroma is MMP-1/PAR-1 dependent activation of CXCL12/CXCR4 signaling (Ho et al. 2014). Since CXCL12/CXCR4 cascade is crucial for homing and trafficking in hematopoietic stem cells in bone marrow (Dar et al. 2006), the mechanism attracted interest in MSCs tropism to tumor stroma. Matrix metalloproteinase-1 (MMP-1), as a matrix remodeling protein, is expressed in MSCs in response to cytokines such as interleukin 1 beta (Chen et al. 2018). MMP-1 activates its receptor, PAR-1, by cleaving it at the N-terminal part and the activated PAR-1 leads to activation of several downstream signaling pathways including CXCL12 through interacting intramolecular receptors. Interaction of CXCL12 with its receptor CXCR4 initiates a conformational change of the receptor, which in turn leads to exchange of guanosine diphosphate with guanosine triphosphate and so the activation of downstream pathways including PI3K. These complex signaling pathways ultimately lead to reorganization of cytoskeleton and migration of cells (Chen et al. 2018; Ho et al. 2014; Ho et al. 2009). Insulin-like growth factor-1 and insulin-like growth factor-2 (IGF1/IGF-2) are growth factors regulating MSC migration (Fiedler et al. 2006). IGF-binding proteins form a complex with IGFs and prevent their binding to corresponding receptors (Bunn and Fowlkes 2003; Firth and Baxter 2002). Although insulin-like growth factor-1 was known to enhance MSC migration in a CXCR-4 dependent manner, underlying mechanism for IGF-2 control on MSC migration was unknown until recently (Huang et al. 2012; Li et al. 2007). A recent study has demonstrated that endogenously produced MMP-1 in MSCs as a protease cleaved IGF-2/IGFBP2 complex for releasing free IGF-2, which binds to its receptor IGF-2R on MSC surface and trigger MSC migration (Guan et al. 2018). Once MSCs migrates to the stroma, the tumor microenvironment and fusion of MSCs with cancer cells cause transdifferentiation of MSCs into CAFs, which are fibroblast-like cells characterized by expression of α-Smooth muscle actin (α-SMA), fibroblast-specific protein 1, and fibroblast activation protein (Rasanen and Vaheri 2010) and promote tumor progression by secreting inflammatory cytokines such as interleukins and CXC chemokine ligands and proteinases such as Matrix metalloproteinases (MMPs) (Spaeth et al. 2009). MSCs and CAFs have many similarities such as common surface markers including CD29, CD44, CD73, CD90, CD106, CD117 and differentiation capacity to osteocyte, chondrocyte and adipocyte. CAFs also show some differences including up-regulation of some cytokines levels such as IL4, IL6, IL10,VEGF, TGFβ and tumor necrosis factor α. High IL6 expression by CAFs increased proliferation of CAFs in an autocrine manner (Paunescu et al. 2011; Spaeth et al. 2009). Due to these differences and similarities it has been suggested that MSCs, as the fibroblasts at the resting state, are activated by stimulations from tumor microenvironment and this active state is called CAFs (Kalluri 2016). Although the

mechanism has not been completely revealed, evidences indicated the role of TGFβ at the differentiation of MSCs into CAFs in tumor microenvironment. TGFβ, as an over-expressed factor in cancers, seems to act as a cue to trigger MSCs for CAFs transformation through Smad-dependent pathway (Shangguan et al. 2012; Peng et al. 2013). The pivotal mechanism claimed for the differentiation MSCs to CAFs is CXCL16/CXCR6 signaling. CXCL16, as the unique ligand of CXC Chemokine Receptor 6 (CXCR6), is expressed by MSCs and abundantly in cancers. Interaction of CXCL16 with CXCR6 receptor induces AKT/mTOR pathway and triggers the transformation of MSCs to CAFs. In addition, this differentiation causes increased expression of CXCL12 on CAFs and in turn promotes metastasis by stimulating EMT of cancer cells (Jung et al. 2013; Wang et al. 2008a; Papaccio et al. 2017).

The invasive character of cancer cells is also induced by MSCs activity at the primary tumor site. There are evidences indicating that MSCs in the tumor microenvironment drive tumor cells to metastatic state via EMT, the process in which cancer cells acquire metastatic characteristics through loss of cell-to-cell contact and gain of mesenchymal properties (Floor et al. 2011). MSCs in tumor stroma secrete high level of TGFβ in response to tumor necrosis factor α and interferon ɣ which help cancer cells to gain EMT characteristics, such as migration by leading to down-regulation of E-cadherin and up-regulation of N-cadherin, Vimentin, Snail and Twist (Xue et al. 2015; Martin et al. 2010; Jing et al. 2012).

Chemokine ligand 5 (CCL5 or RANTES) secretion by MSCs was claimed to involve in breast cancer cell and osteosarcoma cell metastasis (Karnoub et al. 2007; Mi et al. 2011; Xu et al. 2009). Osteopontin (OPN), which is an adhesive protein binding to integrins, is secreted by tumor cells and stimulates MSCs for production and secretion of CCL5, which in turn binds to CC Chemokine Receptor 5 (CCR5) on cancer cell (Karnoub et al. 2007; Mi et al. 2011; Ridge et al. 2017). This CCL5/CCR5 axis enhanced expression of matrix Metalloproteinase 9 (MMP9), zinc finger E-box-binding homeobox 1 and CXC chemokine receptor 4 (CXCR4) in cancer cells and resulted in increased metastasis (Ridge et al. 2017; Zhong et al. 2017).

CXCR4 expression in cancer cell and its interaction with CXCL12 induce metastasis. CXCL16, secreted by prostate cancer cells, induce expression of CXCL12, also known as stromal cell-derived factor, in CAFs via Extracellular Signal-Regulated Kinase (ERK) and nuclear factor kappa b (NFκB) signaling. Secretion of CXCL12 to tumor microenvironment triggers EMT conversion of the cancer cells and enhances the expression of its receptor, CXCR4, on cancer cells, which ultimately result in metastasis to secondary sites (Papaccio et al. 2017; Jung et al. 2013).

Origin of the cell is also a determinant of MSCs contribution to cancer metastasis. Some researches state differences at morphology, behavior and differentiation capacity between Adipose-derived MSCs originated from subcutaneous fat and visceral fat. While subcutaneous AD-MSC are favoring to home to cancer cells, visceral adipose-derived MSCs, possessing higher proliferation and differentiation capacity, make cell-to-cell contact and release IL-6 and IL-8 to promote EMT via activation of PI3K/AKT pathway, increase migration capacity and enhance invasiveness of breast cells (Ritter et al. 2015; Papaccio et al. 2017).

Another important issue related to activity of chemokine-cytokine based MSCs-cancer cell interaction is the generation of Cancer Stem Cells (CSCs). CSCs, also called as tumor-initiating cells, are cancer cells carrying characteristics of stem cells, including self-renewal and differentiation into tumor constituting cells (Yu et al. 2012). Although origin of CSCs and the mechanism behind the CSCs formation is not well understood, some evidences indicates the role of MSC-induced EMT as the mechanism for the formation of CSC niche (Ansieau 2013; Biddle and Mackenzie 2012). These evidences indicates that IL-1 secreted by cancer cells causes up-regulation at the expression of cyclooxygenase-2 and microsomal prostaglandin-E synthase-1, which leads to Prostaglandin E_2 (PGE_2) biosynthesis from

arachidonic acid in MSCs. PGE_2 functions both in autocrine manner, in collaboration with IL-1 from cancer cells to induce expression of IL-6, IL-8 and GRO-α in MSCs, and in paracrine manner to trigger EMT of cancer cells. The cytokines, released by MSCs in response to PGE2 induction, trigger the activation of β-catenin signaling in cancer cells (Alcolea et al. 2012). The paracrine acting PGE_2 induces EMT by causing down-regulation of E-cadherin, which in turn sets free β-catenins attached to adherent junctions. β-catenin, then, translocates into nucleus and up-regulates the expression of its target genes including genes regulating pluripotency such as Oct4, Sox2 and Nanog. This way, the stemness properties of the cells increase and CSCs are formed (Li et al. 2012).

Angiogenesis, which is the process of new blood vessel formation from the preexisting vessels (Alcayaga-Miranda et al. 2016), is an irreplaceable element of tumor growth and metastasis and this hallmark of cancer is also regulated by MSCs and chemokine-cytokine interactions. Growth factors such as VEGF and EGF, and cytokines such as TGFβ1 secreted by MSCs contribute to vascularization of tumors (Bellone et al. 2010; Li et al. 2010; Kinnaird et al. 2004). In addition, endothelial cells receiving MSCs-derived extracellular vesicles, carrying signal transducer and activator of transcription 3 and proteins associated NFκB, starts to express pro-angiogenic proteins (Anderson et al. 2016; Shabbir et al. 2015). CXCL16/CXCR6 signaling has also been reported to enhance tumor vascularization by inducing the expression of pro-angiogenic factors, VEGF and IL-8, at the down-stream (Jung et al. 2013; Papaccio et al. 2017).

One of the well-known mechanisms for MSC-mediated angiogenesis involves IL6 secreted from MSCs. Released IL6 up-regulates endothelin-1 (ET-1) secretion in cancer cells which is a mitogenic polypeptide, leads to activation of AKT and ERK in endothelial cells and eventually cause angiogenesis (Lazennec and Lam 2016; Huang et al. 2013). Cancer cells were also reported to secrete pro-angiogenic factors, such as VEGF, in response to stimulation of MSCs and their exosomes. VEGF leads to activation of ERK1/2 pathway and promotes tumor growth (Zhu et al. 2012).

MSCs are recruited to tumor site by cancer cell induction and regulate cancer progression at multiple stages through autocrine and paracrine signaling mechanisms Despite researchers have made a great progress to understand the molecular pathways and MSC interactions with cytokines and chemokines, the complexity of signaling mechanisms needs to be revealed completely. Understanding the underlying molecular mechanism that controls the production and secretion of chemokines-cytokines and their interaction with MSCs is crucial for future therapies.

4 Angiogenesis and MSCs Interaction

MSCs have the ability to trigger angiogenesis which is one of the most well-known and major hallmarks of cancer. Angiogenesis has various sub-steps including remodeling of pre-existing vessels, vascular mimicry, recruitment and differentiation of bone marrow endothelial precursors (Junttila and de Sauvage 2013). MSCs control the microenvironment to modulate the vascularization by secretion of angiogenic factors such as VEGF, angiopoietin (Ang-1) and IL-6 (Spaeth et al. 2009). A previous study demonstrated that MSCs could differentiate into pericytes and endothelial cells to support colorectal cancer angiogenesis and vascularization by producing VEGF and Ang-1 (Papaccio et al. 2017). Moreover, secretion of pro-angiogenic factors' such as IL-6 and Ang-1 triggered the activation of Akt and ERK pathways in endothelial cells leading to vascularization (Mao et al. 2013). Besides, adipose-derived MSCs and endothelial cells collaborate in the development of mature vessels in breast cancer. Ang-1 secreted from MSCs, bound to corresponding endothelial receptors Tie1 and Tie2 which initiates the cascade that activate Akt and ERK (Orecchioni et al. 2013). Differentiation of MSCs into pericytes can also be upregulated by CXCL12 and PDGF-B secretions from cancer cells. It has been shown that radiation therapy

dramatically increases CXCL12 and PDGF-B secretions from cancer cells. Along with the increased angiogenic capability, these soluble factors initiated MSCs migration to tumor site for modifying the tumor environment as discussed in the metastasis part later (Wang et al. 2016). Targeting the CXCL12 and/or PDGF-B might be vital for overcoming the limitations of radiotherapy. It has been reported that, secreted angiogenic factors from glioblastoma, induced glioblastoma oriented MSCs to differentiate into pericytes. MSCs formed tube-like structures on matrigel when cultured in glioblastoma-derived angiogenic factors enriched medium which is an indicator of vascularization *in vitro* (Yi et al. 2018). A recent study on multiple myeloma and MSCs demonstrated that, co-culturing these cells increases the expression of α-SMA protein through the activation of Integrin-linked kinase pathway. Enhanced α-SMA expression can be considered as a marker to CAF differentiation (Zhao et al. 2018). MSCs are crucial regulators of angiogenesis by differentiating into smooth muscle cells and pericytes that are surrounding blood vessels and correlated with vascularization and the vessel maturity. Additionally, VEGF has an autocrine property to increase the synthesis of other angiogenic factors like Ang-1, IL6, IL8 from coronary microvascular endothelial cells (Zheng et al. 2001). Moreover, MSCs have been demonstrated for their paracrine activity through the secretion of VEGF, PDGF, bFGF, Ang-1, IL6, IL-8, TGF- β and FGF-7 to promote tumorigenic angiogenesis (Feng and Chen 2009) by initiating new vessel formation. It has been reported in a previous study, VEGF secretion from MSCs promotes HUVEC (Human umbilical vein endothelial cell) proliferation *in vitro* indicating the potential angiogenic role (Beckermann et al. 2008). Additionally, transforming growth factor alpha is one of the most vital factor of tissue repair, also a key modulator of epithelial malignant transformation. Transforming growth factor alpha modulates epithelial malignant transformation by up-regulating type I collagen expression and production of VEGF in MSCs via mitogen-activated protein kinases (MAPK) and PI3K pathway activation, independent from JNK and ERK (Wang et al. 2008b). Furthermore, exosomes derived from MSCs can trigger VEGF secretion from cancer cells which leads increased tumor proliferation by ERK 1/2 pathway in addition to vessel promoting activity (Zhu et al. 2012). Similarly, IL6 secretion from MSCs promoted endothelial cell-derived ET-1 production and release from cancer cells (Huang et al. 2013) which acts a mitogenic factor on endothelial cells, vascular smooth muscle cells and cancer cells. Huang et al. demonstrated that targeting ERK or IL6/ET-1/Akt pathway in the tumor-stroma environment is efficient for inhibition of tumor growth (Huang et al. 2013). Surprisingly, in another study from the same group showed that, IL6 secretion from MSCs triggered cancer cell marker expression in non-cancer stem cells, therefore increased tumor formation *in vivo* (Tsai et al. 2011). These findings suggest that, secretion of angiogenic factors from MSCs not only promotes angiogenesis by targeting endothelial cells, but also contributes to promotion of angiogenesis by communication with cancer cells.

MSCs possess an anti-angiogenic activity in some situations by controlling paracrine pathways that inhibits endothelial progenitor cell movement and differentiation (Ho et al. 2013). Additionally, restriction of tumor growth by inhibition of pro-angiogenic factors was also observed. MSCs can be described as the natural regulators of angiogenesis through multiple regulatory mechanisms. They either increase angiogenesis if the surrounding tissue is carcinogenic or decrease the angiogenesis based on environment stimulation. It has been shown that, low-dose irradiation of MSCs in GL261 glioblastoma cell line, changes MSCs behavior against angiogenesis. Decreased angiogenic capacity was observed after low-dose irradiation due to decrease in active TGFβ1 (Stefani et al. 2018) which appears to be potential therapeutic target in the battle against cancer.

Further research is required to understand autocrine and paracrine interactions of MSCs and cancer cells individually or together which might enable to solve underlying molecular mechanisms and lead determination of new targets in the clinics.

5 The Role of MSCs in Metastasis

Role of MSCs has been shown in various type of cancers including pancreas (Kallifatidis et al. 2008), colon (Hung et al. 2005; Menon et al. 2007), breast cancer (Chen et al. 2008; Klopp et al. 2007; Dwyer et al. 2007) as well as gliomas (Nakamizo et al. 2005) and melanomas (Studeny et al. 2002; Chen et al. 2008). Most of the observations in those cancers indicate that MSCs might have a regulatory role in cancer metastasis to the other body parts (Joyce and Pollard 2009). As MSCs have the ability to migrate to tumor environment and increase tumor's aggressiveness, there should be certain molecular pathways which control adult stem cell and cancer cell interactions via direct cell-cell contact or paracrine activities. MSCs do not only migrate into tumor tissue but also differentiate into CAFs to improve tumorigenic growth and aggressiveness (Shinagawa et al. 2010). Differentiation into CAF has been verified by the expression of markers such as alpha-actin-2, α-SMA and platelet-derived growth factor receptor-b and is one of the possible mechanisms that contributes to cancer metastasis. Although a-SMA expression was not observed, minor PDGFR-B expression was detected on cultured MSCs. Studies have shown that in prostate cancer cell line, CAF differentiation is correlated with TGF-β expression (Barcellos-de-Souza et al. 2016). Overall, transformation into CAF phenotype through alterations in alpha-actin-2, α-SMA, platelet-derived growth factor receptor-b, desmin, TGF-β, fibroblast activation protein and fibroblast secretory protein expressions followed by vital changes in cancer cells, including MAPK and Wnt pathways, results in enhanced cancer cell metastasis to surrounding tissue and finally to other body parts.

Co-culture strategies that enable the investigation of adult stem cell-cancer cell interactions have been used to study the activity of soluble factors including chemokines. Karnoub et al. were able to enhance metastatic potency of breast cancer cells with poor metastatic properties by using a co-culture system in which they mixed human breast cancer cells with bone marrow derived MSCs (Karnoub et al. 2007). CCL5 was found to be secreted from MSCs by breast cancer stimulation through chemokine receptor CCR5. CCR5 receptor could initiate downstream pathways such as PI3K/Akt thereby IKKalpha/beta and NF-κB that are required for cell migration (Huang et al. 2009). Eventually, CCL5 reversibly increases breast cancer's metastasis and invasion capacity as well as motility. CCL5 could be effective on cancer cell metastasis via participation of other secreted factors including VEGF or IL-8 and other molecular pathways such as PI3K/Akt. Even if, CCL5 overexpression had no significant effect on the tumor growth, it was shown that metastatic potential was enhanced by activated (Ser 473-phosphorylated) AKT. However, anti-apoptotic (BAX or BAD) or pro-apoptotic protein (Bcl-Xl or Bcl-2) levels did not change (Karnoub et al. 2007). It was also shown that CCL5 knockdown of MSCs via shRNA, could not be able to promote migration of breast cancer cell line MDA-MB-435 (Karnoub et al. 2007). In the light of this information, CCL5-CCR5 connection seems to be a crucial target for metastatic cancers' treatment and CCR5 antagonists and CCL5 analogues are also currently in use. MSCs co-cultured with human colon cancer cell line KM12-SM expressed fibroblast activation protein, fibroblast secretory protein, desmin, PDGFR-b and a-SMA indicating the acquired CAF phenotype after cancer cell interaction. Moreover, liver metastasis has been detected only in one experimental group that was co-culture group for colon cancer cell lines and MSCs (Shinagawa et al. 2010).

One of the reasons leading to MSC movement to tumor area is hypoxic conditions which is eventually result of tumor growth. When hypoxic conditions are combined with adjuvants like interleukin 6 (IL6), CCL2 and/or growth factors such as PDGF, vascular endothelial growth factor A and insulin-like growth factor 1, MSCs migration to the tumor area is promoted. Additionally, several factors such as IL1β, which is upregulated by focal adhesion kinase and MAPK pathways and downregulated by TGFβ signaling pathway, are secreted from prostate, breast, colon, lung and

head or neck cancers and have any pleiotropic effects on MSCs (Al-toub et al. 2013). Those factors modulate MSCs via focal adhesion kinase, MAPK and TGFβ pathways by transforming the MSCs into pro-inflammatory cells in cancer stroma. Activation of V-CAM on the surface of MSCs, by TNF-α and interleukin 1 beta which are secreted from tumor cells, promotes the decrease of MSCs recruitment towards tumor bed when hypoxic pathways are active (Uchibori et al. 2013). Cancer cells express high amounts of hypoxia-inducible factors as a reaction to hypoxic conditions. HIF-1α, together with membrane type 1 matrix metalloprotease, is responsible for activation of 3BP2 which mediates oncogenic migration of MSCs (Proulx-Bonneau et al. 2011). A study in 2014 indicates that, hypoxia-inducible factors are responsible for mediating the connection and collaboration between MSCs and breast cancer cells, as a result of high HIF expression in MSCs and metastatic tendency in breast cancer cells. This communication between MSCs and breast cancer cell lines is controlled by CCL5 and chemokine ligand 10 secretions from MSCs. CCL5 and chemokine ligand 10 interact with CCR5/CD44 and CXCR5-CXCR3 respectively on tumor cells to initiate metastatic process (Chaturvedi et al. 2014). Furthermore, Chaturvedi and colleagues monitored colony stimulating factor 1 secretion from cancer cells which stimulates recruitment of MSCs to the tumor and placenta-derived growth factor secreation that increases the expression of VEGFR1 and triggers chemokine ligand 10 expression in MSCs which gives rise to HIF dependent CXCR3 expression on breast cancer cells (Chaturvedi et al. 2013). Promotion of metastasis and mobility via MSC-derived factors such as IL-17B and IL-6 (Chaturvedi et al. 2013) are responsible for stimulation of ERK1/2 pathway for activating CC Chemokine Ligand 2, IL-8, CXCL1 and trefoil factor 1 (Bie et al. 2017). Recently, Du et al. observed that in osteosarcoma, IL-8/CXCR1/Akt pathway which is regulated by MSCs secretions is the key pathway for avoiding anoikis. CXCR1 was defined as a crucial target for regulating pulmonary metastasis of osteosarcoma cells (Du et al. 2018). Another study on gastric cancer displayed that IL15 which is secreted from MSCs increase metastatic properties of gastric cancer cells. Both serum and tissue samples collected from patients had significantly higher amount of IL15 compared with healthy donors. Regulatory T cell (Tregs) ratio was increased by pleiotropic cytokine, IL15 and caused advanced metastasis. Programmed cell death protein-1 also upregulated in CD4 + T cells as a result of IL15 activity to support tumor metastasis (Sun et al. 2018). Furthermore, release of signaling proteins such as Gremlin1 (Hong et al. 2018), β2-Microglobulin (Wang et al. 2018) promoted EMT which support invasion and metastasis (Ishikawa et al. 2014).

MSCs promote cancer cells to synthesize LOX (lysyl oxidase) (El-Haibi et al. 2012) which is known as a tumor suppresser protein. A study in nasopharynged carcinoma indicated that knocking down LOX gene ends up with tumor growth and *vice versa* (Sung et al. 2014). On the other hand, activation of LOX triggers Twist transcription which promotes EMT initiation *via* stimulation of ZEB transcription factors. Additionally, miR10b activation by LOX protein has been strongly interacted with metastasis (Park et al. 2008; Gregory et al. 2008; Ventura and Jacks 2009).

miRNA activation and secretion from MSCs plays crucial role in tumor growth and metastasis. Activation of miR-155 by S100A4 (secreted by MSCs), activates SOCS1, signal transducer and activator of transcription, and eventually MMP9. Activated MMP9 interacts with CD44v6 to promote degradation of extracellular matrix members (Xu et al. 2003; Yan et al. 2013) leading to metastasis. Furthermore, miR-221 cargo of MSCs derived exosomes were also correlated with enhanced metastasis in gastric carcinoma, but the exact mechanism is unclear (Ma et al. 2017).

MSC-derived EVs also have an effect on cancer cells metastasis. A previous study on BALB/c nu/nu mice xenograft model, indicates that MSC-derived EVs could give rise to tumor growth via ERK1/2 and p38 MAPK pathway activation which increases VEGF expression (Ji et al. 2015). However, *in vitro* studies showed that EVs do not exert any significant change in percentages of cell cycle levels (G0/G1, S and

G2/M) or cell proliferation rate of cancer cells. Therefore, MSC-derived EVs might only support tumor environment to enhance tumorigenic growth, not the tumor cell proliferation. On the other hand, bone marrow stem cell-derived EVs were studied on multiple myeloma by Wang and his co-workers. p53, p38, Akt and c-Jun pathways were affected and increased survival capacity was observedin response to EVs (Wang et al. 2014). EVs could have a nucleic acid and protein content leading to communication with other cells. RNA content of Wharton's jelly of umbilical cord MSC derived EVs induced HGF expression in renal cell carcinoma. HGF is responsible for activation of Akt and ERK1/2 pathways that enables G0/G1 to S transition (Du et al. 2014). More surprisingly same scientists have shown that wharton's jelly of umbilical cord MSC derived EVs decreased proliferation and increased apoptosis on bladder cancer cells via upregulation of p-p53, activation of Akt and caspase cascade (Wu et al. 2013).

As MSCs have dual effect on the progression of carcinomas, it is crucial to understand inhibitory role of MSCs on tumor growth. The negative role of MSCs on tumor growth is controlled by glycogen synthase kinase-3β pathway as well as tissue inhibitors of metalloproteinase 1 and tissue inhibitors of metalloproteinase 2 pathways. A study in China discovered that co-expression of interleukin 18 and interferon β by BMSCs can inhibit the glioma cell growth (Xu et al. 2015) by modulating the glycogen synthase kinase-3β molecular pathway and thus inhibits cancer cell metastasis and triggers apoptosis. Several studies demonstrated increased tissue inhibitors of metalloproteinase 1and tissue inhibitors of metalloproteinase 2 expression by MSCs in tumor stroma (Kuvaja et al. 2012; Ries et al. 2007). Tissue inhibitors of metalloproteinase 1-2 expression enhance over time as MMP1, MMP2 and MMP9 ratio due to negative feedback mechanism. Observing the MSCs and breast cancer cell line interaction in a time dependent manner (24–120 h), MMPs have been shown to increase primarily. Subsequently, as a result of MMPs increase, TIMPs were secreted into tumor stroma in order to regulate MMPs' migratory effect (Clarke et al. 2015).

MSCs might be considered as effective players on cancer metastasis in a positive and negative way since they have capability to regulate cancer stroma, cell-cell interactions and thus invasion and metastasis.

6 The Role of MSCs in Drug Resistance

MSCs might be involved in acquired resistance in cancer cells in many cancer types against chemotherapeutic drugs. Resistance to chemotherapeutics impairs the success in clinics by creating a different cancer cell phenotype that is more proliferative, more invasive and stronger against various therapies. Understanding the MSC induced drug resistance in different cancer types might enable the identification of molecular regulation and new targets for therapy. General drug resistance acquisition is conducted by several mechanisms including multi-drug resistance, inhibition of apoptosis, modifying the drug metabolism and drug target, epigenetic changes of the targeted cell, improving DNA repair and amplification mechanisms (Mansoori et al. 2017). The role of MSCs in cancer drug resistance is related to several molecular pathways and downstreat mechanisms. We will try to focus on recent advances about MSC induced drug resistance in cancer in this part of the chapter.

Leukemia is one of the well-known cancer type that has been studied to identify activity of MSCs in drug resistance. Physiologically, bone marrow derived MSCs support the self-renewal and differentiation of hematopoietic stem cells. Malignant hematopoietic cells residing at close areas to MSCs, are affected by the mitogenic contribution of MSCs, and acquire resistance against therapeutic drugs. Lack of ASNS (asparagine synthetase) in acute lymphoblastic leukemia (ALL) generates a more sensitive phenotype to asparagine depletion by therapeutically delivered asparaginase. Nonetheless, high amount of ASNS was observed in bone marrow derived MSCs of ALL patients, to prevent ALL cells against asparaginase cytotoxicity. RNAi based

knockdown of ASNS translation in MSCs, caused a decrease in ALL protection by MSCs while overexpression of ASNS in MSCs ends up with highly protected ALL cells (Iwamoto et al. 2007). Furthermore, chronic myeloid leukemia (CML) cells have been studied to understand drug resistance effects of MSCs more clearly. CML gained resistance to imatinib therapy, which is a specific inhibitor of numerous tyrosine kinase enzymes including abelson proto-oncogene, by the help of MSCs. It has been shown that MSCs are responsible for decreased caspase-3 activation as well as increased Bcl-xl levels via CXCL12/CXCR4 axis. Increased CXCR4 levels in MSCs overcomes apoptotic effect of the imatinib on CML and it has been also proven that AMD3100 (a CXCR4 antagonist) treatment decreases and even restores apoptosis in CML (Vianello et al. 2010). Furthermore, it has been shown that, co-culturing CML cells with MSCs increases imatinib resistance of CML cells. CXCR4 activation in CML cells by stromal cell-derived factor secretion from MSCs, initiates a cascade which leads to activation of various anti-apoptotic proteins such as ERK, AKT and Nf-Kb resulting in resistance to imatinib (Vianello et al. 2010; Jin et al. 2008). Besides, a study on chronic lymphocytic leukemia revealed that, resistance to forodesine (a transition-state analog inhibitor of purine nucleoside phosphorylase) is caused by MSCs which reduce the forodesine-induced ATP depletion and 2′- deoxyguasine triphosphate accumulation. MSCs cells have been demonstrated to be responsible for reduction in inhibitory effect of forodesine on RNA and protein synthesis. Induced myeloid leukemia cell differentiation protein, which is one of the main proteins in inhibition of the intrinsic pathway of apoptosis, was observed to be stabilized in chronic lymphocytic leukemia cells by MSCs (Balakrishnan et al. 2010). In addition hemotologic malignancies, the role of MSCs on acquired drug resistance has also been studied in ovarian carcinoma. It has been reported that hyperthermic intraperitoneal chemotherapy is becoming a promising treatment method for ovarian carcinoma which focuses on replacement of tumors after surgery. After removal of the tumor tissue by surgery, pre-heated chemotherapeutical agent against ovarian carcinoma is delivered to the abdomen directly to target rest of cancer cells that may still exist in the ovarian tissue (van Driel et al. 2018). Notwithstanding the bright future of the hyperthermic intraperitoneal chemotherapy treatment, CXCL12 secretion from bone marrow derived tumor-associated MSCs was shown to generate thermotolerance in ovarian cancer cells through CXCR4 pathway (Lis et al. 2011). As discussed in metastasis chapter earlier, MSCs increase the IL6 levels in tumor environment. Huitao Xu et al. demonstrated that increased IL6 secretion of MSCs caused enriched cisplatin resistance on breast cancer. MSCs derived IL6 activated JNK/STAT pathway and started a cascade that lead to activation of anti-apoptotic proteins, Bcl-2 and Bcl-xl. Activation of signal transducer and activator of transcription pathway enhanced anti-apoptotic proteins and reduced mitochondrial membrane cleavage suggesting the potential of IL6 as a target to overcome cisplatin resistance in breast cancer (Xu et al. 2018).

MSC-derived exosomes have been reported as regulators of MSCs based drug resistance mechanisms in previous studies. A study on gastric carcinoma demonstrated that MSC-derived exosomes triggered Raf/MEK/ERK cascade and Calcium/calmodulin-dependent protein kinases to block 5-flourouracil's effect by increasing the proliferation of cells. Multi-drug resistance correlated proteins, such as multidrug resistance-associated protein and lung resistance-related protein, were also increased by MSC-derived exosomes (Ji et al. 2015). Similarly, mir-9 is also observed to be transferred from MSC to glioblastoma cells to induce temozolomide resistance (Munoz et al. 2013). Both mir-222/223 and mir-9 experiments showed that, receiving miRNAs thorough gap junctional intercellular communication via connexin 43 protein gives rise multidrug resistance protein 1 (also known as to P-glycoprotein 1). Upregulation of MDR1 and P-gp caused efflux of temozolomide outside of the cancer cells leading to resistance.

7 Conclusion

MSCs are derived from various sources in the adult body and widely used in regeneative medicine applications because of their differentiation capacisty and therapeutic role. In addition to their potential in tissue repair in many organs and diseases, they are involved in the carcinogenesis process in terms od cancer initiation, proliferation, metastasis and progression. MSCs have a dual role in cancer pathogenesis by supporting the tumor growth or stimulating the apoptotic pathways.

Identification the regulatory pathways of cancer in which MSCs are involved would be valuable for further understanding the clinical potential of MSCs in cancer treatment. Investigation of MSCs as a target for cancer treatment or using MSCs to target cancer cells could only be possible by analyzing the control mechanisms. It is crucial to understand how MSCs and cancer cells interact to promote or suppress tumor growth. Although a large amount of basic research has been conducted to understand the role of MSCs and MSC-derived factors in different cancer stages, further studies are required. Detailed researches at the molecular level would enable investigation of MSC-cancer relationship and generation of gene and stem cell based clinical therapy tools.

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