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Kursad Turksen *Editor*

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Stem Cells, Bio-materials and Tissue Engineering

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Preface

The diverse developmental potential of stem cells has been recognized for several decades. However, clinically relevant approaches for placing stem cells in compromised and even hostile environments, while maintaining their ability to express their inherent potential to achieve repair and regeneration, are still challenging. Advanced biomaterials and multifaceted tissue engineering methods are increasingly coming into play. The identification of appropriate cells and, in many cases, other required biological and chemical mediators, along with the optimal biomaterials for their encapsulation and delivery to stimulate regenerative processes, continues to be explored and developed. With the multiplicity of challenges and advances occurring in this very active field, I have recruited several experts in the area to provide summaries of their ongoing research studies.

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, ON, Canada — Kursad Turksen

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Definitive Erythropoiesis from Pluripotent Stem Cells: Recent Advances and Perspectives

Selami Demirci and John F. Tisdale

Abstract

Derivation of functional and mature red blood cells (RBCs) with adult globin expression from renewable source such as induced pluripotent stem cells (iPSCs) is of importance from the clinical point of view. Definitive RBC generation can only be succeeded through production of true hematopoietic stem cells (HSCs). There has been a great effort to obtain definitive engraftable HSCs from iPSCs but the results were mostly unsatisfactory due to low, short-term and linage-biased engraftment in mouse models. Moreover, ex vivo differentiation approaches ended up with RBCs with mostly embryonic and fetal globin expression. To establish reliable, standardized and effective laboratory protocols, we need to expand our knowledge about developmental hematopoiesis/erythropoiesis and identify critical regulatory signaling pathways and transcription factors. Once we meet these challenges, we could establish differentiation protocols for massive RBC production for transfusion purposes in the clinical setting, performing drug screening and disease modeling in ex vivo conditions, and investigating the embryological cascade of erythropoiesis. More interestingly, with the introduction of relatively efficient and facile genome editing tools, genetic correction for inherited RBC disorders such as sickle cell disease (SCD) would become possible through iPSCs that can subsequently generate definitive HSCs, which then give rise to definitive RBCs producing β-globin after transplantation.

Keywords

Embryonic stem cells · Erythrocytes · Hemogenic endothelium · β-Globin

Abbreviations

AGM	Aorta-gonad-mesonephros
BMPs	Bone morphogenetic proteins
BMT	Bone marrow transplantation
EBs	Embryoid bodies
EHT	Endothelial-to-hematopoietic transition
EMPs	Erythromyeloid progenitors
EryD	Definitive erythrocytes
EryP	Primitive erythrocytes
ESCs	Embryonic stem cells
FGF2	Fibroblast growth factor 2
FLT-3	Fms-like tyrosine kinase 3
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
ILs	Interleukins
iPSCs	Induced pluripotent stem cells
RBCs	Red blood cells

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SCD Sickle cell disease
SCF Stem cell factor
TPO Thrombopoietin
VEGF Vascular endothelial growth factor

1 Developmental Hierarchy of Erythropoiesis

The knowledge for mammalian embryonic hematopoiesis has mostly been obtained through mouse experimentation and a limited number of human studies. During embryonic development, 10 different blood cell types are produced, and of those, erythroid cells providing essential nutrients for embryonic growth, regulating blood viscosity and forming shear stress required for vascular network development are the most abundant cell linages. Red blood cells (RBCs) are produced by a series of highly regulated and tightly orchestrated events during embryonic development (Barminko et al. 2016). RBC production takes place in at least 3 sequential and overlapping waves. The first wave emerges in the yolk sac within the blood islands, leading to the first morphologically identifiable embryonic hematopoietic cells, primitive large nucleated erythrocytes (EryP) primarily providing the needs of the embryo such as oxygen, along with macrophages and primitive megakaryocytes (Tavian and Peault 2003; Tober et al. 2007). EryP are detected at day 7.25–8.75 in the mouse embryo and 3–4 weeks of human gestation (Van Handel et al. 2010). The origin of primitive EryP is believed to be derived from mesodermal progenitors that are in close proximity to the visceral endoderm, that is required for efficient hematopoietic and endothelial transformation (Baron 2005). Soluble factors secreted from this region including Bone morphogenetic proteins (BMPs), Indian hedgehog, and Vascular endothelial growth factor (VEGF) are confirmed to regulate emergence and expansion of EryP cells during gestation (Barminko et al. 2016). The identification and tracking of EryP cells have been complicated due to lack of specific markers. CD31, Tie-2, endoglin, CD34 and VE-cadherin have been shown to be expressed in mouse EryP cells, but these markers are also expressed by endothelial cells (Ema et al. 2006). While EryP cells share several common properties with their definitive counterparts including cell proliferation capacity, hemoglobin accumulation, decrease in cell size and RNA content, their globin chain expression profiles are unique (Palis 2014). There had been long-held belief that EryP were nucleated throughout the gestation, that was overturned by the study reporting nuclear extrusion of EryP by E12.5 (Kingsley et al. 2004). This enucleation process of EryP was confirmed by others using different approaches while EryP cell numbers have also been shown to remain stable over the gestation (Baron 2013).

The second wave also starts in the yolk sac, that produce definitive erythropoiesis (EryD) at E8.25 in mice (Palis et al. 1999) and around week 4 in human (Migliaccio et al. 1986), indicating partial overlap between primitive and definitive hematopoiesis in the yolk sac. In mice studies, the progenitors generated by the second wave of hematopoiesis were shown not to be hematopoietic stem cells (HSCs) seeding the fetal liver and organizing the hematopoietic system of the adult body but erythromyeloid progenitors (EMPs, $CD41^+$ c-kit^+ $CD16/32^+$) expressing adult globins and translocating to fetal liver to establish early myeloerythropoiesis before leaving their places to the true owners, HSCs (McGrath et al. 2015). HSCs are produced by the third wave of hematopoiesis that occurs in a much more complex manner and in various sites of the embryo including aorta-gonad-mesonephros (AGM) region, major blood vessels, and placenta (reviewed in (Baron 2013; Dzierzak and Speck 2008; Ditadi et al. 2017)). Definitive hematopoiesis in the AGM region starts at E11 followed by the initiation of HSC generation in the yolk sac at E12, which likely contributes to fetal liver HSC population (Kumaravelu et al. 2002; Rowe et al. 2016). After specification of the definitive HSCs, they move to the fetal liver, spleen, thymus, and finally bone marrow in mammals. There is not consensus for a cell surface marker expression profile for HSCs while

HSC enrichment in subpopulations have been reported. The general appreciation for HSCs is that they are present within the population of cells with the expression profile of $CD34^+$ $CD38^-$ $Thy1^+$ $CD45RA^-$ (Doulatov et al. 2012). However, stage-specific HSC enrichment were reported in different subpopulations as CD34+ CD38-lo/$^-$ $CD90^+GPI\text{-}80^+$ for fetal liver HSCs (Prashad et al. 2015) and $CD34^+$ $VE\text{-}cadherin^+$ $CD45^+$ $C\text{-}KIT^+$ $THY\text{-}1^+$ $Endoglin^+$ $CD38^-$/lo $CD45RA^-$ $RUNX1^+$ cell population was presented to be enriched for HSCs at the dorsal domain of aorta at 4–6 weeks of human embryo (Ivanovs et al. 2014). Along with this difference, HSCs derived from different sources have diverse expansion and engraftment ability. E9.5 and E10.5 embryo derived HSCs preferentially engrafted neonates better, while HSCs derived from E14.5 fetal liver or adult bone marrow more robustly engrafted adult recipients (Arora et al. 2014). Blood progenitor cells at different time of embryo have different gene expression patterns and phenotypic characteristics including proliferation and cell surface profile probably due to being exposed to different niche populations at various stages (Rowe et al. 2016). These differences likely determine the characteristics of EryP and EryD. One of the main distinct feature between EryP and EryD is their globin expression profile. EryP mainly expresses embryological globin chains (ζ- and ε-globins) with a small levels of definitive hemoglobin subunits (γ- and α-globins) (Iarovaia et al. 2018). Then, β-like globin chain expression switched to fetal globin expression towards the end of first trimester of gestation, that is driven by a large upstream sequence element called the locus control region (LCR) (Bender et al. 2000; Bungert et al. 1995). While the exact molecular mechanism of globin switching is not yet well characterized, involvement of various transcriptional factors, epigenetic modifications and structural organizations in globin switching have been reported (reviewed in (Iarovaia et al. 2018; Sankaran et al. 2010; Tallack and Perkins 2013)). After the first trimester of gestation, fetal globin subunits, γ1 (Aγ) and γ2 (Gγ), are the most predominant β-like globin chain in the embryo (Fig. 1) (Stamatoyannopoulos 2005; Grosso et al. 2012). Fetal globin is switched to adult globin (β- and δ-globins) after the birth, and its contribution to hemoglobin is less than 1% and not pancellular but concentrated in some specific cells referred to as F-cells.

Using the knowledge of developmental erythropoiesis, scientist have been trying to establish ex vivo models for basic research, drug screening

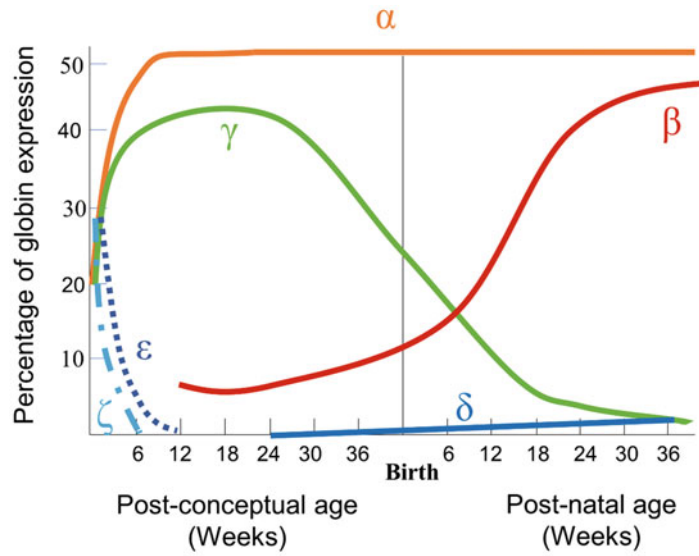

Fig. 1 Globin switching in human embryo. The first switch is from ζ- and ε-globins to α- and γ-globins, respectively, during the first trimester of gestation. The second switch is from γ-globin to β-globin immediately after the birth. Adapted from (Grosso et al. 2012)

and disease modelling. In addition, having mature, functional and high-quality blood cells from progenitor cells with robust expansion capacity is a dream goal for the treatment of blood related diseases including sickle cell disease (SCD).

2 Derivation of RBCs from Pluripotent Stem Cells (PSCs)

The main idea to treat blood-related disorders is to eradicate all diseased/mutated cells and transplant healthy long-term repopulating HSCs. As the bone marrow is the primary organ for HSCs that replenishes blood development throughout life, bone marrow transplantation (BMT) has been widely used for the treatment of various blood disorders including SCD and β-thalassemia. While there are 26 million adult marrow donors registered in the Bone Marrow Donor Worldwide system, around 37,000 patients are still waiting for a matched donor (Batta et al. 2016; Gratwohl et al. 2015). Besides, graft rejection, graft-versus-host disease, and poor reconstitution remain serious issues for BMT, resulting in significant transplant related morbidity and mortality (Fitzhugh et al. 2017). After the introduction of relatively facile and effective genome editing tools, particularly CRISPR/Cas9 technology, scientist have focused on patient-derived HSC-based therapies, especially for monogenic diseases such as SCD. However, ex vivo modification methods for HSCs are not well established, and often lead to diminish multilineage capacity compared to fresh HSCs, and the additional effects of editing approaches are still unknown raising not only efficacy but also safety concerns (Walasek et al. 2012; Yu et al. 2016). In theory, embryonic stem cells (ESCs) with unlimited proliferation and differentiation abilities offer great possibility to obtain HSCs that can be subsequently differentiated into RBCs. As ethical considerations remain for ESCs derived from human embryos, the discovery of induced pluripotent stem cells (iPSCs) obtained by genetic reprogramming of somatic cells avoiding ethical problems associated with ESCs provides a rationale alternative. In just a few weeks, skin cells can easily be conferred pluripotent characteristics from which a variety of cell linages can be generated. This technology has already proven valuable for gaining insights into hematopoiesis, and hold the great potential to be utilized for the definitive cure for many blood related disorders. In particular, patient-derived iPSCs can be genetically corrected and selected to be used to produce HSCs that are subsequently transplanted, or used for patient-specific blood cell production (*i.e.* RBCs).

2.1 HSC Generation from PSCs

Cell engineering strategies are currently available for patient specific hematopoietic precursor development and cell-based therapeutic modalities of hematological disorders. While cell differentiation protocols and molecular-based genetic strategies have enabled the generation of multipotent hematopoietic precursors, derivation of therapeutic grade hematopoietic lineages is still problematic due to the lack of functionality and self-renewal problems in the long term (Rowe et al. 2016). Establishment of efficient protocols and understanding the regulatory molecular mechanisms might move us from basic research to clinical therapy to obtain fully differentiated erythrocytes that are able to transport adequate oxygen, maintain homeostasis, express adult globin and be immune tolerant. Among other hematopoietic cells, RBCs have therapeutic importance as they are required for transfusion in massive bleeding situations, surgical operations and chronic hematological diseases such as SCD (Ebihara et al. 2012). There has been an extensive research to produce sufficient number of RBCs from various blood progenitors, but the efficiency of RBC generation remains disappointing for transfusion purposes (Fujimi et al. 2008; Neildez-Nguyen et al. 2002; Giarratana et al. 2011). In this manner, PSCs with limitless expansion capability offer great advantage. Moreover, establishment of ex vivo systems for derivation of functional RBCs from PSCs, expressing mostly adult globin would constitute a proper

model to elucidate developmental erythropoiesis and investigate potential ideas for RBC-related diseases including SCD prior to animal experiments.

To enable the use of RBCs derived from iPSCs, differentiation system should allow efficient enucleation and β-globin expression similar to adult RBCs, and great number of cell derivation (10^{12}) that is needed for a single transfusion unit. It is well-appreciated that to have functional RBCs carrying β-globin, definitive HSCs should be first generated from pluripotent cells. Recent progress on HCS generation from PSCs have been reported in detail (Ferreira et al. 2018; Hwang et al. 2017; Wahlster and Daley 2016); therefore, the focus of this review is going to be current progress for definitive HSCs derived from PSCs that can transform into functional and adult globin expressing RBCs. Human iPSCs subcutaneously implanted into immunocompromised (NSG) mice was provided proof-of-principle revealing that in theory, functional engrafting HSCs are obtainable from PSCs in proper experimental conditions (Amabile et al. 2013; Suzuki et al. 2013). In these studies, $CD34^+CD45^+$ cells were sorted from iPSC driven teratomas and could reconstitute hematopoietic system in serial transplantations that was comparable to cord blood-derived HSCs. While further functional analysis, molecular and genetic evaluations are needed, these reports urge an international focus to investigate vital environmental parameters and media components to produce clinical grade HSCs ex vivo. In ex vivo conditions, there are three general methods to obtain HSCs from PSCs; through (i) co-culturing with stromal cells, (ii) forced aggregation of cells forming 3-D embryoid bodies (EBs), and (iii) monolayer cultures inoculated on extracellular matrix protein-coated plates. In 2001, it was first reported to derive HSC-like cells from ESCs co-cultured with murine bone marrow stromal cells (S17) or yolk sac endothelial cell line (C166), that had the myeloid, erythroid, and megakaryocyte potential (Kaufman et al. 2001). Vodyanik et al. reported a further improved method for $CD34^+$ derivation from hESCs after co-culturing with OP9 (murine bone marrow stromal cells) in monolayer culture without supplementation of any growth factors (Vodyanik et al. 2005). However, these cells were lacking pan-leukocyte marker (CD45), indicating that co-culturing with OP9 cells recapitulates the early stage of erythropoiesis. Similarly, ESCs co-cultured with mouse fetal liver-derived stromal cells (mFLSCs) produced $CD34^+CD45^-$ cells that gave rise to β-globin expressing and enucleating RBCs (Ma et al. 2008). In a different study, murine stromal cells obtained from AGM or fetal liver were compared as feeder cells for hematopoietically differentiated ESCs (Ledran et al. 2008). While ESC-derived erythroid colonies (CFU-E and BFU-E) did not express adult globin but mostly embryonic and fetal globins, and limited hematopoietic reconstitution in NSG mice was noted in the bone marrow, hESC-derived cells after co-culturing with AGM-derived stromal cells provided the highest primary and secondary hematopoietic engraftment levels for short-term periods (12 weeks). As these co-culture techniques are strictly dependent on cell-associated and secreted components derived from feeder layers, it is not clinically relevant and the results are variable due to difference in lots of feeder cells and animal serum that includes poorly defined growth and differentiation factors.

The necessity of robust feeder- and serum-free differentiation system was partly met with the establishment of EB protocol that undergoes a transient ex vivo gastrulation stage, leading to the transient expression of mesodermal genes and a subsequent HSC emergence. Several methods have been presented for EB formation including suspension culture, hanging drop, and forced aggregation by spinning. Cells forming the EBs undergo rapid differentiation, decrease expression of pluripotent markers including *Oct4* and *Nanog*, and eventually form three germ layers (Poh et al. 2014). To direct the differentiation towards hematopoietic lineage, some growth factors and cytokines including Thrombopoietin (TPO), Fms like tyrosine kinase 3 (FLT-3), Stem cell factor (SCF), BMP4, VEGF, Fibroblast growth factor 2 (FGF2) and Interleukins (IL) are included in the

differentiation media to activate required pathways involved in hematopoiesis (Gil et al. 2015; Smith et al. 2013; Vanhee et al. 2015; Sweeney et al. 2016). Mouse and human ESC studies have shown that right combination of growth factors, activating/inhibition of critical signaling pathways, and the duration of the application are necessary to have long-term engrafting HSC phenotype (Carotta et al. 2004; Sturgeon et al. 2014). While a great effort has been taken on this subject and encouraging improvements have been reported, mostly short-term engrafting or linage-biased and limited engraftment have been reported so far for clinically relevant HSCs derived from PSCs (Table 1).

A vast amount of research was conducted to understand the difference in the transcriptome between HSCs derived from different sources including PSCs (McKinney-Freeman et al. 2012; Sugimura et al. 2017; Sauvageau et al. 1994; Meader et al. 2018; Kartalaei et al. 2015). In light of these reports, some critical pathways such as Notch and Wnt signaling pathways, and vital gene expressions including homebox family genes were noted to be important for definitive HSC specification (Sturgeon et al. 2014; Kyba et al. 2002; Burns et al. 2005). While several reports have shown the improvements of HSC-like cell generation from PSCs by some gene addition methods, their engraftment potential remained disappointing. Daley's group, however, has recently showed that transduction of PSCs with a combination of transcription factor cocktail (*ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1* and *SPI1*) is sufficient to generate definitive HSCs that engraft myeloid, B and T cells (albeit with notable B cell bias) in primary and secondary mouse recipients, that was analyzed for up to 16 weeks (Sugimura et al. 2017). While the method requires intermediate phase ($CD45^{+}CD43^{-}$) followed by respecification into induced HSCs, and ectopic expression of several transcriptional factors that remains to be analyzed in terms of safety, the reports set out the actual possibility of engraftable HSC derivation from iPSCs. A year later, Tan et al. presented that single factor (*MLL-AF4*) was sufficient to respecify the PSCs into long-term engrafting iHSCs (Tan et al. 2018).

2.2 Hemogenic Endothelium Derived HSCs

Endothelial and blood cells have been recognized as two cell types with many common features for a long time (Sabin 1920; Maximow 1924; Crosby et al. 2000). Lineage-tracing and time-lapse imaging analysis showed that some specialized endothelial cells, referred to as "hemogenic endothelium", produce blood cells thorough endothelial-to-hematopoietic transition (EHT) (Eilken et al. 2009; Lacaud and Kouskoff 2017). While yolk sac EMPs, T and B progenitors and HSCs have been shown to derive from hemogenic endothelium in mouse studies, the origin of first primitive wave (E7.5) hematopoiesis is not proven yet (Lacaud and Kouskoff 2017). As the first wave takes place before the establishment of the vascular network, it is not likely that primitive hematopoiesis is generated through hemogenic endothelium. On the other hand, primitive hematopoietic progenitors were also found to be expressing endothelial markers including TIE2, VE-cadherin, and CD31 (Ema et al. 2006; Lancrin et al. 2009; Fraser et al. 2002), indicating that there is a close relationship between endothelial and hematopoietic linages in all phases of hematopoiesis. Therefore, hemogenic endothelium has been adapted to ex vivo culture systems in an attempt to have proper model for hematopoiesis and derive definitive HSCs. Keller's group showed the presence of these common progenitors by reporting that BMP4 stimulated EBs can give rise to transient endothelial progenitors that can differentiate into primitive erythroid cells expressing ε- and γ-globins, macrophages, and endothelial cells (Kennedy et al. 2007). It was later presented that using canonical Wnt signaling can activate definitive hematopoiesis through hemogenic endothelium stage as evidenced by gamma globin expression in RBCs, and T-lymphoid differentiation ability of HSC-like cells derived from PSCs (Sturgeon et al. 2014). The T-lymphoid potential of progenitors is one parameter used for definitive hematopoiesis evaluation (Kennedy et al. 2012); however, β-globin expression in these RBCs and

Table 1 Selected reports for engraftment of hematopoietic stem cell derived from pluripotent

Application	Differentiation method	Globin expression profile	Engraftment	References
In vivo teratoma formation	Injection of ESCs or iPSCs to mouse with or without OP9 feeder cells and cytokines	ε, γ, ζ, β, δ globins in CFU-E colonies derived from PSC driven teratomas	Long term B-, T- cells and myeloid	Amabile et al. (2013; Suzuki et al. (2013)
Direct differentiation with feeder cells	Differentiation of iPSCs with stromal cells derived from aorta-gonad-mesonephros (AGM) region	ε, γ, and ζ globins in CFU-E and BFU-E colonies	Short-term myeloid and lymphoid (12 weeks)	Ledran et al. (2008)
Coculturing with endothelial cells of hESCs and monkey iPSCs	Embryoid body formation for HSC derivation. Sorted cells were cocultured with endothelial cells + hematopoietic cytokines	Predominantly β globin and limited γ globin in CFU-E colonies derived from engrafted multipotent progenitor cells	Long-term myeloid, lymphoid and erythroid	Gori et al. (2015)
Ectopic expression of *HOXA9, ERG, RORA, SOX4* and *MYB* in iPSCs	Embryoid body	Mostly ε and γ, little or no β globin in ex vivo derived cells. Hemoglobin switching after transplantation to NSG mice (γ and β-globin)	Short-term erythro-myeloid (4–8 weeks)	Doulatov et al. (2013)
Ectopic expression of *ERG*, *HOXA5*, *HOXA9*, *HOXA10*, *LCOR*, *RUNX1* and *SPI1* in hemogenic endothelium cells derived from iPSCs	Respecification of HSCs from iPSCs through hemogenic endothelium	γ and β globins in engrafted human erythroid cells with limited enucleation	Long term B-, T- cells and myeloid	Sugimura et al. (2017)
Extopic expression of MLL-AF4 in iPSCs	Monolayer differentiation	NA	Long-term B-, T- cells, erythroid and myeloid	Tan et al. (2018)

engraftment ability of HSC-like cells in NSG mice were not reported. These endothelial progenitors have been shown to be restricted to a certain population ($CD34^+CD73^-CD184^-DLL4^-$) that generates multipotent hematopoietic progenitors and distinct from vascular endothelium progenitors (Ditadi et al. 2015). In addition, the report showed that the activation of EHT is strictly dependent on Notch signaling. The same group also presented strong *CDX4* gene expression within Wnt-activated definitive hematopoietic mesoderm, showing the critical roles of transcriptional regulatory network in HSC specification (Creamer et al. 2017). These reports, however, did not show any engraftment ability of the HSC-like cells derived from hemogenic endothelium. As mentioned above, a recent study, however, reported that hemogenic endothelium derived progenitors transduced with 7 transcription factors could produce primary and secondary engraftable HSCs (Sugimura et al. 2017). Taken together, while the complete molecular mechanisms of hematopoiesis in not completely elucidated, ex vivo and in vivo experiments indicate that we need time-dependent activation/inhibition of critical signaling pathways and transcription factor gene expressions to have safe, functional and engraftable HSCs to be used in clinical setting. After establishing the differentiation system, definitive cell lineages such as RBCs would be generated for clinical purposes.

2.3 RBC Derivation from PSCs

Definitive RBC generation from PCSs would allow limitless production of RBC for transfusion purpose as well as establishment of proper models for diseases (*i.e.* SCD), drug screening and elucidating the embryological cascade of erythropoiesis. It has been suggested that 150 iPSCs produced from homozygous human leukocyte antigen (HLA)-typed volunteers could match 93% of the UK population with a minimal requirement for immunosuppression (Taylor et al. 2012). As nucleated RBCs are separated from the RBC concentrates during the transfusion, and RBCs express low levels of HLAs, iPSCs can be used to generate universal O and rhesus (RhD)-negative blood types (Xie et al. 2014). In addition, after the introduction of relatively easy genome editing approaches, correction of mutations responsible for inherited red blood cell (RBC) disorders such as SCD become possible through iPSCs that can subsequently generate definitive HSCs in proper laboratory conditions, which then give rise to definitive RBCs producing β-globin after transplantation.

From the clinical perspective, the quality and quantity of RBCs generated are of importance as well as expressed hemoglobin type. Most of the erythrocyte generation from PSC demonstrate only primitive erythropoiesis with high levels of ε- and γ-globin expressions (Chang et al. 2006; Hatzistavrou et al. 2009). As stated before, to have RBCs with adult globin derived from PSCs, definitive HSCs should be generated that can subsequently activate the globin switching mechanism during RBC differentiation to produce β-globin. Treatment of EBs with VEGF along with basic hematopoietic growth factors (SCF, Flt3, BMP-4, GSCF, IL-6 and IL-3) resulted in higher erythroid marker expression (Cerdan et al. 2004). While the EB derived progenitor cells expressed embryonic globins (ε and ζ) erythroid clones derived in methylcellulose media expressed both adult (γ and β) and embryonic (ε) globins. Interactions, particularly direct cell-cell interactions, within the niche is critical for normal hematopoiesis. For this reason, AGM or fetal liver were used to derive stromal cells to be used as feeder cells in hematopoiesis from PSCs studies. Accordingly, Ma et al. showed that hESC derived HSCs expressed ε-globin in the first phases of differentiation while most of the cells expressed β-globin in later times upon co-culture with murine fetal liver derived stromal cells, revealing there could be a switching mechanism similar to in vivo (Ma et al. 2008). Surprisingly, treatment with conditioned media derived from AGM and fetal liver derived stromal cells also induced β-globin expression, indicating that some key proteins secreted from fetal stromal cells are also important for globin switching along with cell-cell interactions within the niches (Lee et al. 2010). Recently, we have shown that when ES cells cultured on murine stromal cells (C3H/10T1/2), ES-sacs (hemangioblast-like structures) forms, that concentrate phenotypic HSPCs ($CD34^+CD45^+$), and more definitive ($CD235a^-CD34^+$) and primitive ($CD235a^+CD34^-$) erythroid precursor cells, which can differentiate into β-globin expressing erythroid cells (Fujita et al. 2016). Definitive erythropoiesis occurred successfully during ES sac maturation with mostly γ- and β-globin expression. These findings were extended to SCD patient derived iPSCs that were differentiated into erythrocytes with detectable sickle globin expression (Uchida et al. 2017) while the engraftment ability of these cells remains to be tested. As the main goal for such genetic diseases is correction of the mutation(s) and generation of functional HSCs from patient specific iPSCs, Huang et al. presented adult beta globin expression at the protein level and partial enucleation in SCD patient-specific iPSCs-derived RBCs after correction of the mutation (Huang et al. 2015).

Although the complete mechanisms are not clear yet, it is well-appreciated that developmental hematopoiesis and lineage specifications are strictly controlled by a set of transient signaling pathway activation/inhibition, and various expression levels of transcriptional regulatory elements. Leung et al. has recently showed that key roles of stage specific controlling of Notch and the aryl hydrocarbon receptor (AHR) pathways in the derivation of definitive

hematopoietic cells (Leung et al. 2018). They concluded that Notch signaling is important for the putative HSC specification from PSCs at the early mesodermal differentiation of PSCs, and later for emergence of definitive erythrocytes with adult globin expression although the expression levels were scant. In addition, AHR signaling was presented to affect the number of HSC generation. Other than signaling pathways, several genes including Gata1, Gata2, Eklf/Klf1, and Lmo2 have been reported to be involved in regular erythropoiesis (Palis 2014). To apply stage-specific expression, tamoxifen-inducible KLF1 expression system were activated at day 10 of differentiation, revealing higher RBC production and more detectable enucleated cells in differentiated iPSCs but mostly fetal and embryonic globin expression (Yang et al. 2017). More impressively, transcription factor cocktail transduced PSCs were respecified to definitive HSCs with long-term myeloid, B and T engraftment ability in primary and secondary transplants (Sugimura et al. 2017). Although the specification is synthetic and safety questions remained to be answered from a clinical perspective, engrafted RBCs with significant β-globin expression (comparable to RBCs differentiated from transplanted cord blood derived progenitors) and enucleation are encouraging for future optimizations. Accumulating evidence like these reports suggest that if the critical pathways and regulatory elements including transcriptional networks could be closely adapted to ex vivo systems, it should be possible to obtain satisfactory amounts and quality of definitive progenitor cells from PSCs.

3 Conclusion and Future Perspectives

Although there remains a tremendous focus, derivation of clinically usable *bona fide* HSCs from PSCs remains to be demonstrated. Several researches have presented generation of HSC-like cells as analyzed phenotypically similar to HSCs, reconstitution of hematopoietic system in immunocompromised mouse and long-term engraftment were limited. This is probably due to generation of primitive progenitors or EMPs, and lack of specific cell surface markers for real stem cells. Transplantation with $CD34^+$ in the clinical practice works well, however, $CD34^+$ cells are heterogeneous population and only a small fraction reconstitutes the whole hematopoietic system. More work on identifying the characteristics of this small stem cell fraction would allow scientist to focus on the generation and investigation such particular cell types in haematopoietically differentiated PSCs.

In theory, definitive hematopoiesis should provide stem cells with this engrafting stability. Although there are several claims of the establishment of protocols for definitive hematopoiesis as evidenced by T-cell differentiation potential and cell surface protein analysis, long-term engraftment in immunocompromised mice models are not satisfactory due to being mostly limited and lineage-biased engraftment. Evaluation methods of definitive HSCs should be confirmed with more reliable methods. One way would be to extend and define the minimum criteria of definitive HSCs providing multilineage long-term engraftment and erythropoiesis with adult globin expression. As such, the ex vivo method would be to investigate the potential of HSC-like cells derived from PSCs for RBC production with mostly β-globin expression. But there is not any well-established protocol to derive definitive erythropoiesis from PSCs; hence, this theory is yet to be tested.

Developmental hematopoiesis is far more complex than our understanding. There are several transient signaling pathway activation/inhibition phases that are tightly controlled by growth factors, cytokines, small molecules, extracellular matrix proteins, etc., and complicated interactions among cell types. Other than signaling pathways and transcription factors, microRNAs, long non-coding RNAs and epigenetic factors are being discovered to take place all phases of hematopoiesis (Wahlster and Daley 2016). To mimic in vivo hematopoiesis in ex vivo conditions, we need to expand our knowledge and establish well-orchestrated cell culture protocols. When these challenges are met, we will be able to use these

approaches for disease modeling and drug screening, production of limitless patient-specific HSCs to be used in the treatment of blood related diseases, and generation of required hematopoietic lineages such as RBC and platelets for transfusion purposes. However, generation of enough high-quality cells with required maturity (*i.e.*, RBCs with β-globin expression) is not solely sufficient for clinical applications. The next issue for PSC-derived HSCs will be the establishment of standardized, clinical-grade cell production (GMP) methods that do not include any xenogeneic protein during cell culture and possess any safety issue.

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Comparison of Hematopoietic and Spermatogonial Stem Cell Niches from the Regenerative Medicine Aspect

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Abstract

Recent advances require a dual evaluation of germ and somatic stem cell niches with a regenerative medicine perspective. For a better point of view of the niche concept, it is needed to compare the microenvironments of those niches in respect to several components. The cellular environment of spermatogonial stem cells' niche consists of Sertoli cells, Leydig cells, vascular endothelial cells, epididymal fat cells, peritubular myoid cells while hematopoietic stem cells have mesenchymal stem cells, osteoblasts, osteoclasts, megacaryocytes, macrophages, vascular endothelial cells, pericytes and adipocytes in their microenvironment. Not only those cells', but also the effect of the other factors such as hormones, growth factors, chemokines, cytokines, extracellular matrix components, biomechanical forces (like shear stress, tension or compression) and physical environmental elements such as temperature, oxygen level and pH will be clarified during the chapter. Because it is known that the microenvironment has an important role in the stem cell homeostasis and disease conditions, it is crucial to understand the details of the microenvironment and to be able to compare the niche concepts of the different types of stem cells from each other, for the regenerative interventions. Indeed, the purpose of this chapter is to point out the usage of niche engineering within the further studies in the regenerative medicine field. Decellularized, synthetic or non-synthetic scaffolds may help to mimic the stem cell niche. However, the shared or different characteristics of germ and somatic stem cell microenvironments are necessary to constitute a proper niche model. When considered from this aspect, it is possible to produce some strategies on the personalized medicine by using those artificial models of stem cell microenvironment.

Keywords

Bone marrow niche · Hematopoietic stem cell · Microenvironment · Niche · Regeneration · Spermatogonial stem cell

Sevil Köse and Nilgün Yersal contributed equally to the chapter.

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Abbreviations

2-AG	2 arachidonoyl glycerol
ABP	Androgen Binding Protein
ADAM	A Disintegrin and Metalloprotease
AEA	(anandamide), *N*-arachidonoyl ethanolamine
AGM	Aorta-Gonad-Mesonephros
BADGE	Bisphenol A Diglycidyl Ether
bFGF	Basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
BTB	Blood Testis Barrier
CB_1	Cannabinoid receptor targets type-1
CB_2	Cannabinoid receptor targets type-2
CLEC-2	C-type lectin-like receptor-2
CNS	Central Nervous System
CSF1	Colony Stimulating factor 1
CSFR1	CSF1 Receptor
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	Chemokine receptor type 4
EC	Endothelial Cell
ECM	Extracellular Matrix
ECS	Endocannabinoids
ES	Ectoplazmic
EWAT	Epididymal White Adipose Tissue
FAAH	Fatty Acid Amide hydrolase
FGF	Fibroblast Growth Factor
FGFR2	FGF Receptor 2
FSH	Follicle-Stimulating Hormone
G-CSF	Granulocyte Colony-Stimulating Factor
GDNF	Glial cell-line Derived Neutrophic Factor
GFRA1	GDNF-Family Receptor α1
GPCR	G Protein-Coupled Receptors
hCG	Human Chorionic Gonadotropin
HSC	Hematopoietic Stem Cells
HSPC	Hematopoietic Stem/Progenitor Cells
IM	Interstitial Macrophage
KDR	Kinase Insert Domain Receptor
LC	Leydig Cell
LH	Luteinizing Hormone
MAGL	Monoacylglycerol lipase
MAPK	Mitogen-Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
MMPs	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cells
Nes+	Nestin Positive
NO	Nitric Oxide
PGC	Primordial Germ Cell
PM	Peritubular Macrophage
PMC	Peritubular Myoid Cell
PN	Postnatal
PPAR-γ	Proliferator-Activated Receptor-γ
PPR	Parathyroid hormone protein receptor
RA	Retinoic acid
RET	Receptor Tyrosine Kinase
Runx2hi	Runx2 high
SC	Sertoli Cell
SCF	Stem Cell Factor (KIT ligand)
SFK	Src Family Kinase
SSC	Spermatogonial Stem Cell
STO	SIM mouse embryo-derived thioquanine – and- quabian –resistant cells
TJ	Tight Junction
TPO	Thrombopoietin
VE	Vascular Endothelial
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor-2

1 Introduction – Stem Cells and the "Niche Concept"

Stem cells maintain their specific/undifferentiated characteristics and pool size throughout their lifespan by means of the "stem cell niche". Basically, the niche is the specialized microenvironment that helps the maintenance of the stem cells and supplies their stemness function. In other words, stem cells' quiescence, self-renewal and survival capacity are controlled by the microenvironment in which they are embedded (Bardelli and Moccetti

2017). This situation makes very important to understand the content and the complexity of the microenvironment for the researches on the regenerative medicine field. There are lots of elements that regulate the function of the stem cell niche. The cellular components, which include blood vessels with endothelial cells, differentiated/undifferentiated or progenitor neighboring cells and with their paracrine signals, are the first members of the system. There is also an autocrine regulation of stem cells by themselves. Secondly, the extracellular matrix components containing glycoproteins, proteoglycans, adhesion molecules and collagen/elastic/reticular fibers provide a cell-to-cell connection, which is crucial for the regulation of the niche. Besides the cellular components, there is also a chemical regulation in the form of secreted soluble factors such as hormones, cytokines, growth factors, chemokines and also neurotransmitters. In addition to chemical factors, every niche has a special physical condition constituted by temperature, pH and the amount of oxygen. Also, niches from various tissues show difference in the elasticity, shear stress, bending, compression and tension and they provide specific biomechanical setting for the preservation of stem cell function (Fig. 1) (Redondo et al. 2017).

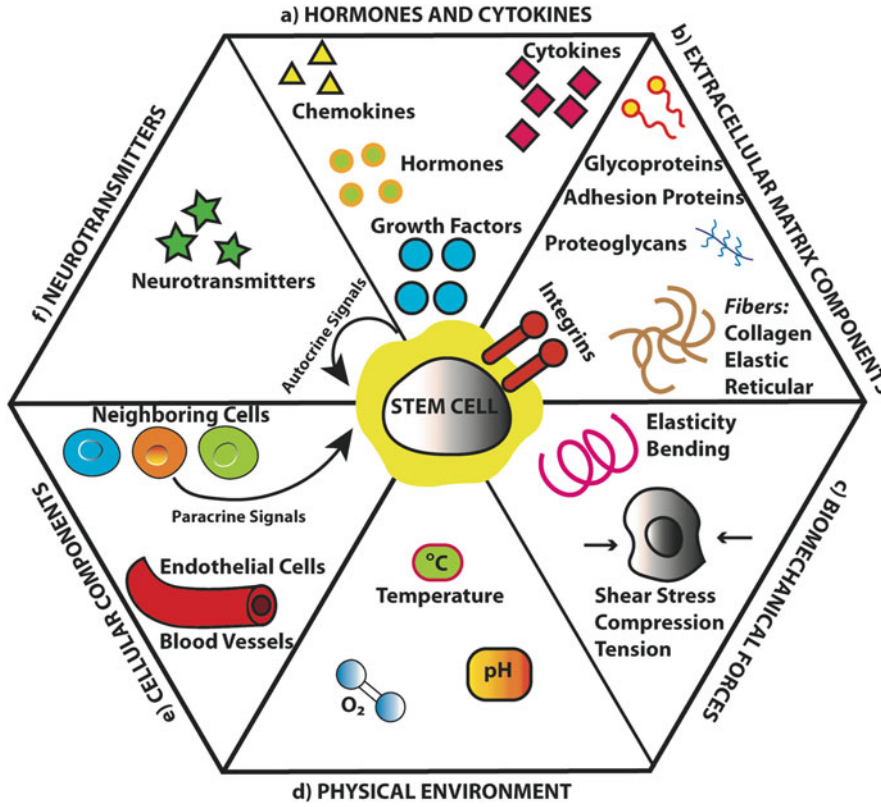

Fig. 1 The members of the niche are shown in general. (*a*) Hormones, chemokines cytokines and growth factors are crucial in elements which are responsible for the stem cell survival, quiescence, self-renewal and differentiation. (*b*) The communication of the stem cells with their cellular and chemical environment is operated by extracellular matrix components such as collagen fibrils, elastic fibrils, reticular fibrils, proteoglycans, glycoproteins and, the adhesion molecules. (*c*) Elasticity, bending, shear stress, compression and tension are the biomechanical forces applied by the microenvironment to the stem cells and they also affect the stem cell fate. (*d*) Physical environment is the other factor in the system. Temperature, oxygen level and pH are the special characteristics of stem cell niche. (*e*) Differentiated or undifferentiated neighboring cells, endothelial cells (coming from blood vessels), adipocytes and macrophages are the cellular components of the microenvironment. (*f*) Finally, neurotransmitters are other chemical factors influencing the stem cell maintenance and differentiation

The sum of all those components creates a microenvironment in which maintenance of the quiescence or the progenitor production –in other words differentiation- are decided. It is the space that is provided for the reproduction of the stem cells and their sustainability. The preservation of the stem cell phenotype is only possible by the connection of the stem cells with their niches. If they loose their contact with their niche, they are not able to receive the inhibitory signals for the differentiation and they begin to differentiate. To understand why the stem cells require a special support from their microenvironment for the maintenance of the stem cell pool is important. There is a feedback mechanism provided by the environmental factors. The elements like growth factors or extracellular matrix components such as cell surface molecules are necessary for this mechanism and the control of the stem cell pool (Dong et al. 2015a).

It is possible to compare the germ and the somatic stem cell niches in terms of their structures. It is known that there is a heterogeneity in the stem cell niche types while there are lots of similarities indeed (Muzes and Sipos 2016). The similarities of somatic and germ stem cell niches can be summarized by this way; the existence of the specialized and unspecialized cells in the microenvironment, physical anchoring function of the niche for the stem cells, functioning to regulate the stem cell behavior (like self-renewal, differentiation or quiescence) according to the signals from the body, the availability of the blood vessels found near the niches and the presence of a dynamic structure of the niche which includes extracellular matrix, chemical factors, cell to cell contacts and mechanical stimuli. A somatic stem cell type "hematopoietic stem cells" and a germ stem cell type "spermatogonial stem cells" will be compared in this review according to the properties of their niches with a regenerative perspective. Both stem cell microenvironments have their own specialized stem cells supported by similar accessory cells. The accessory cells consist of sertoli cells, leydig cells, peritubular myoid cells, macrophages, vascular endothelial cells and epididymal fat cells in the spermatogonial stem cell niche (Garcia and Hofmann 2015) (Fig. 2). The mesenchymal stem cells, vascular endothelial cells and pericytes and the adipocytes, represent the accessory cell population for hematopoietic stem cell microenvironment

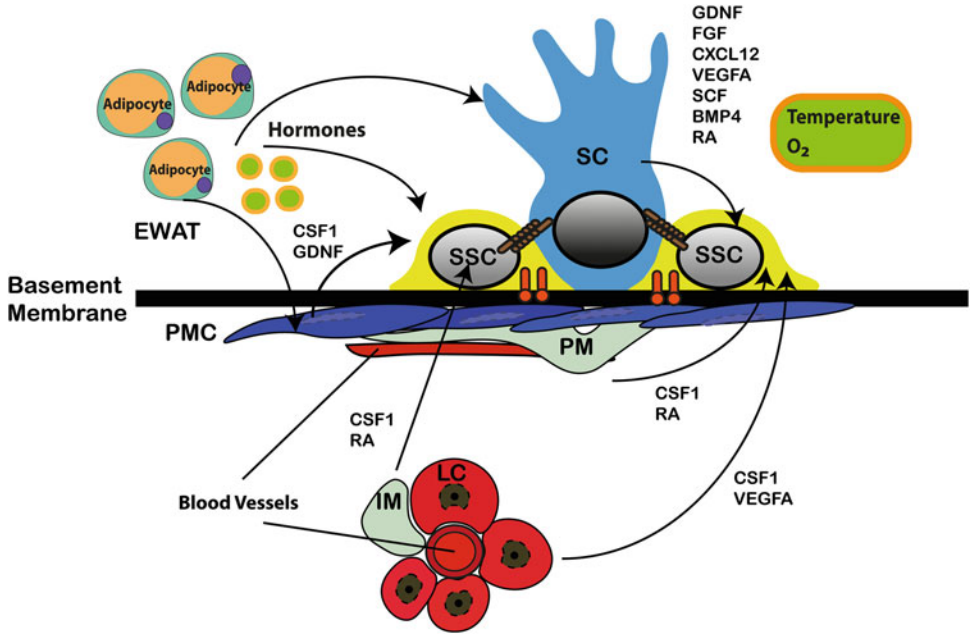

Fig. 2 The SSCs niche is presented with its cellular and extracellular matrix components. Proliferation and differentiation of SSCs are regulated by soluble factors, biomechanical forces and, the physical elements interacting with each other. Compare the similarities with Fig. 3

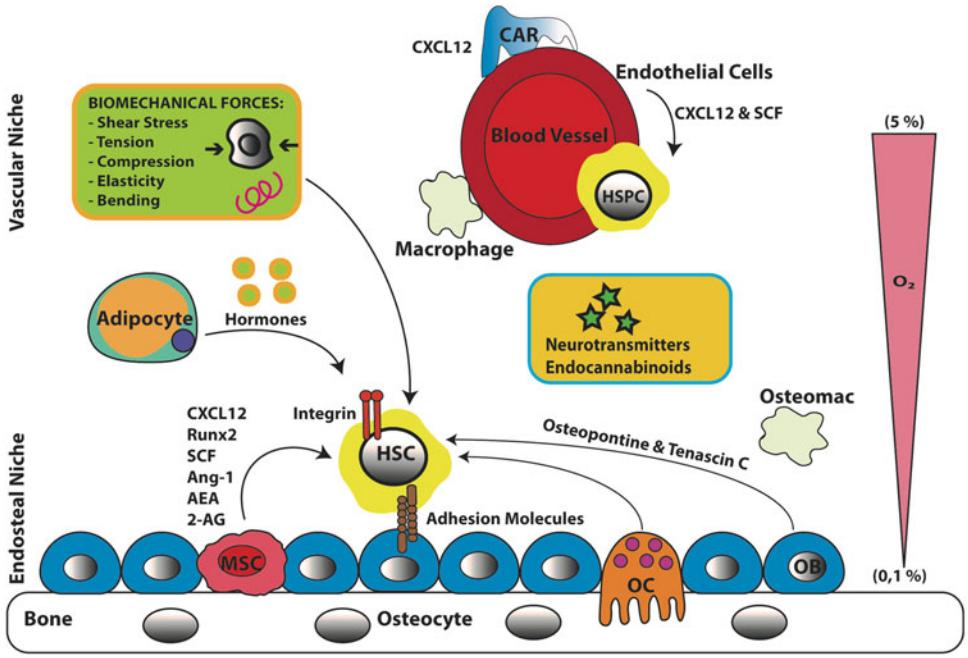

Fig. 3 Hematopoietic stem cell niche components in the adult BM are presented. Note the presence of cellular and extracellular matrix components, soluble, physical and biomechanical factors crosstalking with each other. Compare the similarities with Fig. 2

(Asada et al. 2017) (Fig. 3). The cellular and extracellular players are exposed to divergent mechanical stresses among those niches because of their different systemic location in the body. All of these associations and the others will be clarified during the chapter in detail. To understand the components of the microenvironments and how the niche content changes according to the niche type and disease conditions may help to develop new strategies for the regenerative medicine (Sugimura 2016; Kirkpatrick 2015). In this review, the hematopoietic and the spermatogonial stem cell niches will be compared in terms of the regenerative medicine perspective.

2 Hematopoietic Stem Cell Niche/Microenvironment

The hematopoietic stem cell 'niche', provides a specialized microenvironment that preserves their repopulating capacity, as proposed by Schofield (Schofield 1978). Hematopoietic stem cells (HSCs) repopulating activity is usually evaluated by transplantation assay, in which cells from tested tissue are transplanted into irradiated recipients (Frisch and Calvi 2014). For the past few decades, considerable efforts have been devoted to elucidate the key components of this niche, with recent evidence showing that the HSC niche is composed of ECM components, cytokines, hormones, autonomic innervation, physical biomechanical forces and most importantly diverse cell types that have specific regulatory roles, working together to support HSC maintenance (stemness), proliferation, migration and differentiation (Lucas 2017). However, many questions remain to be answered about the HSC niche, such as how the many cell types within the BM niche contribute to HSC heterogeneity. In the following section, HSC niche components will be mentioned in a regenerative perspective.

2.1 Embryogenesis of Hematopoietic Stem Cells

Hematopoietic stem cells are a limited cell population that found on the basis of the hematopoietic

system; they have the capability to self renew and differentiation to give rise to all blood cells of the hematopoietic system (Lucas 2017). The hematopoietic system components are of mesodermal origin (Dharampuriya et al. 2017). Definitive HSCs being capable of reconstituting the entire hematopoietic system, emerge from the hemogenic endothelium (endothelial cells that can give rise to multilineage HSPCs within AGM; a region of embryonic mesoderm. The hematopoietic stem cells then migrate into the fetal liver through the circulation before colonizing the adult BM. Heamtopoietic stem cells can also migrate to extramedullary sites (sites outside of the BM) to bring about hematopoiesis in response to stress. As mature blood cells are dominantly short lived, HSCs are involved entirely the lifetime of an individual to replenish the blood system (Julien et al. 2016). Functionally, they are defined by their capacity to reconstitute/regenerate the entire blood in an irradiated recipient by stem cell transplantation, a method now widely used clinically to treat patients with hematological diseases, including leukemia, lymphoma, and sickle cell disease (Lin et al. 2015). However, given the limited number of matching donors and of cord blood derived HSCs, obtaining sufficient numbers of compatible HSCs remain as limiting factor for BM transplantation therapy. Thus, there is a major need to develop new strategies to expand HSCs ex vivo efficiently for transplantation therapies.

2.2 Cellular Constituents of the Hematopoietic Stem Cell Niche

The bone marrow found inside the trabecular bone is the main site of hematopoiesis (Sarkaria et al. 2018). The work done for many years showed that, putative HSCs have been found next the endosteal surface of trabecular bone (*endosteal* niche/microenvironment), in the central marrow cavity (*central or medullary* niche/microenvironment), and near to vascular tissues such as the sinusoidal endothelium or arterioles (*perivascular, arteriolar* niche/microenvironment) (Kumar et al. 2018). Due to their close proximity, these theoretical niches are likely to be overlapping or mutually exclusive microenvironments. Some researchers do not accept this regional difference, but they are available to researchers who distinguish BM niches into more regions. However, it is known that there are special areas in the BM that trigger the silencing and differentiation of HSCs.

To mimic the HSCs and their niche to remodeling the hematopoietic related diseases or overcome the lack of donors, HSCs can also be obtained directly or reprogrammed from other cells like as mature hematopoietic cells, endothelial cells, fibroblasts and pluripotent stem cells (Sugimura 2016). In order to avoid an immune reaction, it is important to make HSC differentiation from cells to be isolated from the individual. Therefore, it is very important in this aspect that the properties of the cells in the niche, such as the functional profiles, are well defined.

2.2.1 Osteoblasts

Osteoblasts have been played a crucial role in regulating HSC maintenance. Osteoblast progenitors (bone lining cells), which are located in endosteum, can enhance the HSC maintenance by supporting the quiescence characteristics of the cells (Calvi et al. 2003). Furthermore, the presence of HSCs near endosteal surfaces may in part be due to a need for osteoblast-derived products in the maintenance and expansion of early hematopoietic precursors. Researchers showed that human osteoblasts assist human HSPCs in *in vitro* BM cultures (Taichman et al. 1996). At the same time, HSC fate was affected differently by various subsets of osteoblasts. Parathyroid hormone protein receptor (PPR) activation on mature osteoblasts have no impact on HSC self renewal mechanism or differentiation (Calvi et al. 2012). Osteoprogenitor cells like as Nes+ MSCs (Mendez-Ferrer et al. 2010) or Runx2hi progenitor osteoblasts (Chitteti et al. 2010) were able for HSCs maintenance by secretions as SCF, Ang-1, CXCL12 (SDF-1). These factors assist HSC self-renewal and/or quiescence (Levesque

et al. 2010). And also, primitive HSPCs show preferred homing, lodgement and engraftment to positions close proximity to the endosteum and adjacent to the osteoblasts and osteoprogenitor cells (Nombela-Arrieta et al. 2013).

2.2.2 Mesenchymal Stem Cells

Mesenchymal Stem Cells, the multipotent stem/stromal cells have important roles in HSC niche. Firstly, these cells give rise to adipocytes and chondrocytes and most importantly osteoblasts, as active components of the HSC microenvironment (Zhou et al. 2017). Secondly, they have extensive chemokine/cytokine secretion profile. These signals have a variety of effects on HSC that vary according to the area in which the cells are located (endosteal or vascular niche). The best example for this is CXCL12, which is a chemokine that is constitutively secreted by native MSCs at endosteal niche, and it is known to play an crucial role in controlling HSC quiescence and retention in the BM and repopulating activity. The most fundamental role of donor HSCs in migration and engraftment of recipient BM is the SDF-1 released from CAR cells. CXCL12-abundant 'reticular' cells are adjacent to sinusoids in vascular niche and co-localize with HSCs/HPSCs throughout the BM (Sugiyama et al. 2006). Repress of CXCL12-expressing BM cells deplete HSCs and also osteogenic and adipogenic capacity decrease (Omatsu et al. 2010). Thirdly, *in vitro* and *in vivo* data show that MSCs have anti-inflammatory effects, interactions with immune cells modulate/suppress immunologic responses, and home to damaged tissues to involve in regeneration. And also, secretion of ECM components from these cells is very crucial for the HSC maintanence (Grimaldi et al. 2013).

All these features of the MSCs make these cells indispensable for the sustainability of the HSC niche processes through their diverse biologic properties (Battiwalla and Hematti 2009). In some current BM transplant protocols, using donor or recipient MSCs, it is aimed to increase HSC migration, lodgment and engrafment to the appropriate BM niches, repair damaged BM microenvironment and most importantly prevent GVHD (Battiwalla and Hematti 2009).

2.2.3 Vascular Endothelial Cells

Endothelial cells form the all blood vessels and help supply oxygen and nutrients to tissues/organs throughout the body, including the BM. Hematopoietic progenitor (CD34+) and stem cells and especially differentiated hematopoietic cells reside close to BM sinusoidal endothelial cells. Blocking of endothelial cells angiogenic properties by blocking VEGFR2 and VE-cadherin neutralizing supporting function of ECs to long-term HSCs is impaired (Butler et al. 2010). The role of endothelial cells in the BM modulate of HPSC and long-term culture initiating cells proliferation by enrichment of lineage specific cytokines (Rafii et al. 1995). Niche factors, like CXCL12 or SCF which are important for HSC maintenance also expressed from BM endothelial cells. Endothelial cells have been shown to modulate HSC quiescence through an adhesion molecule E-selectin expression. Ablation of E-selectin stimulated HSC quiescence and supported survival (Winkler et al. 2012). The expression of Notch ligands by endothelial cells also promotes HSC proliferation and differentiation *in vitro* (Ishige-Wada et al. 2016). *In vivo* studies showed that Notch signaling in endothelial cells also expanded HSCs *in vivo*, and the reactivation of Notch signaling in endothelial cells repair the BM microenvironment in aged mice but did not repair the aged HSCs (Kusumbe et al. 2016). Furthermore, permeability of BM arterial/sinusoidal endothelial cells can regulate the quiescence, proliferation and consequently differentiation of HSCs. Permeable sinusoidal endothelial cells, can activate HSCs as a result of more plasma flow and stimulate high level of ROS in HSCs; less permeable arterial endothelial cells maintain HSCs at quiescent state (Itkin et al. 2016).

2.2.4 Macrophages

Several macrophage phenotypes have been identified in BM microenvironment (Yona et al. 2013). In endosteal niche F480+ macrophages (osteomacs) are described and these cells settled close to bone lining cells and osteoblasts. And

also, these cells and CD169+ macrophages associated with Nestin+ mesenchymal stromal cells (Chow et al. 2011). Not only MSCs, macrophages importantly improve the production of mature osteoblasts *in vitro* and *in vivo* (Chang et al. 2008). Ablation of macrophages (80–90%) in MAFIA mice is resulting in osteoblasts suppression (Chang et al. 2008), HPSC mobilization to the peripheral blood, reduction of osteoblast cell number and decreased expression of common stem cell niche factors, including KitL, SDF-1 and Ang-1 (Chow et al. 2011; Winkler et al. 2010). Importantly, GCSF that is a glycoprotein stimulates the BM to stem and progenitor cell release through into the bloodstream and treatment results in a remarkable loss of monocytes (Christopher et al. 2009) and osteomacs (Winkler et al. 2010) in BM.

2.2.5 Megakaryocytes

Megakaryocytes that are the hematopoietic cell that produce platelets have been introduced to consist one of the elements of HSC niche. Immune fluorescent labeling showed that a group of HSCs particularly are placed next to megakaryocytes (Bruns et al. 2014). Megakaryocyte depletion caused loss of quiescence of HSCs, and the injection of CXCL4 that is oscillated by megakaryocyte increased HSC survive (Bruns et al. 2014). In another study supporting this data, the ablation of megakaryocytes resulted to an increased HSC proliferation and number (Zhao et al. 2014). This effect of megakaryocytes on HSCs was found to be mediated by TPO produced and released by megakaryocytes through CLEC-2 signaling. CLEC-2 signaling in megakaryocytes is crucial for HSC maintenance in BM (Nakamura-Ishizu et al. 2015).

2.2.6 Adipocytes

Adipocytes form a large part of the human BM increase in volume with age (Horowitz et al. 2017). In parallel with this, there is a decrease in hematopoietic system elements (Patel et al. 2018). This negative relationship between adipocytes and HSCs have been proved by *in vivo* studies. Naveiras et al. showed that BM healing was enhanced after irradiation in PPAR-γ inhibitor treated BADGE mice, which inhibits adipogenesis or fatless mice (Naveiras et al. 2009; Zhu et al. 2013). At the same time, escalated adipogenesis *in vivo* did not affect the HSC number in BM (Spindler et al. 2014). However, initially adipocytes were supported of BM HSCs. Adipocytes secrete adipokine which is the adiponectin and its receptor is expressed on HSCs. Adipokine has been shown to promote HSC proliferation (DiMascio et al. 2007). The function of BM adipose tissue is also yet to be firmly reported. Some reports have been demonstrated that BM adipocytes have brown adipocyte like properties which are a distinct type of adipocytes found in mammals and specialized for thermogenesis. Instead, BM adipocytes may have more white adipocyte-like properties that are specialized to store excess energy as endocrine functions and lipid storage. A third opinion is that BM adipocytes have a characteristic beige adipocyte, a mixture of these two types of adipocytes (Scheller et al. 2016; Suchacki et al. 2016).

2.3 Extracellular Matrix Components of the Hematopoietic Stem Cell Niche

Hematopoietic stem cell DNA contains genetic information required to lead HSC fate as quiescence, proliferation, self renewal, lineage specification, differentiation and/or apoptosis (Choi et al. 2015). But, signals for the activation of these events are required and they are provided from surrounding niche cells, ECM which is local matrix environment and ECM bound or its unengaged molecules are required to trigger these events (Morrison and Scadden 2014). The extracellular matrix which is a complex formation of various proteins including laminin, fibronectin and collagen and also ECM remodeling proteins such as MMPs that defines the mechanical and structural environment, is another crucial component of the BM microenvironment (Sagar et al. 2006). Integrins linking cells to ECM such as αLβ2, αMβ2, α4β1 and α5β1 and their signaling pathways have been involved in HSC

maintenance, differentiation, and mobilization (Klamer and Voermans 2014). Researchers showed that heparin sulfate which is found in the BM ECM and secreted from BM MSCs, is necessary for the adhesion of HSC to the microenvironment. The ablation of this proteoglycan triggers HCSs to the peripheral blood (Saez et al. 2014). Tenascin C and osteopontin, of the ECM secreted by stromal, endothelial and osteoblastic cells, assist hematopoiesis (Li et al. 2018; Ma et al. 2016).

Engineering a simulated BM that restructures ordinary marrow microenvironment and function could be a strong platform to study hematopoiesis and screen new therapeutics. Three-dimensional biomaterial platforms to simulate the HSC microenvironment as a coordinated issue of action needs agreement for HSC fate determination in response to supporting niche cells, biophysical, biomechanical and molecular signals. 'Organ on a chip' is 3D microfluidic cell culture system that mimics the physiological and mechanical conditions that answer complete tissues/organs and/or their environments (Kim et al. 2015). 'BM on a chip', was recently built to mimic as much of the whole HSC niche as possible. Bone matrix chip is embedded under mouse skin, so that native HSCs, MSCs and vascular cells migrate to the matrix chip and finally restructure BM. In this way, vascularization problems could be solved by adding these cells. After that, matrix chip is removed from animals and cultured in microfluidics device (Kim et al. 2015). "BM on a chip" carries out like as a stand to screen drugs and assess therapeutic effect of chemotherapy or irradiation caused damage. The next step will be the humanization and xeno-free design of these matrix chips.

2.4 Physical Factors Affecting the Hematopoietic Stem Cell Niche

Hematopoietic stem cell fate can also be affected by biophysiological stimulants such as circadian rhythms (Ieyasu et al. 2014), hormonal signals (Hoffman and Calvi 2014), sympathetic innervations (Katayama et al. 2006; Kose et al. 2018), and oxygen tension or hypoxia. Circadian HSC network is regulated in the CNS by group of clock genes (Bmal1, Npas2, Clock) that modify HSC movement to BM microenvironment by rhythmic secretion of epinephrine from nerve terminals, activation of the adrenergic receptor, degradation of Sp1, and down regulation of SDF-1 (Mendez-Ferrer et al. 2009; Giudice et al. 2010). These findings show that the CNS can directly adjust the function of a HSC niche in BM. The circadian guided infusions in clinical trials have reported promising results (Levi and Schibler 2007). Another important physiologic modulator in the fate of HSC is hypoxia. Deletion of the Hif1a gene promotes, HSC proliferation was supported, while increases HSC quiescence is increased by pharmacological stabilization of HIF-1a protein. This data demonstrated that HIF1a is a critical regulator of HSC fate (Takubo et al. 2010; Forristal et al. 2013). Genetic deletion of Hif1a gene affects bone formation in osteoblasts (Wang et al. 2007).

Combination of small molecules such as SR1 and UM171 which inhibits AHR pathway and suppresses transcripts related to differentiation of megakaryocyte and erythroid cells expanded human cord blood HSCs (Sugimura 2016). Rapamycin, valproic acid, lithium and PGE2 (in phase II trial) are the most remarkable examples of successful maintain, expansion and engraftment interventions for HSCs by preventing their differentiation (Sugimura 2016).

2.5 Biomechanical Forces Affecting the Hematopoietic Stem Cell Niche

Like many tissues, there is significant biomechanical and structural heterogeneity within the BM. Indeed, recent studies showed that HSC subsets respond to changes in topography (Kurth et al. 2011) and material elasticity (Choi and Harley 2012). Blood flow is a critical determiner for the vascular remodeling, arterial lineage specification, and hematopoiesis (Chouinard-Pelletier et al. 2013). Three types of hemodynamic forces have been defined for the hematopoietic and vascular cell development; shear

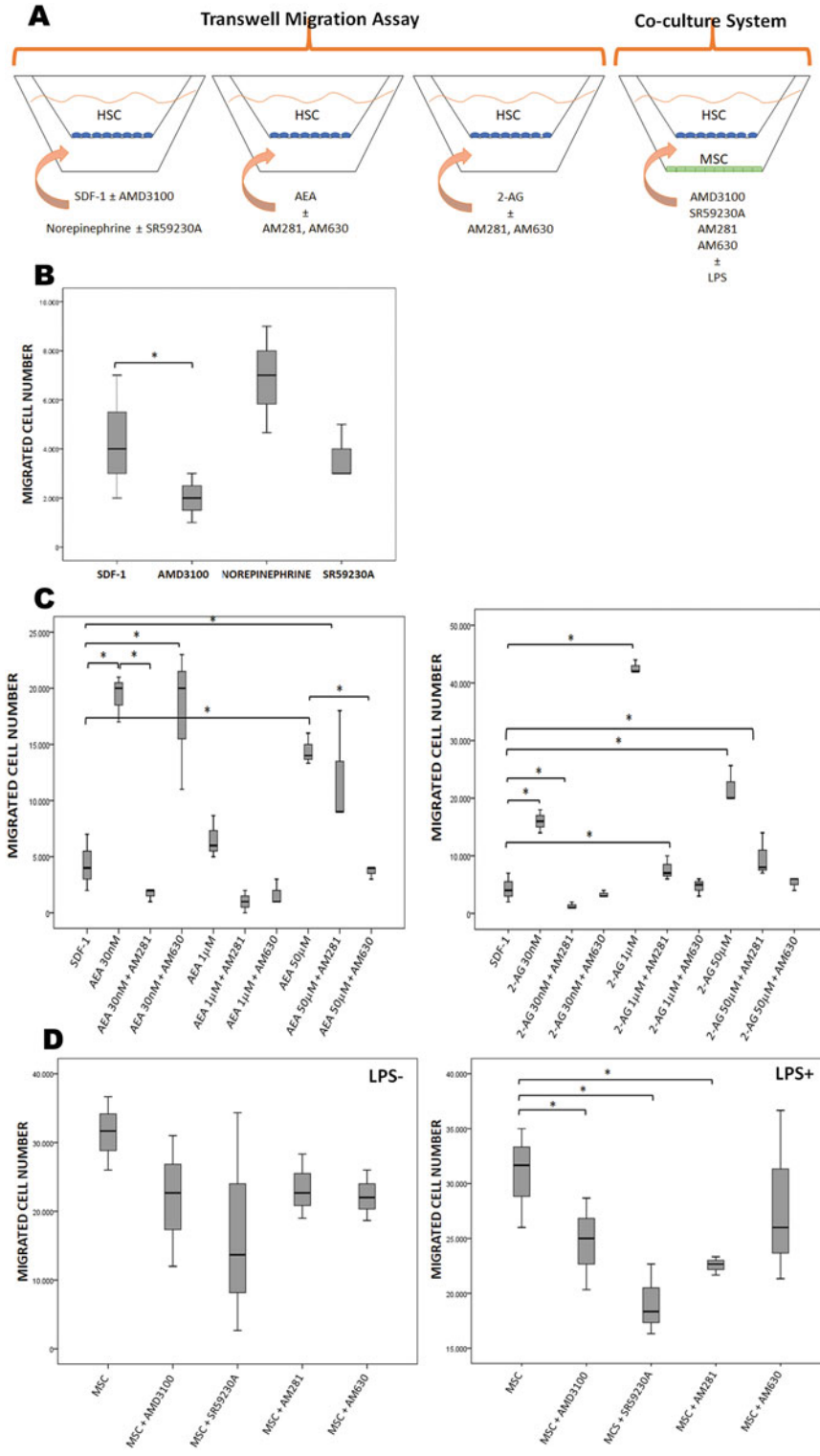

Fig. 4 Endocannabinoids (AEA and 2-AG) stimulate CD34 + HSC migration to MSCs and this migration effect is blocked by beta adrenergic receptor and cannabinoid receptor 1 and 2 antagonists (AM281 and AM630, respectively). (**a**) Experimental design for transwell migration assay is shown. The co-culture system allows CD34+ HSC migration toward

stress, hydrostatic pressure and stretching. Especially, shear stress has important involvement for hematopoietic signaling (Adamo et al. 2009). The peripheral blood pressure/shear stress has been reported to be in the range of 110–140 mmHg in animals, whereas intramedullary pressure is about 30 mmHg (Gurkan and Akkus 2008). Hematopoietic cells would not be directly exposed to fluid forces of these magnitudes in the endosteum but could be impacted by osteocyte mechanotransduction and biochemical signaling. Pericytes and endothelial cells are also likely to connect with forces of fluid and either directly or indirectly deliver signals to HSC/HSPCs that tightly regulate cell cycling and mobilization. These signals can be received from hematopoietic cell by mechanosensors and signaling pathways such as cell adhesion molecules (especially integrins), ADAM family, NO signaling and GPCR superfamily (Even-Ram et al. 2006). It has also been determined that the MSCs from the support cells vary according to the surface topography of the material produced on the differentiation potentials (KÖSE et al. 2016).

Open-cell foam biomaterials made using different materials (scaffolds) have been adopted as analog of BM physical environment (Bello et al. 2018). The biomechanical signals provided by these materials have been used to stimulate various cell behaviors such as proliferation, migration, differentiation or cell fate and also apoptosis (Sugimura 2016).

2.6 Autonomic Innervation of the Hematopoietic Stem Cell Niche

The sympathetic activation and the subsequent beta adrenergic system involvement is well described in BM under physiologic and stress conditions and stimulation of HSC mobilization (Beiermeister et al. 2010). Sympathetic noradrenergic stimulus suppresses microenvironmental functions of nestin negative stromal cells via β3 adrenergic receptors and adjust rhythmic release of HSCs to blood stream (Mendez-Ferrer et al. 2008). Sympathetic signaling is also comprised in the HSC mobilization from the niche supported cells stimulated by G-CSF (Katayama et al. 2006; Asada and Katayama 2014). Although endocannabinoids functioning as neurotransmitters playing role in HSC migration/mobilization, similar to mobilization is activated as a result of stress induced sympathetic activity in the human BM niche. In our study, elements of the endocannabinoid system and their interaction with adrenergic receptor subtypes were demonstrated on HSCs and MSCs of G-CSF treated and untreated healthy donors *in vitro* (Fig. 4). Data revealed that endocannobinoids might be potential candidates to induce or modify G-CSF-mediated HSC mobilization (Kose et al. 2018).

2.7 Age or Disease Related Decline of Hematopoietic Stem Cell Niche Support

Similar to other stem cell niches, the hematopoietic system members are sensitive to the harmful effects of aging. Identifying mechanisms that rely on hematopoietic and/or hematopoietic niche aging are critical for understanding hematopoietic system related diseases (Latchney and Calvi 2017; Kovtonyuk et al. 2016). Aged HSCs exhibit enhanced mobilization from the BM into the peripheral blood in response

Fig. 4 (continued) SDF-1, norepinephrine, AEA, 2-AG or MSCs respectively. (**b**) The CD34+ HSC migration toward SDF-1 and NE are inhibited by specific beta-adrenergic receptor antagonists (AMD3100 and SR59230A, respectively). (**c**) Migration of CD34+ HSCs to endocannabinoids; AEA and 2-AG. CD34+ HSCs exhibited significantly higher migration to 30 nM and 50 μM doses of AEA, and 30 nM, 1 μM and 50 μM doses of 2-AG, when compared to SDF-1. This migration effect is blocked by cannabinoid antagonists. (**d**) CD34+ HSCs effectively migrated towards LPS stimulated (LPS +) and unstimulated (LPS-) MSCs. Migration effect is blocked by CB1 antagonist AM281, CXCR4 antagonist AMD3100 and the beta adrenergic receptor blocker SR59230A significantly (* $p < 0.05$, $n = 6$) (Kose et al. 2018)

to cytokines and chemotherapy compared to young HSCs and reduced homing and/or engraftment to the BM. However, researchers reported that young and aged HSCs choose different anatomical niches *in vivo* at BM (Latchney and Calvi 2017; Florian et al. 2012). Additionally, there is increased mobilization of aged HSCs to the blood in response to chemotherapy and cytokines comparing to young HSCs (Xing et al. 2006; Geiger et al. 2007). Aged HSCs have also decreased adhesive properties (Geiger et al. 2007). With age, there is increased adipocyte cell and fat tissue content in the BM attributed to the differentiation of BM MSCs. This is reciprocally correlated with SDF-1 plasma levels in the elderly (Tuljapurkar et al. 2011). These data demonstrate that there is a pivotal interaction between HSC and their niche. Consequently, modification in niche composition such as ECM composition, cell to matrix adhesion and thus aberrant interaction between HSCs and their niche can cause HSC aging. The bone marrow microenvironment may be responsible not only for aging but also for hematopoietic system diseases, either directly or indirectly. Therefore, within the regenerative perspective, it is very important that the BM niche can be well understood and mimicked for a regenerative perspective strategy for diseases or for the improvement of HSC transplantation.

3 Spermatogonial Stem Cell Niche/Microenvironment

3.1 Embryogenesis of Spermatogonial Stem Cells

Spermatogenesis is a complicated and coordinated process which takes place in the seminiferous tubules of the male testis by which spermatozoa are produced daily from SSCs (de Rooij 2017). Like all other stem cells, SSCs have self-renewal and differentiation abilities and are the only type of cells that transmit genetic information to the upcoming generations. (Mei et al. 2015).

In mice, development of SSCs begins with PGCs derived from epiblast cells. The primordial germ cells migrate from their original location into the hindgut endoderm at embriyonic day 7.5 (E7.5) (Dong et al. 2015b; Cantú and Laird 2017). The primordial germ cells then move through dorsal mesentery to colonize the gonadal ridges at E 11.5 (Cantu et al. 2016). They develop into gonocytes at E12.5, then enter mitotic arrest in the G0/G1 at E13.5 and stay quiescent till approximately PN 1-2. After birth, the gonocytes migrate from initial location of the seminiferous cord to the basal membrane and transform into the SSCs (Chassot et al. 2017). In mice, SSCs or Asingle (As) spermatogonia localized at basal compartment of seminiferous tubules either divide into two single cells or into a pair of spermatogonia (Apr). The Apr spermatogonia divide to produce 4, 8 or 16 Aaligned (Aal) spermatogonia. The Apr and Aal spermatogonia are called "undifferentiated spermatogonia". Differentiation starts from A1 spermatogonia. The Aal cells differentiate without mitotic division into A1 spermatogonia undergoing five mitotic divisions to form A2, A3, A4, intermediate and B type spermatogonia. Type A1-A4, intermediate and B spermatogonia are called as "differentiating spermatogonia". The type B spermatogonia divide into primary spermatocytes undergoing two meiotic divisions to form spermatids. The spermatids undergo a series of differentiation steps to develop into mature spermatozoa (Potter and DeFalco 2017) (Fig. 5).

Human PGCs can be identified during the fourth week of gestation within the endodermal layer of yolk sac. Between 4 and 6 weeks, PGCs move from the yolk sac to hindgut endoderm and then migrate via dorsal mesentery to gonadal ridges. PGCs (usually called gonocytes) remain dormant from the sixth week of embryonic development until puberty. Seminiferous tubules mature and the PGCs differentiate into spermatogonia at puberty (Mamsen et al. 2012). In humans, spermatogonia can be categorized into three types: Adark, Apale and type B spermatogonia. Whereas Adark spermatogonia are the reserve stem cells, Apale spermatogonia are called as renewing stem cells. During the prepubertal period, SSCs differentiate

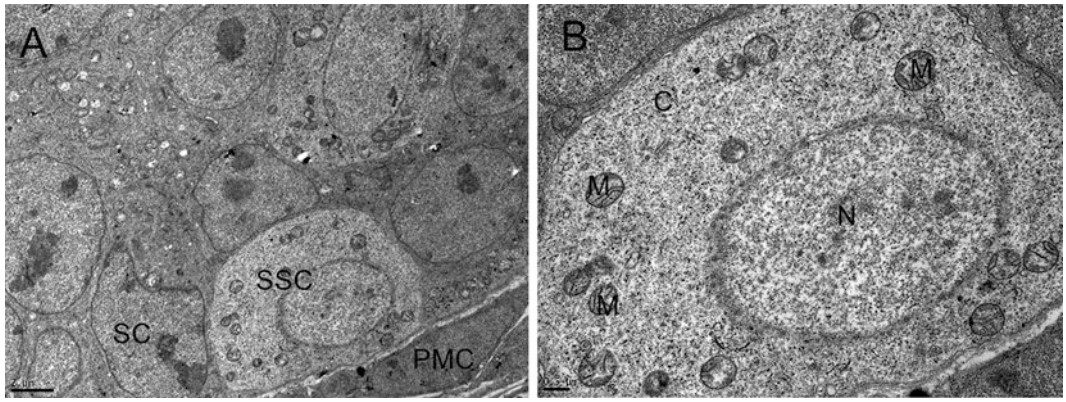

Fig. 5 Transmission electron micrographs of 6-day-old-mouse testis. Undifferentiated spermatogonial cells are shown with their spherical nucleus (*N*) exhibiting small clumps of heterochromatin. Note numerous mitochondria (*M*) within the cytoplasm (*C*) of SSCs. Peritubular miyoid cells (*PMC*) surround seminiferous cords. Sertoli cell (*SC*). Uranyl acetate and lead citrate. (**a**) 6000×, (**b**) 20000×

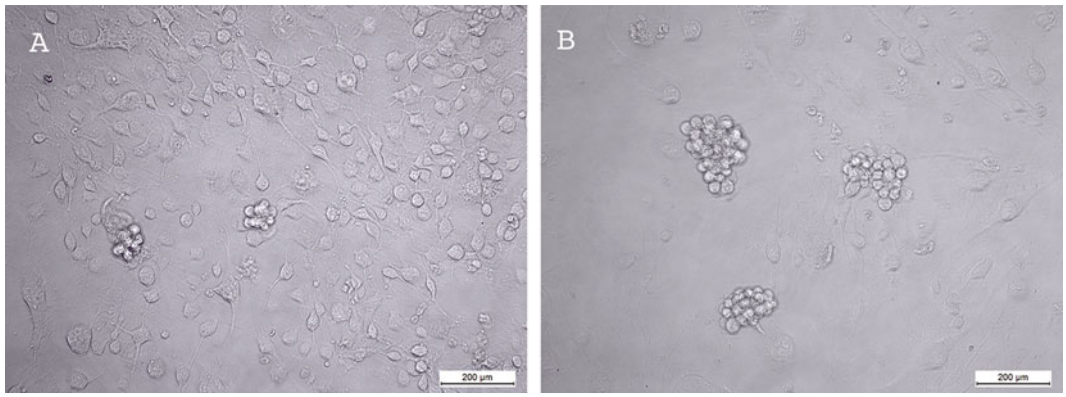

Fig. 6 Micrographs of of cultured SSCs. Thy-1(+) SSCs were placed on STO feeder cells. Colonies of 6-day-old mouse SSCs on 3th day of culture is observed in (**a**);. Colonies belong to 6-day-old mouse SSCs on 10th day of culture in (**b**). (**a**) 200×, (**b**) 200×

into B spermatogonia (Hai et al. 2014; van den Driesche et al. 2014).

In our laboratory we isolated SSCs from 6-day-old C57BL/6 mouse testis. We used a combination of different techniques which include enzymatic digestion, 30% percoll gradient and MACS separation with Thy1.2 microbeads to isolate SSCs. The Thy1.2 (+) SSCs are maintained on SIM-STO feeder layer in mouse serum-free medium containing 1 ng/mL human bFGF, 150 ng/mL GFRα-1 and 20 ng/mL GDNF. In the first day of culture, SSCs are single and adhere to the feeder layer and on the 3rd day the cells start to form colonies. The spermatogonial stem cell colonies continuously proliferate under same culture conditions (Fig. 6).

3.2 Cellular Constituents of the Spermatogonial Stem Cell Niche

The spermatogonial stem cells reside in a special niche similar to other HSCs. Testicular niche cells consisting of SCs, LCs, PMCs, PMs, IMs and vascular ECs determine the fate of SSCs (Potter and DeFalco 2017).

3.2.1 Sertoli Cells

Sertoli cells are polygonal, non-replicating cells resting on the basement membrane of the seminiferous epithelium. The Sertoli cells play central role to regulate spermatogenesis by secreting several factors and support the SSCs survival since they are located adjacent to SSCs in epithelium of the seminiferous tubules (Hai et al. 2014). Sertoli cell to SC junctions create BTB which physically separate the epithelium of seminiferous tubule into two partition: basal and adluminal (Hai et al. 2014). The blood-testis barrier is formed by several types of junctions consisting of TJs, basal ES and desmosome-gap junctions (Zhang et al. 2014). Undifferentiated spermatogonia including SSCs and preleptotene spermatocytes are found in the basal part, while the leptotene, zygotene, pachytene and diploten spermatocytes, and all post-meitotic spermatids are found at the adluminal part of the seminiferous tubule. The junctional complexes between SCs undergo remodeling to permit differentiating germ cell moving to the adluminal part during spermatogenesis. In addition, intermediate filaments make desmosome-like junction between SCs and SSCs. Sertoli cells do not only provide physical support but also regulate spermatogenesis via their paracrine factors (Schrade et al. 2016).

The most important paracrine growth factors secreted by SCs are GDNF, FGF and, the BMPs. These factors are indispensable for determining fate of SSCs both in *in vitro* and *in vivo* conditions (Hai et al. 2014). Sertoli cells can be used as feeder layer to support SSCs *in vitro* conditions. Numerous studies have shown that the colony number and diameter of SSCs increase when SCs act as feeder layer compared to other feeder cells like MEF and STO (Hai et al. 2014).

The binding of GDNF to GFRA1 and RET receptor initiates several pathways like PI3K/AKT pathway, SFK pathway or MAPK pathway to promote self-renewal of SSCs and maintain their undifferentiated properties. Fibroblast growth factor 2 which is also known as bFGF stimulates self-renewal of SSCs. FGF2 activate PI3K/AKT and MAPK pathway via FGFR2 located on the cell surface of SSCs. Apart from that, FGF2 may contribute to SSC maintenance by stimulating GDNF released from SCs (Sargent et al. 2016).

The CXCL12/CXCR4 pathway promotes also proliferation of SSCs and, blocks RA-induced differentiation of SSCs. Sertoli cells express CXCL12 and, binds to its receptor CXCR4 on SSCs. This signaling has significant role to regulate migration of SSCs following transplantation into recipient testes. (Yang et al. 2013).

Sertoli cells not only stimulate several pathways to promote proliferation of SSCs but also promote differentiation of SSCs through various molecules including BMPs, SCF, and, the RA. BMP4 has effects on differentiation of SSCs through its receptor ALK3 and SMAD5 expressed by undifferentiated spermatogonia. In addition, BMP4 may induce expression KIT receptor in spermatogonia (Rossi and Dolci 2013). Stem cell factor (KIT ligand) secreted SCs promote differentiation of SSCs through KIT receptor tyrosine kinase present on the cell surface of differentiating spermatogonia. The transition of Aal spermatogonia into A1 spermatogonia via KIT ligand/KIT pathway is crucial to expand differentiating spermatogonia pool (Rossi and Dolci 2013). The KIT receptor can be used as a marker to distinguish differentiating spermatogonia from undifferentiated spermatogonia including SSCs. In addition to these factors, RA induces the differentiaon of Aal into type A1 differentiated spermatogonia (Meistrich and Shetty 2015). Soluble factors released from SCs effect each other through different ways on differentation of SSCs. Retinoic acid signaling induces the production of BMP4 in Sertoli cells, then BMP4 may induce expression of KIT receptor in spermatogonia.

3.2.2 Leydig Cells

The testicular interstitial tissue consists of Leydig cells, blood vessels and macrophages**.** Cytoplasm of Leydig cell is eosinophilic due to presence of lipid droplets. The activation of Leydig cells change during lifetime. They are active during early development of the male fetus, then they are inactivated from about 5 months of fetal life to

puberty. They again become androgen-secreting active cells at puberty and remain active through life.

Hormonal interactions between SCs and LCs control spermatogenesis. The Leydig cells and SCs stimulate gonadotropic hormones LH and FSH respectively. Luteinizing hormone binds to the LH receptor expressed by LCs and then, LCs produce and secrete testosterone. After FSH stimulation, SCs produce ABP that binds testosterone so it increases accumulation of testosterone in the abluminal part of the seminiferous tubule (Shiraishi and Matsuyama 2017). Leydig cells secrete also CSF1 to control SSCs' renewal mediated by CSF1R expressed by SSCs. When culture medium with GDNF/FGF2 is supported by CSF-1 increase the numbers of SSCs in *in vitro* conditions (Potter and DeFalco 2017).

3.2.3 Peritubular and Interstitial Macrophages

Peritubular and interstitial macrophages have roles in determination of SSCs fate by either supporting self-renewal or beginning differentiation. While PMs are associated with PMCs and blood vessels, IMs are associated with LCs and blood vessels. Peritubular macrophages differ from the interstitial macrophages by level of CSFR1 and MHCII expression (Meistrich and Shetty 2015). Both peritubular and IMs produce SSC renewal factor CSF1 and differentiation-inducing factors such as enzymes involved in RA biosynthesis (DeFalco et al. 2015).

3.2.4 Peritubular Myoid Cells

Peritubular cells are very thin, smooth muscle-like cells and they have very important role in male infertility by transporting immotile sperm by using their contractile abilities. Unlike the single layer of PMCs surrounding the seminiferous tubule in rat, the peritubular wall in human testes consists of several layers of PMCs. The peritubular myoid cells can also contribute to SSC niche with their secretory factors since they are are only separated by a basal lamina from the SSCs (Mayerhofer 2013). The testosterone-regulated GDNF secretion by PMCs supports SSCs' self- renewal. Peritubular cells secrete also CSF1 that regulate SSC activity through CSF1R.

3.2.5 Vascular Endothelial Cells

Principal source of blood to the testis is from the testicular artery, which derives from the aorta. Vascular network between seminiferous tubules effects location of SSCs. While SSCs reside on the basement membrane close to vascular network and interstitial cells, differentiating cells move away from basement membrane to lumen (Kusumbe et al. 2016).

VEGFA have roles in endothelial cell proliferation, survival, migration and permeability. While VEGFA is found on chromosome 6 in humans and four distinct isoforms (VEGFA206, VEGFA189, VEGFA165, and VEGFA121) have been demonstared, VEGFA is found on chromosome 17 and isoforms (VEGFA120, VEGFA205, VEGFA188, VEGFA164) have been identified that are homologous to those found in humans (Sargent et al. 2016). Some of these isoforms are angiogenic, whereas others are anti-angiogenic. Anti-angiogenic isoforms lead to reduction number of SSCs either by promoting differentiation or by interfering with SSC formation. Angiogenic isoforms stimulate SSC self-renewal. VEGFA is produced by SCs and LCs and its receptor KDR is expressed in spermatogonia. Production of VEGFA by LCs and SCs is stimulated in response to hCG/LH and FSH respectively.

3.2.6 Epididymal White Adipose Tissue

Epididymal white adipose tissue has important effects on gonadal function by means of local factors. It has been showed that removal of EWAT causes spermatogenic failure and testicular degeneration. Adipocyte within the EWAT express androgens affecting the other niche cells through androgen receptors located on SCs and PMCs (Hansel 2010; Jalali 2017). The removal of EWAT leads to significant decrease in GDNF expression (Jalali 2017). Decrease in GDNF expression causes loss of SSCs since this is the essential growth factor expressed by Sertoli cells to stimulate SSC self-renewal. Testosterone secreted by EWAT promotes GDNF secretion by PMCs. According to these studies EWAT regulates spermatogenesis via release of

androgens that directly acts on SCs and PMCs. In addition to androgens, EWAT produces leptin (Hansel 2010). Leptin is a peptide hormone regulating food intake, body metabolism and reproductive function. Leptin acts through its specific receptors located in hypothalamus, liver, lung, kidney, pancreas, hematopoietic cells and gonads (Fasshauer and Bluher 2015). The neonatal mouse SSCs express leptin and leptin receptors. Leptin may stimulate proliferation of SSCs via both paracrine and autocrine mechanism (Landry et al. 2013). Leptin deficiency impairs spermatogenesis and leads to loss of germ cells in mouse (Bhat et al. 2006).

3.3 Extracellular Matrix Components of the Spermatogonial Stem Cell Niche

Each testis is surrounded by dense connective capsule, the tunica albuginea and is divided into lobules by septa that project from the tunica albuginea. Each lobule is composed of one to four seminiferous tubules surrounded by tunica propria containing PMCs. Seminiferous epithelium consists of SCs and spermatogenic cells. Between the seminiferous tubules interstitial compartment consists of LCs, macrophages, other immune-competent cells and blood vessels. In 6-day-old mice, seminiferous cords only contain SCs and spermatogonia (Fig. 7).

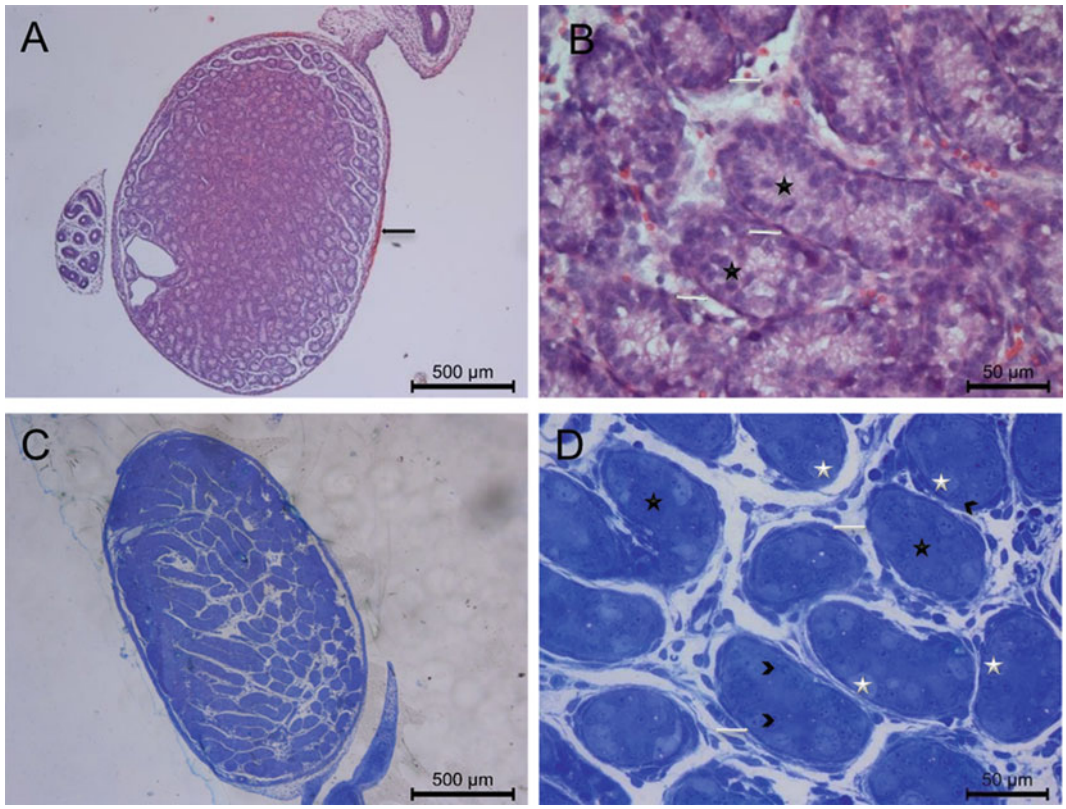

Fig. 7 Light micrographs of 6-day-old-mouse testis sections stained with hematoxylen-eosin (H&E) (**a**, **b**) and methylene blue- azur II (**c**, **d**). The testis is covered by thick connective tissue capsule, Tunica albuginea (black arrows). The bulk of testis is composed of seminiferous cords (black asterisks) and interstitial tissue. Each seminiferous cord is surrounded by tunica propria; consists of myoid cells (white arrows). Sertoli cells have small ovoid nuclei and are organized perpendicular to the basement membrane (black arrowheads). Undifferentiated SSCs with large spherical nuclei are located at the basal compartment of the seminiferous epithelium (white asterisks) (**a**) 50×, (**b**) 400×, (**c**) 50×, (**d**) 400×

Extracellular matrix has a significant role to regulate spermatogenesis (Eslahi et al. 2013). Sertoli cells and the PMCs secrete collagen α1(IV), α2 (IV), α3 (IV) that builds the basement membrane of seminiferous tubule with the other components such as laminin, entactin and heparin sulfate proteoglycan. Spermatogonial stem cells express both α6- and β1-integrin (laminin receptor components) providing homing of SSCs adjacent to the basement membrane. Recent studies have demonstrated that impaired β1-integrin expression disrupts the reestablishment of spermatogenesis following transplantation of SSCs, however the SSCs translocate to the basement membrane. Since their structural abnormalities are associated with infertility, this problem can be solved using scaffolds that mimic ECM. The scaffolds contribute three-dimensional biomimicking and send appropriate signals to the cells, thus may provide physiologically relevant cellular phenotype. Several artificial carbon nanotubes, poly-L-lactic acid nanofibers, 3D soft agar culture systems, human serum albumin/tri calcium phosphate nanoparticles and electrospun polyamide nanofibers have been used to enhance the self-renewal of SSCs (Yadegar et al. 2015). Recently decellularized matrices have been used as biomimicking niche engineering strategy (Yu et al. 2016). It has been demonstrated that adult and pubertal testicular cells can self-organize into human testicular organoids within a decellularized scaffold (Baert et al. 2017). These findings indicate that tissue compatible bioscaffolds can be used in regenerative medicine, tissue engineering, assisted reproductive technology for treatment of infertility in adult males and pediatric cancer patients to restore spermatogenesis.

3.4 Physical Factors Affecting the Spermatogonial Stem Cell Niche

The spermatogenesis is not only controlled by extrinsic factors delivered by niche cells but also regulated by physical factors including temperature and O_2 level (Jankovic Velickovic and Stefanovic 2014). Testicular temperature is maintained as 4–5 °C below body-core temperature for normal spermatogenesis. Since testis is a naturally O_2-deprived organ, undifferentiated SSC self renewal may be enhanced in the range of 3–5% O_2 in *in vitro* culture condition. While physiological hypoxia maintains SSC self-renewal and spermatogenesis, pathological low oxygen pressure or content causes male infertility. Degeneration of germinal epithelium, increase in germ cell apoptosis, poor vascularization and, decrease in testicular mass can be observed in pathological conditions (Jankovic Velickovic and Stefanovic 2014).

3.5 Biomechanical Forces Affecting the Spermatogonial Stem Cell Niche

Provision of nutrients and oxygen by capillaries surrounding the tissue and removal of waste products regularly, promote the homeostasis of the tissue in the body. Even homeostasis is facilitated by microvascular system *in vivo*, imitating this system as *ex vivo* is difficult. Until today, several methods have been used to culture tissue or small organs. Although among these methods, interphase method is an effective method in which tissues or small organs are positioned between the culture medium and a gas layer, the method doesn't have any microcirculatory system. To overcome this problem microfluidics in which a porous membrane segregates a small organ or tissue spread in the chamber from the flowing medium flowed through reservoir tank has been applied into cell culture experiments. Both nutrients and waste products diffuse between porous membrane and oxygen reach the small organ or tissue through oxgen–permeable polydimethylsiloxane. The fragments of testis are cultured and successfully maintain spermatogenesis by this method. Then, fertility is succeeded by microinsemination. In addition, testis produces testosterone for a long time and responds to stimulation of LH. These findings show that microfluidic system can be

used to mimic *in vivo* conditions. Although microfluidic device is useful to culture the tissues, it has drawbacks. One of the major drawbacks of the device is power-pump to supply flow of medium. It has been demonstrated that pumpless microfluidic device using hydrostatic pressure provides slow, longer lasting medium flow. So, this device induces spermatogenesis from SSCs up to haploid cell in organ culture system compared to pump-driven methods similarly. (Komeya et al. 2016, 2017).

3.6 Autonomic Innervation of the Spermatogonial Stem Cell Niche

Numerous reports have showed that intratesticular nerves have significant effects on the functions of the testis. The testis receives only autonomic nerve via superior and the inferior spermatic nerves. Most testicular nerves seem to be catecholaminergic. It has been indicated that LCs, SCs, PMCs possess alpha adrenergic receptors (α-ADRs*)* and beta adrenergic receptors (β-ADRs*)*. Catecholamines via these receptors have important role in controlling testicular function. This pathway regulates both LC stereodiogenesis and contraction of PMCs. Numerous peptidergic fibers have been found in superior and inferior spermatic nerve in addition to catecholaminergic fibers in different proportions. The distribution of these two types of nerve changes according to age (Rossi et al. 2018).

Recently, it has been indicated that endocannabinoids are critical regulators of male reproductive system. Endocannabinoids act via CB_1 and CB_2 cannabinoid receptors and specific enzymes regulate level of endocannabinoids. The cannabinoid system consists of cannabis ligands, their receptors and enzymes. In male reproductive system, endocannabinoids affect both niche cells and germ cells. Regulation of SCs function, proliferation of LCs, differentiation of germ cells, motility, capacitation and acrosome reaction of sperm are the important roles of endocannabinoids. The two best known endogenous cannabinoids are AEA and 2-AG (Grimaldi et al. 2013). Endocannabinoids are hydrolyzed by two enzymes: FAAH and MAGL. While anandamide is cleaved by FAAH into arachidonic acid and ethanolamine, 2-AG is transformed into arachidonic acid and glycerol by MAGL. Especially, 2- AG and CB_2 have a pivotal role in mouse spermatogenesis. Level of 2-AG change during spermatogenesis process; spermatogonia have high level of 2-AG, it declines in spermatocytes and spermatids. Activation of CB_2 via autocrine 2-AG in B spermatogonia provide the maintenance of meiosis. Elements of this system effect also SCs and LCs. Cannabinoid receptor type-2, an AEA membrane transporter and FAAH are expressed by SCs and they stimulate apoptosis of SCs. Hormonally up-regulated FAAH expression in SCs by FSH decrease in apoptosis of SCs. Cannabinoid receptor type-1 mediated LC beheviour is regulated during development and it has negative effect on division of LCs. The immature mitotic LCs express CB_1, immature non-mitotic LCs do not. These findings show that CB_1 have negative effect on proliferation of LCs (Grimaldi et al. 2013).

3.7 Age or Disease Related Decline of Spermatogonial Stem Cell Niche Support

The tissue-specific stem cells are considered as immortal due to their endless self-renewal and long life. On the other hand, the niche cells' ability to supply enough microenvironment decrease and this interrupts SSC functions with aging. Testicular aging leads to decrease in LCs function or changes in the pulsatility of LH. Therefore, testosterone secretion decreases from LCs. Decrease in production of testosterone causes decline in GDNF expression by PMCs. GDNF is the most important growth factor regulating spermatogenesis through promoting self renewal of SSCs. Since SCs produce GDNF in response to FSH, decrease in FSH responsiveness leads to reduction of GDNF expression by SCs with aging. It has been showed that transplantation of SSCs from old males into testes of young males improves SSC capacity to

reestablish the spermatogenesis at a normal level. These results indicate that insufficient microenvironment impairs the balance between self-renewal and differentiation of SSCs resulting in decline of spermatogenesis.

The most important side effects of chemotherapy and radiotherapy in pediatric cancer patients are testicular dysfunction and germ cell loss. Since pediatric cancer patients don't have mature sperm, the numerical and functional preservation of SSCs to keep up fertilization is the only manner for patients to have their biological children after cancer treatment. The number of SSCs in the testis is very low (Potter and DeFalco 2017). Thus the isolation and proliferation of these cells are extremely important for pediatric cancer patients.

About 1% of men in the population and 10–15% of infertile men are azospermic (Esteves 2015). Azospermia is described as the lack of sperm in semen and, can be classified as OA or NOA. Nonobstructive azoospermia is characterized by spermatogenic failure and can be subclassified as Sertoli cell only, early or late maturation arrest mixed atrophy, or complete hyalinization of the seminiferous tubules (Gassei and Orwig 2016). Although testicular sperm extraction is possible in NOA patients, if sperm can't be retrieved by this method, SSCs from infertile patients can be used to restore spermatogenesis. The development of SSC isolation and proliferation methods will be very useful for restoring fertility in pediatric cancer patients after cancer treatment and NOA infertile patients.

4 The Hematopoietic and Spermatogonial Stem Cell Microenvironments from Regenerative Medicine Aspect

The system of the body consists of cellular or non-cellular materials and, the interactions occur in between cells and the environment. The sum of all those elements gives us the hierarchial relationships in the organism, and the aim of the modelling in basic, to simulate the physiology of organism such *in vivo*. Despite of their advantages in *in vitro* studies, 2D cultures do not present the real situation about cell to cell and, cell to matrix interactions. Because the 2D system is needed to be manipulated manually, the cells cultured in this system are prone to lose their phenotype (Yin et al. 2016). They may not have the similar ability for the signaling pathways that take place in tissues. Recent stem cell studies have been focusing on how much favorable to create a 3D self-organized culture environment. These 3D systems are close to mimic a tissue/organ model (Yin et al. 2016). The use of different secretomes of accessory niche cells in order to promote proliferation and differentiation of stem cells within 3D *in vitro* environments give promising results as regenerative strategies. While GDNF and RA are used for the proliferation and differentiation of SSCs to spermatozoa in the treatment of infertility (Song and Wilkinson 2014), CXCL12, GCSF, SCF are used for HSC tranplantation in the treatment of hematologic diseases in the same way (Omatsu and Nagasawa 2015).

The mesenchymal stem cells of BM and Sertoli cells of testicular niche constitute the chief supporting cellular elements of the HSC and SSC niches respectively. Both cells mainly behave via several growth factors but similar ECM components in order to mediate their self renewal, differentiation and the subsequent mobilization of the stem and progenitor cell lineages. Integrins are the major cell to matrix adhesion molecules that regulate those decisions. Thus the HSCs and SSCs attach to ECM via different integrin chains and keep their pool. Integrins such as αLβ2, αMβ2, α4β1 and α5β1 and their signaling pathways have been involved in HSC self renewal (Klamer and Voermans 2014). The SSCs and HSCs may undergo differentiation by spermatogenesis and, by making colony forming units respectively when detached form ECM respectively.

The vascular endothelial cells and the osteoblasts (bone lining cells) act similar to MSCs in BM mediating the mobilization by using several ECM adhesion protein expressions. Tenascin C and osteopontin of endothelial and osteoblastic cells assist hematopoiesis (Li et al. 2018; Ma et al. 2016). Pericytes and vascular endothelial cells deliver signals to HSCs that regulate cell cycling and mobilization. The signaling pathways relate to

cell adhesion molecules, glycoproteins and, fibrillar components. Similarly Sertoli cells and the PMCs secrete collagen α1(IV), α2 (IV), α3 (IV) that builds the basement membrane of seminiferous tubule with the other components such as laminin, entactin and heparin sulfate proteoglycan in testicular niche. Vascular endothelium belonging to vessels between seminiferous tubules locates the SSCs in testis. While SSCs rest on the basement membrane close to vessels and interstitial Leydig cells, differentiating cells move away from basement membrane to lumen (Kusumbe et al. 2016). Thus the vascular endothelial cells mediate the selfrenewal and the differentiation of SSCs in the same way with BM vascular endothelial cells via their molecular secretome.

A well-balanced and sustained vascularization is the challenging issue to overcome in *ex vivo* 3D tissue/organ cultures. Use of the foaming scaffolds with bioreactors, decellularized organs/tissues or microfluidic devices is solving this problem (Kim et al. 2015; Komeya et al. 2017). Those bioengineering interventions are widely investigated in order to sustain BM and testicular environments in a reproducible manner. The microfluidic systems work well for maintaining the BM niche in disease modeling studies for hematologic disorders. Recently testis strips are cultured by using microfluidic systems and successfully maintained spermatogenesis. This system provided fertility by microinsemination. These promising findings open the way for new microfluidic technologies in order to mimic *in vivo* conditions for regeneration of BM and the testicular niches.

Engineered synthetic ECM components replacing local matrix environment and the restoration of physical and biomechanical conditions may allow superior imitating and regeneration of BM and testicular niche *in vivo*. Adhesion molecules of ECM and the cytokines are generally used with synthetic matrix platforms in maintenance of stem cell function. Differentiating HSCs and SSCs move away from their initial location to vascular niche and to the lumen of seminiferous tubules respectively. During this process, the mechanical adaptation of the ECM by reorganization of adhesion molecules supports the movement. Both the HSC and SSC populations react to ECM topographical alterations. The shear stress, hydrostatic pressure and stretching mediate the hematopoietic and vascular cell development. The pumpless microfluidic devices with hydrostatic pressure provide spermatogenesis from SSCs up to spermatids in organ culture systems. Different biomaterials may biomechanically stimulate various cell behaviors such as proliferation, migration, differentiation or cell fate and, apoptosis (Sugimura 2016). Thus biomechanical manipulations are potential tools to restore and improve the regenerative capacity of the somatic and germ stem cell niches.

Adipocytes are critical components for somatic and germ cell microenvironments. Both the adipocytes of EWAT and the BM secrete hormones having direct or indirect roles in sustaining the pool of the SSCs and HSCs respectively. Several studies (Hansel 2010; Jalali 2017) report the importance of signaling factors initiated or derived by adipocytes, however, these cells haven't been integrated in 3D culture systems yet. Especially, the cooperation of EWAT with SSCs may be a potential tool in designing biomimetic *in vivo* systems for the maintenance of SSC niche. On the other hand, the possibility of the negative effect of the increasing amount of adipocytes on stem cell pool should be considered, since the BM is an example for this situation. The bone marrow adipocytes increase and the HSC pool naturally decreases with aging (Patel et al. 2018).

Both the testis and BM receive autonomic innervations. Catecholamines of the sympathetic nervous system are induced in stress conditions and regulate the fate of HSCs and the SSCs through adrenergic receptors. They also provide the PMC contraction and LC sterodiogenesis through their receptors on the niche cells. Our group recently reported the induction of a new regulatory pathway operating with beta adrenergic receptors in BM niche. This is the endocannabinoid system. The

endocannabinoid system consists of ligands, their receptors and enzymes. Endocannabinoid ligands act via CB_1 and CB_2 cannabinoid receptors and specific enzymes regulate their level within the body. Endocannabinoids are critical regulators of both hematopoietic and male reproductive system. The accessory cells of both HSC and SSC niches; the MSCs, SCs and LCs promote stem cell differentiation and/or migration via endocannabinoids and. Our group demonstrated that exogeneous and endogeneous 2-AG (a major endocannabionid) secreted from BM MSCs is a potent mobilizing agent that induces the differentiation and migration of of HSCs from BM (Kose et al. 2018). Endocannabionids have also major roles on the regulation of SCs function, proliferation of LCs, differentiation of germ cells, motility, capacitation and acrosome reaction of sperm. The spermatogonia have high levels, but the spermatocytes and, spermatids have low levels of 2-AG. Activation of CB_2 receptors via autocrine 2-AG in B spermatogonia provides the maintenance of meiosis. Thus the endocannabinoid system may be a new potential regulatory system for both HSC and SSC microenvironments deserving further investigation.

5 Conclusion- Future Perspectives for Clinical Medicine

The somatic and germ stem cell microenvironments like BM and testicular niches are complicated systems that regulate quiescence, proliferation, migration and differentiation of their stem cells. The crucial challenge in the *ex vivo* expansion of HSCs or SSCs is to mimic all chemical, biologic and physical systemic constituents in a complete way for restorative and regenerative purposes. Several artificial systems partly imitate *in vivo* conditions. Better understanding of HSC and SSC niche biology by making an analogy between those two environments with a regenerative perspective would be beneficial in order to create the whole orchestra or at least minorize the problems. Therefore, the review has focused on the comparison of SSC and HSC niches on which the team is concentrating their experimental work. Further basic and translational studies may provide new regenerative perspectives for the personalized treatment of infertility, auto-immune diseases, leukemia and, metabolic diseases related to stem cell niches.

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Dental Stem Cells and Tooth Regeneration

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Abstract

Dental stem cells are a minor population of mesenchymal stem cells existing in specialized dental tissues, such as dental pulp, periodontium, apical papilla, dental follicle and so forth. Standard methods have been established to isolate and identify these stem cells. Due to their differentiation potential, these mesenchymal stem cells are promising for tooth repair. Dental stem cells have been emerging to regenerated teeth and periodontal tissues, ascribe to their self-renewal, multipotency and tissue specific differentiation potential. Therefore, dental stem cells based regeneration medicine highlights a promising access to repair damaged dental tissues or generate new teeth. In this review, we provide an overview of human dental stem cells including isolation and identification, involved pathways and outcomes of regenerative researches. A number of basic researches, preclinical studies and clinical trials have investigated that dental stem cells efficiently improve formation of dental specialized structure and healing of periodontal diseases, suggesting a great feasibility and prospect of these approaches in translational medicine of dental regeneration.

Keywords

Dental stem cells · Mesenchymal stem cells · Tooth regeneration

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Abbreviations

3-D	3-dimensional
ALP	alkaline phosphatase
bFGF	base fibroblast growth factor
BMMSCs	bone marrow mesenchymal stem cells
BMP2	bone morphogenetic protein 2
BSP	bone sialoprotein
DFCs	dental follicle cells
DKK1	Dickkopf 1
DMP1	dentin matrix protein1
DNCPs	dentin noncollagenous proteins
DPSCs	dental pulp stem cells
ECM	extracellular matrix
EMD	enamel matrix derivate
GCN5	general control nonrepressed protein 5
G-CSF	granulocyte colony-stimulating factor
GTR	guided tissue regeneration
HA/TCP	hydroxy apatite/tricalcium phosphate
ICAM1	intercellular adhesion molecule 1
IGF-1	insulin-like growth factor-1
iPS	induced pluripotent stem cells
ITGB1	integrin b1
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEPE	matrix extracellular phosphoglycoprotein
OCN	osteocalcin
PDL	periodontal ligament
PDLSCs	periodontal ligament stem cells
PRP	platelet rich plasma
SCAPs	stem cells from apical papilla
SHEDs	stem cells of human exfoliated deciduous teeth
TDM	treated dentin matrix; GMP: Good Manufacturing Practice.
TERT	telomerase reverse transcriptase
TNF-α	tumor necrosis factor-α

1 Introduction

Teeth are composed of hard tissues including outer layers of enamel of the crown/cementum of the root and an inner layer of dentin which enclose the soft pulp tissue containing blood vessels and nerves, etc. Tooth-supporting structures consist of gingival, periodontal ligament and alveolar bone.

Various dental stem cells have been identified from different teeth and tooth-supporting tissues which shared similar *in vitro* properties with bone marrow mesenchymal stem cells (BMMSCs) such as dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAPs) and dental follicle cells (DFCs) (Sharpe 2016) (Fig. 1). Current treatments with artificial materials for tooth defect and tooth loss can restore the esthetic and function of tooth to a certain extent, still several complications following the treatments can be a big headache for dentists. Thus, tooth regeneration with dental stem cells has been studied for many years and achieved great progress. A better understanding of the properties of different dental stem cells and their possible application in tooth regeneration is necessary. This review provides an overview of key findings and advances of dental stem cells and tooth regeneration.

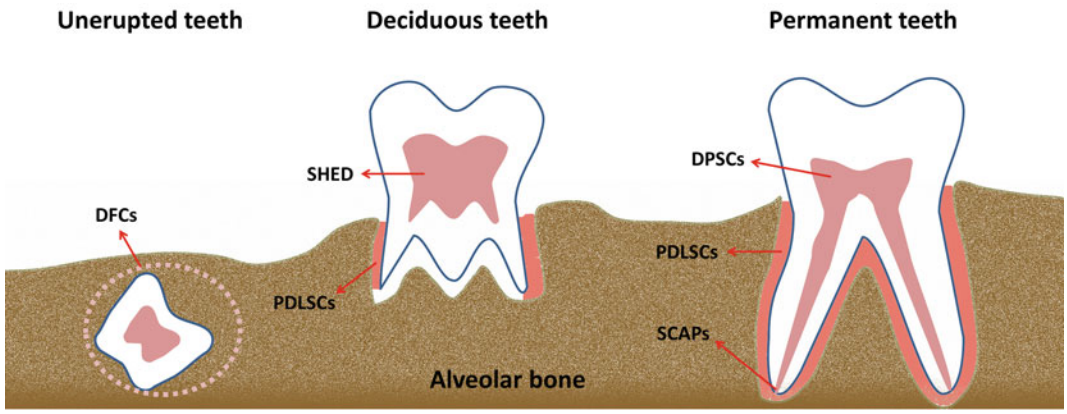

Fig. 1 Location and origin of dental stem cells

2 DPSCs and Dental Pulp Regeneration

Dental pulp tissue consists of odontoblasts, fibroblasts, nerves, immune cells and stem cells, etc. which work as a pulp-dentin complex and hold the function of tooth development, nutrition supply, dentin mineralization, sensory and immune response (Ajay Sharma et al. 2015). It is a very vulnerable soft tissue to different stimulations such as infection and trauma which requires effective clinical treatments. Conventional endodontic treatments including dental pulp capping and root canal therapy merely maintain the structure and function of teeth for prolonged periods of time. However, they fail to sustain the vitality of dental pulp and bring about complications such as lack of capacity of forming reparative dentin, vulnerability to mastication and discoloration, etc. (Zhang and Yelick 2010). Therefore, maintaining dental pulp vitality would be the aim and challenge of future endodontic treatments.

2.1 DPSCs Isolation and Identification

Human dental pulp stem cells (DPSCs) were first isolated and identified from impacted third molar in 2000 by Gronthos et al. with clonogenic and dentin-like structure forming capacity (Gronthos et al. 2000). Human deciduous teeth can also be a resource of dental pulp stem cells and these cells are known as SHED (stem cells of human exfoliated deciduous teeth) (Miura et al. 2003). Explant culture and enzymatic digestion methods of isolating DPSCs have been applied and compared and results indicated that both methods are efficient to yield stem cell populations capable of colony formation and muti-differentiation (Hilkens et al. 2013). Several markers of DPSCs have been reported and used to identify DPSCs including STRO-1, CD29, CD44, CD73, CD90, CD105 and CD146, etc. as positive and CD34, CD45 and CD71,etc. as negative (Suchanek et al. 2009; Kawashima 2012). Different resources of DPSCs have been investigated intensely. DPSCs can be obtained from both permanent teeth and primary teeth, especially impacted third molar and exfoliated deciduous teeth, also supernumerary tooth has been used (Gronthos et al. 2000; Miura et al. 2003; Huang et al. 2008). Growth rate and differentiation capacity of DPSCs and SHEDs have been compared and it showed that SHEDs hold higher proliferation and differentiation capacity while DPSCs possess higher inflammatory cytokines levels which suggested SHED might represent a more proper source for tooth regeneration (Kunimatsu et al. 2018). Extensive expansion *in vitro* of DPSCs and SHED can alter stem cell properties such as proliferation and differentiation, thus proper passages of DPSCs and SHED shall be carefully chosen before being

applied in clinics (Wang et al. 2018a). Long-term cryopreservation have been proved to be an effective way to preserve tissue and stem cells as stem cells from dental pulp after 2 years' cryopreservation still express stem cell surface antigens and hold their differentiation capacity and cryopreserved dental pulp tissues from exfoliated deciduous teeth owned similar stem cell properties (Papaccio et al. 2006; Ma et al. 2012). Therefore, cells and tissues after long-term cryopreservation can be a useful and reliable resource for regenerative medicine.

2.2 DPSCs Properties and Pathways

Numerous pathways are involved in DPSCs differentiation thus regulating their regenerative capacity. DNA microarray was performed to analyze the gene expression profile of DPSCs and SHEDs and results showed that genes that participate in pathways related to cell proliferation and extracellular matrix were expressed higher in SHEDs than DPSCs (Nakamura et al. 2009). Canonical Wnt signaling inhibited odontoblast differentiation capacity of DPSCs (Scheller et al. 2008). IGF-1 could enhance proliferation and osteogenic differentiation of DPSCs and mTOR pathway was involved (Feng et al. 2014). Odonto/osteogenic differentiation of DPSCs can be regulated by estrogen level, LPS stimulation, TNF-α stimuation via NF-κB pathway (Wang et al. 2013; He et al. 2015; Feng et al. 2013). Biological materials hold the capacity of regulating DPSCs properties via different pathways. Natural mineralized scaffolds promote odontogenic differentiation and dentinogenic potential of DPSCs via MAPK pathway (Zhang et al. 2012). With better understanding of DPSCs molecular mechanisms especially pathways involved in their proliferation and differentiation, methods to increase DPSCs regenerative capacity would be chosen more wisely.

2.3 Dental Pulp Regeneration

As DPSCs hold the ability to differentiate into odontoblasts, they have been used directly for dental pulp regeneration or in vitro study for optimizing biocompatible materials. Dental pulp regeneration research and clinical trial have been the focus for years to replace the conventional treatments.

Cell-based therapy has been widely used in both animal studies and clinical trials which is isolation and *ex vivo* expansion of stem cells and transplantation into dental pulp. Studies indicated that vascularized pulp-like tissue was generated by transplantation of DPSCs or SHEDs seeded in biodegradable scaffolds in immunodeficient mice (Cordeiro et al. 2008; Prescott et al. 2008). Following studies showed that both DPSCs and SHEDs seeded onto some scaffolds, were able to form vascularized pulp/dentin-like tissue in an emptied human root canal which had been subcutaneously transplanted into immunodeficient (SCID) mice (Huang et al. 2010; Rosa et al. 2013). In large animals, reparative dentin was formed after autologous transplantation of DPSCs pellets stimulated by BMP-2 onto the amputated pulp of dog teeth (Iohara et al. 2004). Autologous transplantation of DPSCs mobilized by granulocyte colony-stimulating factor (G-CSF) in dog pulpectomized tooth was taken and proved to be able to regenerate complete pulp/dentin tissue with an apical opening of 0.6 mm (Iohara et al. 2013). With animal studies above, DPSCs application in endodontic treatment is quite promising and of great potential. In the first clinical trial of dental pulp regeneration in 1961, scientists intentionally induced blood from apical into root canal by over-instrumenting which led to mineralization along the root canal walls (Ostby 1961). In the following years, various improvements including disinfection of root canal have been explored and successfully applied. The blood clot induction presumably induced stem cells from apical papilla (SCAPs)

into dental pulp for pulp regeneration. A pilot clinical study showed that human DPSCs of passage 9 or 10 with G-CSF in atelocollagen successfully formed tooth pulp tissue after being transplanted in human pulpectomized teeth and some patients even formed functional dentin after 24 weeks (Nakashima et al. 2017). Our latest study demonstrated very successful outcomes in clinical trial applying autologous DPSCs in premature teeth with crown facture with regeneration of three-dimensional dental pulp tissue, consisting of whole dental pulp with odontoblast layer, blood vessels and nerves (unpublished data). No transplantation rejection and inflammation response was observed during the treatment which indicates that this method could be a potential and effective way for dental pulp diseases (unpublished data). Thus, using DPSCs holds great potential for endodontic treatment and extensive clinical trials to evaluate efficacy and safety and optimize the treatment are required.

Manipulation of DPSCs using different methods to enhance its regeneration capacity has been studied. DPSCs from human third molars cultured in 3-dimensional (3-D) scaffold materials including a spongeous collagen, a porous ceramic, and a fibrous titanium mesh were proved to benefit DSPP-expressing tissue formation both in vitro and in vivo (Zhang et al. 2006). Also it has been reported that three-dimensional pellet culture system of dental pulp progenitor/stem cells stimulated by BMP2 effectively promoted dentin formation (Iohara et al. 2004). Application of DPSCs, collagen as scaffold and DMP1 as growth factor on mice by subcutaneous transplantation could induce dental pulp-like tissue (Prescott et al. 2008). Optimization of DPSCs's application in clinics is necessary and crucial to improve the therapeutic efficacy and more optimization work would be the focus of future study.

3 PDLSCs and Periodontal Regeneration

Periodontitis is a multifactorial inflammatory disease characterized by destruction of tooth-supporting tissues including the periodontal ligament (PDL), alveolar bone and root cementum (Pihlstrom et al. 2005). As a prevalent disease, periodontitis not only causes periodontal attachment and bone loss which finally leads to tooth loss but also is closely related to systemic diseases (Winning and Linden 2017). Conventional interventions and treatments including bone grafts (Hjorting-Hansen 2002), enamel matrix derivate (EMD) (Miron et al. 2016), platelet rich plasma (PRP) (Needleman et al. 2006) and guided tissue regeneration (GTR) (Andrei et al. 2018) are effective in partially restoring periodontal tissue but failed to regenerate the whole functional periodontal tissue. Periodontal tissue repair and regeneration in clinics is of great difficulty. Therefore, a better understanding of tissue specific stem cell-based regeneration seems to be crucial for periodontal tissue remodeling or repair.

3.1 PDLSCs Isolation and Identification

Periodontal ligament stem cells (PDLSCs) are a small population of mesenchymal stem cells isolated periodontal ligament that have self-renewal capacity and hold the capacity of differentiating to osteoblasts, adipocytes and chondrocytes under specific differentiation inductions (Seo et al. 2004). Periodontal ligament obtained from normal impacted third molars or extracted orthodontic teeth are most frequently used for PDLSCs isolation following established explant culture or enzymatic digestion methods. In addition, residual periodontal ligament on retained deciduous teeth has been proposed to be a new resource of PDLSCs (Silverio et al. 2010). Apart from comparative osteogenic differentiation capacity, PDLSCs derived from deciduous teeth showed higher self-renewal ability compared to PDLSCs obtained from permanent teeth (Ji et al. 2013). Moreover, it has also been reported that PDLSCs can be provoked from cryopreserved human periodontal ligament and maintain tissue specific stem cells features, including the expression of surface markers,

colony formation capacity, pluripotent differentiation ability and specialized tissue regeneration, thereby providing another access for PDLSCs isolation using frozen tissues (Seo et al. 2005).

Surface markers similar to BMMSCs and DPSCs have been also applied to identify PDLSCs, containing both positive (CD13, CD29, CD44, CD49d, CD73, CD90, CD105, CD166, etc.) and negative (CD19, CD34, CD45, etc.) markers (Trubiani et al. 2005). Recent evidence also suggests that highly osteogenic subpopulations of PDLSCs incline to express ascending levels of integrin b1 (ITGB1), intercellular adhesion molecule 1 (ICAM1) and telomerase reverse transcriptase (TERT) (Sununliganon and Singhatanadgit 2012). Although PDLSCs express an array of alkaline phosphatase (ALP), osteocalcin (OCN), matrix extracellular phosphoglycoprotein (MEPE) and bone sialoprotein (BSP) after osteogenic induction, the newly formed mineralized nodules are much fewer compared to BMMSCs and DPSCs, which credits to a lower calcium content in extracellular matrix (Seo et al. 2004). However, a higher expression of tendon specific scleraxis highlights the unique identity of PDLSCs to regenerate periodontal tissues among various postnatal mesenchymal stem cells (Seo et al. 2004). In addition, PDLSCs rarely express MHC class II antigen and co-stimulatory molecules (CD40, CD80 and CD86) which indicate low immunogenicity of PDLSCs (Wada et al. 2009). Although PDLSCs exhibit stem cell properties with colony formation and pluripotent differentiation, the property disorders during long-term in vitro expansion cannot be ignored.

3.2 PDLSCs' Properties and Pathways

Numerous mechanisms related to PDLSCs' degenerative properties under periodontitis have been reported, which is commonly regarded as a chronic inflammatory microenvironment. TNFα and IL-1β have been acknowledged as crucial inflammatory factors to destroy periodontal tissues and to block functions of PDLSCs (Xue et al. 2016). WNT pathway exerts its critical role in periodontal homeostasis, and dysregulation of β-catenin is largely related to the disorders of PDLSCs in inflammatory microenvironments (Napimoga et al. 2014). Dickkopf 1 (DKK1), a specific WNT inhibitor, could improve function of PDLSCs in periodontitis with diabetes mellitus by mediating WNT signaling (Liu et al. 2015). NF-κB signaling, MAPK signaling and BMPs signaling are also involved in inflammation induced PDLSCs dysfunction (Mao et al. 2016). In recent years, microRNAs such as miR-17 and miR-21 have been frequently reported to regulate PDLSCs functions at posttranscriptional level, whereas mechanisms mediated by microRNAs remain poorly understood (Liu et al. 2011; Yang et al. 2017). Epigenetically, histone acetyltransferase GCN5 has been proved to be able to regulate PDLSCs' osteogenesis through WNT signaling and Osthole could restore function of PDLSCs from inflammatory tissue via epigenetic regulation (Li et al. 2016; Sun et al. 2017a). Moreover, abnormality of subcellular structures has been verified to affect PDLSCs functions. Autophagy and edoplasmic reticulum stress were both reported to be involved in periodontitis-associated chronic inflammation and proper manipulation of such pathways could alleviate inflammatory condition of periodontitis (Xue et al. 2016; An et al. 2016).

3.3 Periodontal Regeneration

As PDLSCs exhibit multi-potency with differentiation into osteoblasts, fibroblasts and tooth cementoblasts, they have been used alone or combined with biomaterials for periodontal tissues regeneration.

When the PDLSCs were discovered, a typical cementum/PDL-like structures regenerated by PDLSCs-aggregate, which are different from specialized structures generated by BMMSCs and DPSCs, have been verified using a subcutaneous transplantation assay (Seo et al. 2004). Meanwhile, newly formed collagen fibers were also observed to connect with regenerated cementum/PDL-like structures, mimicking

physiological attachment of Sharpey's fiber (Seo et al. 2004). Furthermore, PDLSCs transplanted into artifical periodontal defects in immunocompromised rats were observed to integrate into the surfaces of alveolar bone and teeth roots, bi-directionally (Seo et al. 2004). Additionally, it has been reported that PDLSCs can effectively generate periodontal tissue in a swine or canine model of periodontitis (Liu et al. 2008; Ding et al. 2010), and combination of stem cells from apical papilla (SCAPs) and periodontal ligament stem cells has successfully formed root/periodontal structure (Sonoyama et al. 2006). Transplantation of PDLSCs and BMMSCs was able with to form alveolar bone in a canine peri-implant defect model (Kim et al. 2009). Besides of animal researches, human studies have also been conducted. Recently, a randomized clinical trial has been designed to repair periodontal intrabony defects on patients using autologous PDLSCs, resulting in a marked elevation of alveolar bone height with high biological safety (Chen et al. 2016). However, the therapeutic effects showed no statistically differences between the therapies using and not using PDLSCs (Chen et al. 2016). Therefore, further studies are needed to develop modified strategy for advancement of PDLSCs based periodontal regeneration. Addition of exogenous protein signalings has been verified to promote PDLSCs regenerative capacity. When treated with dentin noncollagenous proteins (DNCPs) or bone morphogenetic proteins (BMPs), PDLSCs presented an improved proliferation, adhesion capability and cementoblastogenesis, which are indicated by changes of morphology, enhancement of ALP activity, improvement of matrix mineralization and upregulation of osteogenic genes (Ma et al. 2008; Wang et al. 2017). Although autologous PDLSCs are tolerated by hosts' immune system and safe for therapy, the limited resource restricts their large scale clinical application. Thus, it is urgently needed to research and develop allogeneic PDLSCs based regeneration medicine, whereas their therapeutic safety has not been totally defined. Recent studies have demonstrated that allogeneic PDLSCs engaged in immune-modulatory function similar to BMMSCs and finally reconstructed the experimental periodontal bone defects, indicating that allogeneic PDLSCs based therapy might be an efficacious and safe alternative for the treatment of periodontal diseases (Ding et al. 2010; Han et al. 2014). Furthermore, extracellular matrix (ECM) derived from periodontal ligament cells has been reported to induce the differentiation of induced pluripotent stem cells (iPS) to PDLSC-like cells, suggesting a novel approach to obtain enough seed cells for periodontal bioengineering (Hamano et al. 2018).

For these results, PDLSCs are generally regarded as the optimum selection of seed cells for periodontal repair and regeneration, not only because of pluripotent stem cell features, but also due to their unique potential to organize three-dimensional periodontal tissues. More studies involving in the underlying mechanism of PDLSCs and periodontal regeneration are greatly required.

4 Other Dental Stem Cells and Tooth Regeneration

4.1 SCAPs and Tooth Regeneration

Stem cells from apical papilla (SCAPs), a type of dental stem cells essential for the developing dental pulp-dentin complex, alveolar bone and tooth root (Sonoyama et al. 2008; Bakopoulou et al. 2011), have been isolated from root tips of growing teeth, and are similar to DPSCs but with a markedly higher proliferative capacity and mineralization potential. SCAPs express high level of STRO-1, CD-146, and negatively express CD34 and CD45 (Bakopoulou et al. 2011). Studies showed that SCAPs had a greater capacity for dentin regeneration compared to DPSCs (Bakopoulou et al. 2011). Furthermore, SCAPs also exhibit a higher proliferation and better tooth regeneration capacity compared to PDLSCs (Han et al. 2010). However, it has been reported that SCAPs and PDLSCs with a HA/TCP carrier can produce a functional biological tooth root in a swine model and finally resemble a functional tooth with an artificial crown (Sonoyama et al.

2006). In addition, besides of healthy SCAPs, SCAPs derived from inflamed root tips also exhibit high proliferation and multipotency. Further researches are essential to identify regenerative properties of inflammation derived SCAPs. Complex molecular mechanisms underlying SCAPs differentiation and proliferation have been investigated extensively. bFGF has been reported to enhance stemness of SCAPs and differentiation capacity under certain conditions (Wu et al. 2012). Canonical WNT signaling also participate in osteo/odontoblastic differentiation of SCAPs (Zhang et al. 2015). MicroRNAs play vital roles in regulateing odonto/osteogenic differentiation capacity of SCAPs (Sun et al. 2014; Wang et al. 2018b). MAPK pathway, NF-κB pathway, etc. are involved in this process as well (Li et al. 2014a, b). As stem cells from developing stage, SCAPs hold superior potential for regenerative medicine, and more mechanism study and clinical trial are expected in the future in order to make better use of them.

4.2 DFCs and Tooth Regeneration

The dental follicle, a loose ectomesenchyme origined connective tissue, surrounds tooth germ during tooth development and plays important roles in tooth eruption and tooth root development. Undifferentiated ectomesenchymal cells known as dental follicle stem cells or dental follicle cells (DFCs) can be obtained from impacted third molars or ectopic impacted teeth, and express high level of STRO-1, CD44, CD105, Nestin and Notch-1 (Yao et al. 2008; Morsczeck et al. 2005). DFCs are multipotent stem cells dental follicle cells are precursor cells of periodontal fibroblasts, osteoblasts and cementoblasts during the process of periodontal tissues development. It has been reported that DFCs hold the properties similar to MSCs, which were able to form a connective tissue-like structure with mineralized clusters after being induced in osteogenic differentiation medium (Sowmya et al. 2015). After transplantation of DFCs with treated dentin matrix scaffold, root-like tissues stained positive for markers of dental pulp and periodontal tissues were found in the alveolar fossa (Guo et al. 2012a). Also data showed that rat DFCs formed a tooth root when seeded on scaffolds of a treated dentin matrix (TDM) and transplanted into alveolar fossa (Sun et al. 2017b). Apart from generating periodontium alone, DFCs have also been observed to improve regenerative capacity of healthy PDLSCs and even rescue degeneration of inflamed PDLSCs, indicating that DFCs could assist PDLSCs to regenerate periodontal tissues via ameliorating local microenvironment (Liu et al. 2014). Additionally, human dental follicle tissue after cryopreservation has been proven to be a reliable resource for regenerative medicine (Park et al. 2017). As DFCs support bone regeneration in defect models of the calvaria of immunocompromised rats, they are also a promising cell medication for bone regeneration (Guo et al. 2012b).

5 Dental Stem Cells Banking

Although dental stem cells have been reported to well regenerate dental tissues, a long period procedure of tooth extraction, primary culture and in vitro cell expansion limits their usage at the time of clinical requirements. Therefore, long-term storage and timely application of dental stem cells remain to be settled. Recently, dental stem cell banking has been emerging to cryopreserve dental stem cells, which highlights the potential to realize a novel approach to support large scale of dental stem cells based regenerative medicine. Several banks provided dental stem cells have been prepared, such as BioEDEN (Austin, USA, http://www.bioeden.com/), Store-A-ToothTM (Lexington, USA, http://www.store-a-tooth.com/), Teeth Bank Co., Ltd., (Hiroshima, Japan, http://www.teethbank.jp/), Advanced Center for Tissue Engineering Ltd., (Tokyo, Japan, http://www.acte-group.com/) and Stemade Biotech Pvt. Ltd., (Mumbai, India, http://www.stemade.com/). Recently, a National Dental Stem Cells Bank (http://www.kqgxb.com/) has been established in People's Republic of China, which is the first high-tech organization of dental stem cells research, storage and translational

medicine development according to Good Manufacturing Practice (GMP) around the world.

Apart from evaluating therapeutic effects of dental stem cells on tooth regeneration, it might be crucial to formulate legislation, industry standard, quality control, bio-insurance, checks and audits for dental stem cells banking development. With these problems solved, dental stem cells banking will be a prospective industry in regenerative medicine.

6 Conclusion

This review concentrated on stem cells from dental tissues and how their current advancement in tooth and periodontal tissues regeneration. Although dental stem cells possess colony formation, proliferation and multipotent differentiation capacity to generate osteogenic, adipogenic and chondrogenic lineages in vitro similar to BMMSCs under certain conditions, they also displayed their own distinctive regenerative potential different from each other in vivo, suggesting that tissue specific stem cells might be the optimal choice for self-tissues repair and regeneration. Basic researches and clinical pilot studies in regenerative medicine highlight the promise of dental stem cells dependent translational medicine. Although the frame of dental stem cells dependent translational medicine has been primarily and successfully constructed, a proper quality control and efficacy in the clinic, and a better understanding of underlying mechanisms regulating dental stem cells regenerative capacity are generally regarded as problems remaining to be urgently solved.

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Challenges in Bio-fabrication of Organoid Cultures

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Abstract

Three-dimensional (3D) organoids have shown advantages in cell culture over traditional two-dimensional (2D) culture, and have great potential in various applications of tissue engineering. However, there are limitations in current organoid fabrication technologies, such as uncontrolled size, poor reproductively, and inadequate complexity of organoids. In this chapter, we present the existing techniques and discuss the major challenges for 3D organoid biofabrication. Future perspectives on organoid bioprinting are also discussed, where bioprinting technologies are expected to make a major contribution in organoid fabrication, such as realizing mass production and constructing complex heterotypic tissues, and thus further advance the translational application of organoids in tissue engineering and regenerative medicine as well drug testing and pharmaceutics.

Keywords

3D culture · Bioprinting · Organoids · Regenerative medicine · Tissue engineering

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Abbreviations

2D	two-dimensional
3D	three-dimensional
adMSCs	Adipose-derived mesenchymal stem cells
ASCs	adipose-derived stem cell
BioLP	biological laser printing
CXCL	CXC ligand
CXCR	CXC receptor
DBB	droplet-based bioprinting
DPCs	dental pulp cells
EBB	extrusion-based bioprinting
ES	embryonic stem
HA	hyaluronic acid
HER2	human epidermal growth receptor
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HTC	hydrogel tissue constructs
HUVECs	human umbilical vein endothelial cells
LBB	laser-based bioprinting
MAPK	mitogen activate protein kinase
MAPLE-DW	matrix assisted pulsed laser evaporation-direct write
MCS	multicellular spheroids
MSCs	mesenchymal stem cells
pHEMA	poly (2-hydroxethyl methacrylate)
PI3K	phosphoinositide 3-kinase
PNIPAAm	poly (N-isopropylacrylamide)
PVA	polyvinyl alcohol
REF-52	Rat embryo fibroblasts
RGD	arginylglycylaspartic acid
SDF	stromal cell-derived factor
SPIONs	superparamagnetic iron oxide nanoparticles
TCD	tissue culture dish
TE	tissue engineering
TNFα	tumor necrosis factor
VEGF	vascular endothelial growth factor

1 Advantages of 3D Cell Culture over 2D Culture

Cells in three-dimensional (3D) culture are encapsulated in spheroids as *in vivo*, and may proliferate at a different rate to two-dimensional (2D) culture. Besides, 3D models have a minimum depth of 50 μm and possess both stroma and structure, the two features absent in 2D cell culture, which ensure more realistic cell-cell and cell-matrix contact and communication (Eglen and Randle 2015). As a result, the cellular responses to stimulators in 3D cultures have shown to be more similar to what occurs *in vivo* compared to 2D culture. Studies have found that several kinds of tumor cells cultured in 3D models were generally more resistant to chemotherapeutic agents than ones in 2D models, and 3D spheroids at Day 6 were insensitive than those at Day 3, irrespective of various action mechanisms of drugs (Karlsson et al. 2012).

There are some physical and physiological differences between 2D and 3D models. Increased glycolysis in 3D spheroid and hypoxia-induced lower pH in the core of spheroids should also exert influence on physiological differences. Inefficient oxygen diffusion to cells in the core of spheroids upregulated the expression of hypoxia-induced survival factors, such as hypoxia-inducible factor (HIF)-1α (Bhang et al. 2011), which resulted in enhanced secretion of both angiogenic and anti-apoptotic factors. It has been found that the concentrations of these factors could be up to 145-fold higher in 3D spheroid suspension bioreactors than those in monolayer cultures (Kwon et al. 2015). Thus, spheroids preferred to represent tumor units because of their high angiogenic and vasculogenic potential. 3D spheroid culture was observed to facilitate the cartilage-specific phenotype and function maintenance as compared to 2D monolayer culture since this type of cell preferred to hypoxia (Shi et al. 2015). Besides, there are some other differences between the two models.

First, more cell-cell and cell-ECM interactions in 3D models may display different gene expressions and protein phenotype profile. It has been shown that higher levels of stromal cell-derived factor (SDF)-1 [chemokine CXC ligand (CXCL)12] was expressed in 3D spheroids than in monolayer cultures (Bhang et al. 2011). SDF-1 is a small molecular weight chemokine mediating the homing of circulating CXC receptor (CXCR) 4-positive endothelial progenitor cells (Laschke et al. 2011). HepG2 cells spheroids from rotating wall vessel showed upregulation of metabolic and synthetic genes, and higher cytochrome P450 activity and albumin production as phenotypes differences compared to 2D culture. Additionally, maintenance of 3D structure and environment was required for maintaining enhanced liver functions, since transferring of spheroids to a tissue culture dish (TCD) resulted in spheroid disintegration and subsequent loss of function such as cytochrome P450 activity and albumin production (Chang and Hughes-Fulford 2008). Skardal et al. have fabricated a sandwich tissue construct, in which primary hepatocytes were seeded on substrate layer and covered with corresponding gel solution followed by crosslinking. Results have shown that primary human hepatocytes cultured in 3D hyaluronic acid (HA) hydrogels with liver ECM components outperformed paralleled cultures on 2D plastic in viability, mitochondrial metabolism, and albumin production (Skardal et al. 2012). In a polyvinyl alcohol (PVA) scaffold cultured with human hepatocyte cell line C3A, CYP3A4 activities were more effective when compared with 2D monolayer cultures (Stampella et al. 2013). It has been reported (Bartosh et al. 2010) that 3D spheroids of mesenchymal stem cells (MSCs) produced increased amounts of anti-inflammatory factors, such as tumor necrosis factor (TNFα) stimulated gene/protein-6 (TSG-6) and stanniocalcin-(STC)-1. Similarly, compared to monolayer MSCs, 3D spheroids of MSCs have shown to be more effective in anti-inflammation and reduced organ injury in a mouse zymosan-induced peritonitis model (Bartosh et al. 2010), and in a rat ischemia-reperfusion model (Xu et al. 2015).

Second, different expression and spatial location of cellular surface receptors and activation of relative signal pathways in 2D and 3D cultures should lead to different responses to stimulators. A relevant example has been reported (Pickl and Ries 2009). Cancer cells overexpressing human epidermal growth receptor (HER2) could form HER2-HER3 heterodimers when they were cultured in 2D models, and HER2 homodimers in 3D spheroids. The latter led to an enhanced activation of HER2, and consequently induced a signaling pathway switch from phosphoinositide 3-kinase (PI3K) in 2D models to mitogen activate protein kinase (MAPK) in 3D models.

Third, cells show different activity in 2D vs 3D models. Cells in 2D culture are relatively identical in cell activity, while cells in 3D culture show more proliferation on the outside of spheroids, and the ones in the core are less active (Kimlin et al. 2013).

Fourth, 3D culture is convenient for co-culture of different cell types. Interactions of heterotypic cells require different cell types to form a cascade reaction system (Astashkina et al. 2012). In particular, 3D culture is suitable for the co-culture of different cell types with *in vivo*-like cellular architecture and direct cell-cell contact. For examples, stromal cells can induce chemoresistance and metastasis of tumor cells, and endothelial cells may dominate tumor angiogenesis inside the tumor. In a recent report, human female U2OS osteosarcoma cells seeding on 3D silk scaffolds were investigated with or without fibroblasts. U2OS cells in 3D constructs upregulated IL-8 expression, which attracted more human umbilical vein endothelial cells (HUVECs) to migrate into tumor constructs when compared to those in 2D plates. The migration of HUVECs in a 3D model could be dramatically reduced by anti-IL-8. However, 2D co-cultured U2OS-fibroblasts showed no response to anti-IL-8 (Tan et al. 2014).

Fifth, cells in 3D culture systems show different response to materials with different stiffness. Lam et al. created spheroids using 3D agar petri dish, and mixed in turn with different concentrations of collagen type 1, resulting in spheroids being placed in

the interface with different stiffness. Cells in spheroids showed decreased invasions on the stiffer surface (Lam et al. 2014).

Finally, different sensitivities to signals have also been observed within different models. Rat embryo fibroblasts (REF-52) were diluted with neutralized collagen type 1 to form hydrogel tissue constructs (HTC). The HTCs provided cells a more *in vivo*-like 3D microenvironment to imitate the morphology and physiology in native tissues. In addition, optical assays in HTCs demonstrated superior sensitivity to fluorescent indicator, since emission signals were collected by multiple cell layers (Lam and Wakatsuki 2011).

2 Biofabrication Techniques Used in 3D Spheroid Models

The classic approach for tissue engineering (TE) involves seeding living cells onto a biocompatible and eventually a biodegradable scaffold. Then, the engineered tissue construct is cultured in a bioreactor until the tissue achieves the desired cell density and mechanical properties for implantation (Jakab et al. 2008). In general, the application of scaffolds in TE is straightforward, but they still subject to some challenges and limitations (Robert 2007; Jakab and Norotte 2010), such as the lack of precision in cell placement, limited cell density, the need for organic solvents, chemical residues, difficulties in integrating vascular network, insufficient interconnectivity, inability to control the pore distribution and pore dimensions, and difficulties in manufacturing patient-specific implants (Yang et al. 2001; Sachlos and Czernuszka 2003). These drawbacks have led many groups toward the development of new approaches those are able to build tissues with 3D architecture using a bottom-up approach, in which cells are able to self-assemble into more complicated and organized tissue structures (L'Heureux et al. 1998; Jakab et al. 2008; McAllister et al. 2009; Norotte et al. 2010).

Cellular self-assembly, a fundamental mechanism in the origin of life and the evolution of complex biological organs, exists at all levels in living systems. In comparison to cells in monolayer cultures, cells that self-assemble into spheroids achieve elevated gene expression, and at the same time maintain their phenotype. These cells show natural cell-cell interactions and mimic *in vivo* differentiation patterns and spatial cell-cell and cell- matrix interactions (Napolitano et al. 2007). Additionally, the spheroids are comprised of cells in varying states namely hypoxic, quiescent, proliferating, apoptotic and necrotic cells. These multicellular spheroids are thus capable of mimicking native tissues, such as tumors, as they exhibit three distinguishable zones, which are the hypoxic core, quiescent zone around the hypoxic core and the outermost region referred to as the proliferating rim. The outermost region has a rich supply of nutrients, oxygen and other metabolites, whereas all the cell catabolites accumulate in the hypoxic core of spheroids, generating biochemical gradients (Edmondson et al. 2014). In embryonic development and tissue morphogenesis, cell adhesion and differentiation contribute to the formation of multicellular aggregates in a three-step process (Lin et al. 2006). First, loose cells rapidly aggregate via the binding of cell surface integrin to arginylglycylaspartic acid (RGD) motifs in the ECM. A delay phase follows this aggregation, and exhibits up-regulated cadherin expression and accumulation. Finally, homophilic cadherin-cadherin binding between two cells confers strong cell adhesion, forming a compact cellular aggregate. Signal transduction might be initiated through the β-catenin complex, eventually leading to differentiated characteristics observed in multicellular aggregates.

The emerging field of bioprinting and biofabrication seeks to address the problem of large tissue constructs, and uses "cellular aggregates" as building blocks to fabricate tissues and organs *in vitro*. Bioprinting, an additive manufacturing technology, have been used to fabricate living structures via a layer-by-layer printing of living cells in their own ECM. Cellular aggregates, such as spheroids, are printed as the 'bio-ink' along with an ECM substrate. These bioink units are masses of cells in either spherical or cylindrical shapes (Yu et al. 2016). Cell aggregate-based bioinks can be homocellular,

containing a single cell type or heterocellular, prepared by several cell types (Yu et al. 2016).

Cell aggregates can be considered as "living materials" with measurable, evolving and potentially controllable material properties (Mironov et al. 2009). However, they must be standardized in size to the utmost extent, in order to make them processable or be dispensable through a bioprinter nozzle or by other means, without clogging issue and structure destruction. Thus, standardization of the dimension of tissue spheroid is required for continuous dispensing.

In general, multiple methods have been developed to prepare cell aggregates without significant cell injury and/or damage (Norotte et al. 2010; Yu et al. 2016; Mironov et al. 2009; Marga et al. 2007). The most popular methods include the hanging-drop method, microfluidic method, liquid overlay method, rotating flask method, spinner flask method, and micromolding method.

2.1 The Hanging-Drop Method

The hanging-drop method for cell culture was developed previously to induce embryoid bodies from embryonic stem (ES) cells (Keller 1995). It has been modified to be a popular way to culture multicellular spheroids (Kelm et al. 2003).

This method relies on gravity-enforced self-assembly to produce spheroids (Figs. 1a and 2a) (Kelm et al. 2003; Achilli et al. 2012). To make spheroids, small volumes (15–30 μL) of a cell suspension (containing approximately 300–3000 cells) are pipetted onto the inner surface of the lid of a tissue culture plate. The lid is inverted, and the drops stay attached to the lid due to surface tension. Cells settle and concentrate at the bottom of the drops due to the gravity, leading to the formation of the spheroids (Kelm et al. 2003; Timmins and Nielsen 2007). The rounded bottom of a hanging drop is able to provide a good environment for the formation of a spheroid. The speed of the process depends on the strength of cell-cell interaction, which depends on the cell type (Marga et al. 2007). The time for creating spheroids should be minimized in order to ensure high cell viability. Also, the spheroid size can be controlled by adjusting the density of the cell suspension. Although the hanging-drop method provides a good way to control the spheroid size, this method is not very efficient owing to its extremely labor intensive and time consuming procedures, and low throughput generation of spheroids.

This technique is particularly useful for generating cellular aggregates with defined sizes, cell numbers, and compositions (Kelm et al. 2003, 2004; Lin et al. 2006). It is also useful for the investigation of cellular or molecular activities during spheroid assembly, tumor invasion, interaction of two different cell types, and tumor spheroid-induced angiogenesis of stem cell embryoid bodies (de Ridder et al. 2000; Wartenberg et al. 2001; Kelm and Fussenegger 2004; Timmins et al. 2004).

2.2 The Microfluidic Method

In the microfluidic method, a hydrogel-based U-shaped microfluidic chip was used for the formation of cellular aggregates (Figs. 1b and 2b). Cells were trapped into the pocket of the chip with the assistance of the fluid flow and gravity (Fu et al. 2014). Cell trapping was realized by applying fluidic flow against gravity, and the spheroid size could be fine-tuned by adjusting the magnitudes of the U-shaped microstructure. The U-shaped structures prevented cells from the damage induced by shear force, and at the same time allowed free diffusion of nutrient and waste.

In the study reported by Fu et al., the U-shaped microfluidic chip was set at three positions (i.e. horizontal, tilted, and vertical) (Fu et al. 2014). The flow force dominated at the horizontal position, where the cells scattered outside the chip, and only a small percentage of the cells could get into the chip. As compared, in the tilted position, the area right above each U-shaped microstructures had relatively low flow rates. Therefore, cells may be pulled down into the U-shaped microstructure by gravity. Cell accumulated at the vertical position over time. Since the cells were constrained within the chip, they exhibited a high compactness, which facilitated to the cell-cell interactions as compared

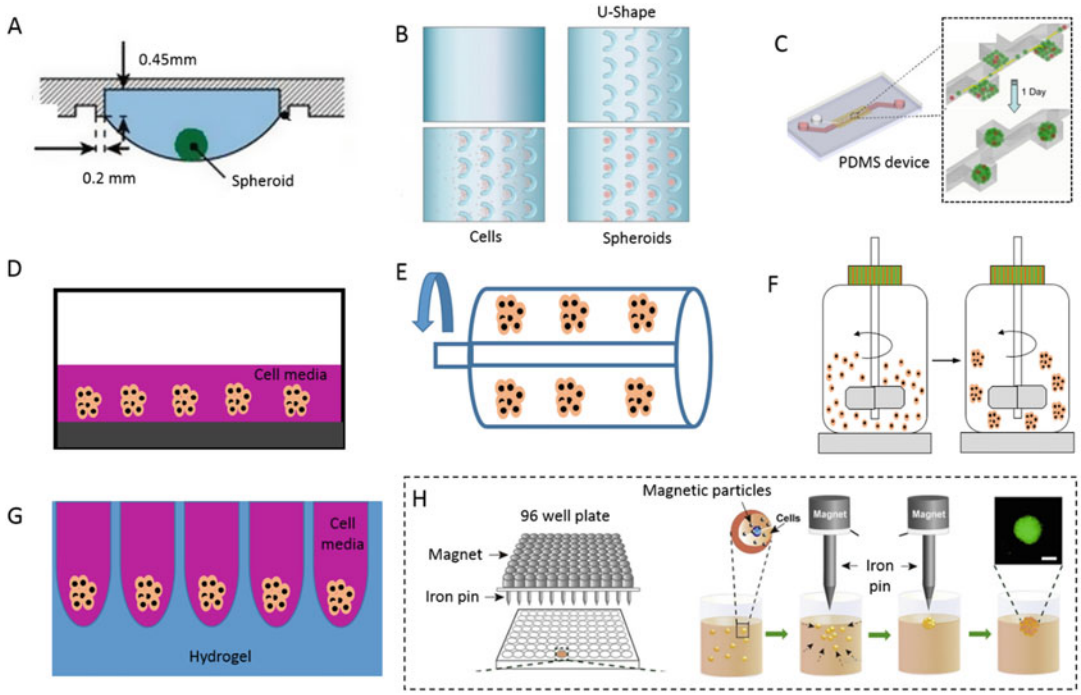

Fig. 1 Spheroid fabrication techniques: (**a**) hanging drop technique (reproduced/adapted with permission from Frey et al. 2014); (**b**) cells were trapped into U-shaped hydrogel microstructure then spheroid formation was obtained in the microfluidic device (reproduced/adapted with permission from Fu et al. 2014); (**c**) metastatic prostate cancer cells (PC-3 cell line), osteoblasts and endothelial cells settle down the wells because of the gravity, then they form co-cultured spheroids after 1 day culture of the cell suspension in the PDMS device (reproduced/adapted with permission from Hsiao et al. 2009) (**d**) liquid overlay system; (**e**) rotating flask technique; (**f**) spinner flask technique; (**g**) micro-molding technique; (**h**) magnetic assembly technique (reproduced/adapted with permission from Kim et al. 2013)

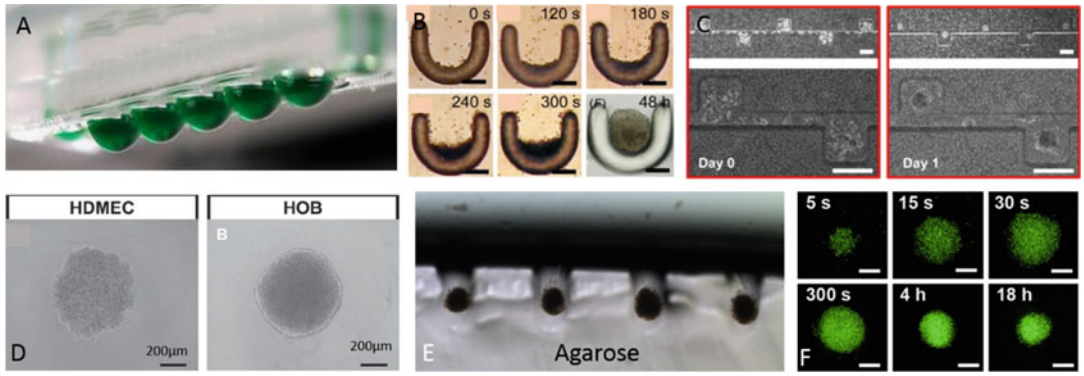

Fig. 2 Fabricated spheroid samples: (**a**) an image showing spheroid fabrication using the hanging-drop technique (reproduced/adapted with permission from Frey et al. 2014); (**b**) cell trapping in the U-shaped microstructure at 0, 120, 180, 240, 300 s. and 48 h (scale bar represents 250 μm) (reproduced/adapted with permission from Fu et al. 2014); (**c**) optical images showing microfluidic spheroid formation device (scale bar represent 200 μm) (reproduced/adapted with permission from Hsiao et al. 2009); (**d**) phase-contract images of human dermal microvascular endothelial cells (HDMEC) and human osteoblasts (HOB) using liquid overlay technique (reproduced/adapted with permission from Metzger et al. 2011); (**e**) spheroid fabrication using agarose micromolding (**f**) time-lapse images of a spheroid using magnetic assembly technique (scale bar presents 200 μm) (reproduced/adapted with permission from Kim et al. 2013)

to those cultured on a plain glass slide or Petri dish. The perfusion flow surrounding the U-shaped microstructure played an interesting role in spheroid formation (Wu et al. 2008). At low flow rates, some cells tended to migrate away through the opening of the microstructure. At high flow rates, cells were prevented from escaping by the flow resulting in the formation of spheroids. Additionally, the perfusion system kept the fluidic shear stresses and the concentration of soluble factors surrounding the spheroids under control (Toh et al. 2007; Agastin et al. 2011).

This method has been successfully used for different cell types, including primary cells, cell lines, and co-culture of multiple cell types (Hsiao et al. 2009; Huang et al. 2009) (Figs. 1c and 2c). Moreover, the microfluidic platforms were usually equipped with biosensors for real-time imaging and monitoring, which provided an approach for high-throughput production of size-controlled spheroids (Agastin et al. 2011; Jin et al. 2011). However, spheroids generated by microfluidic platforms may be difficult to be retrieved for further analysis.

2.3 The Liquid Overlay Method

The liquid overlay method has been reported to inhibit the attachment of cells to tissue culture plates and promote cell-cell aggregation (Figs. 1d and 2d). In this method, a cell suspension was seeded onto flat tissue culture dishes made of low-adhesive surfaces such as agarose (Richard et al. 2001; Metzger et al. 2011) and poly (2-hydroxethyl methacrylate) (pHEMA) (Landry et al. 1985).

This method is based on the principle that cells aggregate, if the adhesive forces between cells are stronger than those between the cells and the substrate on which they are cultured. The success of this model depends on the use of a non-adhesive substrate or a substrate with reduced adhesion (e.g. removal of cellular attachment molecules from the substrate), and the use of a liquid overlay with more nutrient factors than those in the substrate plate (John et al. 1977), which encourages the growth but not the attachment of cell aggregates on the surface of the substrate. However, this technique is time-consuming (1–3 days for most cell lines) (Santini et al. 1998), unable to mass-produce spheroids, and difficult to control the uniformity of the size and shape of spheroids.

2.4 The Rotating Flask Method

The rotating wall vessel creates a microgravity environment that maintains cells in suspension and allows cells to aggregate into spheroids (Figs. 1e). Cell suspension in a rotating wall vessel is slowly rotated to maintain the cells in continuous free fall. Rotation is very slow (~15 rpms) at the beginning. When spheroids begin to form and the mass of the aggregates increases, rotation rate is increased to keep the aggregates in suspension (~25 rpms) (Ingram et al. 1997). Heterotypic spheroids can be formed by co-culture of different cell types. Long term culture is also possible. The method produces aggregates in a low shear environment, and the yield is high. Although there exists variability in spheroid size, spheroids harvested from rotary cultures display a relatively uniform size distribution compared to static cultures. The average spheroid diameter can be controlled by tuning cell-seeding density, medium composition, spinning rate and culture time. However, it is difficult to monitor the assembly of spheroids in real time (Manley and Lelkes 2006).

2.5 The Spinner Flask Method

Spinner flask culture has been the most common technique to culture large quantities of spheroids (Fig. 1f) (Kim 2005). Cells are cultured as monolayer to be almost confluent, followed by being trypsinized and placed in the spinner flask, where the cells are seeded to be a uniform and well-mixed suspension to form spheroids. The fluid environment in the flask is controlled by convective forces generated by an impeller or a magnetic stir bar. A magnetic spinner is used to maintain

the cells in suspension preventing them from adhering to any substrate. A proper rotation speed is critical since the spheroids would settle at the slow rotation speed, and a high rotation speed causes cell damage due to strong fluidic shear stress. Cells begin to aggregate and form spheroids (Sutherland 1988; Santini et al. 1998) when they maintain in suspension. It has been reported that the size and shape of the spheroids produced by spinner flask system were heterogeneous. However, a new platform-based spinner flask has shown better size-controlled properties (Abbasalizadeh et al. 2012). Additionally, the high shear forces exerted on the cells and the substrates that are required for these methods may have an adverse effect on the cellular behaviors.

2.6 The Micro-molding Method

Micro-molding of hydrogels have been used to form spheroids as well as micro-tissues with different shapes (Dean et al. 2007; Napolitano et al. 2007). This method applies computer-aided design software and additive manufacturing to form micro-molds that contain an array of cylindrical or ring-shaped pegs with rounded tops (Figs. 1g and 2e). Non-adhesive hydrogels (agarose or polyacrylamide) are then cast using these micro-molds to form array of micro-wells, which cells can be seeded into. The suspended cells are then loaded onto the micro-wells, redistributed by gravity and hydrodynamic forces, assembled into aggregates according to the geometry of micro-wells, and eventually settle into the recesses of micro-wells. The method is capable of producing spheroids in high-throughput and homogenous shape, size, and cell distribution. Cells can be monitored as they self-assemble, and it is easy to change media and add drugs, antibodies, or growth factors. In addition to aggregates of rounded shape, micro-molds have been designed to guide the self-assembly of cells to generate the aggregates with more complex shapes such as rods, toroids, and honeycombs. However, it is not always possible to deposit cell suspension in each well due to the restricted size of the well, which results in inconsistent cell number in spheroids. Moreover, when this technique is utilized to fabricate heterocellular spheroids, there is limitation to control the ratios of different cell types in each spheroid.

2.7 Others

2.7.1 External Force Method

The external force method uses forces (e.g. electric fields, magnetic force, and ultrasound) to concentrate suspended cells into a high density that facilitates cell aggregation. Electric fields have been utilized to fabricate spheroids based on the action of positive dielectrophoresis in the iso-osmotic solution with low conductivity, which eventually compels cells to adhere to each other and leads to aggregation (Sebastian et al. 2007). To generate spheroids using magnetic assembly technique, cells are incubated with nanoparticles containing a magnetite core-like Fe_3O_4 (Fig. 1h) (Kim et al. 2013). After endocytosis of the magnetic nanoparticles, cells are then attracted to a focal point by an external magnet, resulting in the spheroid formation in a very short span of time (Fig. 2f). In the ultrasound mediated cell aggregation technique, an ultrasound standing wave trap is used to concentrate cells and initiate spheroid formation. However, this technique produces spheroids with non-uniform dimension (Sebastian et al. 2007). The advantages and disadvantages of the abovementioned technologies have been summarized in Table 1.

2.7.2 Cell Sheets

In addition to spheroids, multi-cellular cell sheets have been produced by culturing cells on a polymer, such as poly (N-isopropylacrylamide) (PNIPAAm), which has the potential of thermo-responsive hydrophilic/hydrophobic changes (Park et al. 2005). The hydrophobic polymer may become hydrophilic, when the temperature decreases to 20 °C for 1 h, leading to the release of a contiguous sheet of cells. The released sheet can be further incubated on a non-adhesive surface, where it can compact and form spheroids.

Table 1 Advantages and disadvantages of spheroid fabrication techniques

Spheroid Fabrication Technique	Advantages	Disadvantages
Hanging drop	Better control of spheroid size	Low-throughput
Microfluidic	Easy-to-use	Harvesting spheroids is not easy
	Better control of spheroid size	
	Continuous perfusion	
Liquid overlay system	Easy-to-use	Low-throughput
	Low shear stress	
Rotating flask	Fast production	Costly systems
	Low shear stress	
Spinner flask	Long-term culture	High shear stress forces
	Easy-to-use	
Micro-molded non-adhesive hydrogels	Simple	Low-throughput
	High-throughput	
	Low shear stress	
Magnetic assembly technique	Better control of spheroid size	Low-throughput

Cell sheets obtained from different cell types have been used to generate heterocellular spheroids (Park et al. 2005).

3 Major Considerations in 3D Spheroid Models

3D spheroids fabricated by the aforementioned methods are all scaffold-free. In tissue engineering, spheroids are the minimum units where the cell-cell and cell-ECM contacts and interactions in embryo development are imitated. Spheroids are scalable, and considered promising blocks for tissue engineering with scaffold-based or scaffold-free strategies. For the scaled-up biofabrication of spheroids, several issues have to be taken into account including size control, throughput, heterotype cell co-culture, vascularization, and *in vitro* preconditioning and maturation.

3.1 High-Throughput

Since cells in the core of spheroids suffer from oxygen insufficiency, size of spheroids is generally less than a few hundred micrometers. Considering a large quantity of spheroids are needed for scaled-up tissue engineering, high-throughput and automated methods are required for the fabrication of spheroid with controlled dimensions. Mass production of spheroids using the robot-assisted hanging drop method have been established, which is able to produce up to 384 spheroids per standard 96-well plate (Tung et al. 2011). However, it is difficult to monitor the formation of the spheroids, and laborious to change media or add drugs, which result in low reproducibility (Rezende et al. 2013). However, two companies (i.e. InSphero and 3DBiomatrix) recently modified this method by making hanging-drop droppable, and thus realizing robotic automated dispensing (Tung et al. 2011; Rezende et al. 2013). The micro-molded non-adhesive hydrogels can be scaled up to create up to 822 spheroids in a single mold, with controllable and homogenous shape, size, and cell composition (Achilli et al. 2012). In addition to spheroids, designs of micro-mold also enable the generation of cell aggregates with more complex shapes such as rods, toroids or honeycombs (Napolitano et al. 2007).

One group has developed a robust and cost-effective culture system for mass production of size-controlled human pluripotent stem cell aggregates in stirred suspension bioreactor (Abbasalizadeh et al. 2012). This novel bioprocess utilized the stepwise optimization of both static and dynamic suspension culture conditions to produce aggregates with particular sizes. The hydrodynamic conditions of the bioreactor were optimized by the combinations of

different agitation rates and shear protectant concentrations. This platform is suitable for large-scale generation of hepatocyte-like cells (Vosough et al. 2013) and cadiomyocyte (Fonoudi et al. 2015) from human pluripotent stem cells.

3.2 Co-culture

Heterotypic cell-cell interactions are essential for differentiation and maintenance of normal architecture and function of tissues. Co-culture of different cell types can be realized by some methods including hanging-drop, rotary wall vessel, spinner flask, micro-molding, and micro-fluidics. Spheroid composition is controlled by adjusting the ratio of different cell types.

Enhanced hepatic functions of primary hepatocytes were observed when they were co-cultured with fibroblasts (Takezawa et al. 1992; Lu et al. 2005). Spheroids were obtained by culturing hepatocytes for 3 days, which were subsequently co-cultured with NIH/3 T3 cell. NIH/3 T3 fibroblasts attached to the periphery of the hepatocyte spheroids and proliferated around them. Co-cultured hepatocyte spheroids exhibited significantly up-regulated liver-specific functions such as higher albumin secretion level and 3-methylcholanthrene-induced cytochrome P450 enzymatic activity as compared to spheroids with single cell type (Lu et al. 2005). Besides, freshly isolated rat hepatocytes, which were co-cultured with activated stellate cells, aggregated rapidly to form well-defined viable spheroids. These co-culture spheroids were further found with a specific hepatic ultrastructure with bile canaliculi, tight junctions, desmosomes, lipid storage and superior cytochrome P450 activities relative to hepatocytes monoculture (Thomas et al. 2005). Interestingly, co-culture of hepatocyte and pancreatic islet cells promoted the metabolic functions in comparison to the monotypic spheroids (Lee et al. 2004).

Co-culture of parenchymal cells and mesenchymal stem cells have shown increased parenchymal activities of spheroids. Enrichment of pseudoislets by bone marrow cells enhances vascularization after transplantation and increases the amount of insulin-producing tissue. Accordingly, bone marrow cell-enriched pseudoislets may represent a novel approach to increase the success rate of islet transplantation (Wittig et al. 2013). Similarly, combining hepatocytes with MSCs to create hepatic tissue spheroids exhibited good viability and metabolic activity despite a limited hepatocyte number (Murakami et al. 2004). On the other hand, differentiation commitment of mesenchymal stem cell spheroids also can be induced in co-culture models. Hepatic differentiation of mesenchymal stem cell spheroids can induce hepatocyte-like cells by being co-cultured with primary liver cells (Qihao et al. 2007).

3.3 Vascularization

Vascularization is a prerequisite for transplantation of engineered constructs (Datta et al. 2017; Hospodiuk et al. 2018). The reconstruction of blood vessel network in artificial macro-tissue has been a critical topic for regenerative medicine with some constructs/scaffolds having been fabricated. As the major "raw materials" for engineered constructs, multicellular spheroids with the potential of angiogenesis and developing sprouts to interconnect the surrounding microvessels, are helpful for the grafts. As aforementioned, 3D spheroids represent promising vascular units because of their high angiogenic and vasculogenic potential, which resulted from the up-regulated expression of relative genes such as HIF-1α, vascular endothelial growth factor (VEGF), and SDF-1. Controlling the size of spheroids can modulate the hypoxic levels. Spheroids which are larger than 100 μm in diameter exhibit a more pronounced upregulation of HIF-1α and VEGF secretion when compared to smaller ones. However, low oxygen levels may significantly compromise the cell viability of larger spheroids. Therefore, it is necessary to determine the ideal size of spheroids that balance both cell survival and cytokine production for the paracrine stimulation of angiogenesis (Skiles et al. 2013).

Co-culturing of spheroids with endothelial cells and endothelial progenitor cells has been reported to accelerate the vascularization process (Walser et al. 2013; Dissanayaka et al. 2015). For example, Zhang et al. (Dissanayaka et al. 2015) fabricated 3D spheroids of dental pulp cells (DPCs) co-cultured with HUVECs. Capillary network within spheroids formed by HUVECs sustained for a prolonged period, even after the micro-tissues transformed into a macro-tissue. Those induced prevascularized macro-tissues showed enhanced differentiation capacity compared with DPC only macro-tissues, which was indicated by higher osteo/odontogenic gene expression levels and mineralization.

Incorporation of stem cells in spheroids may be helpful for vascularization. Adipose-derived mesenchymal stem cells (adMSCs) have exhibited a high angiogenic activity and their incorporation into tissue constructs represented a promising vascularization in tissue engineering. Spheroids of adMSCs seeded in porous polyurethane scaffolds were found with formation of the potent initiators of blood vessel after implantation, suggesting that adMSC spheroids might serve as individual vascularization units for simultaneous development of neo-vascular network in the implanted constructs (Laschke et al. 2013). Mineda et al. (2015) reported that human adipose-derived stem cell (ASCs) spheroids cultured in non-crosslinked hyaluronic acid (HA) gel eventually differentiated into vascular endothelial cells, and contributed to the newly formed vascular network. Compared with monolayer culture, upregulated hepatocyte growth factor (HGF) levels in ACSs spheroids may have helped neo-vascularization in HA spheroid-treated models.

3.4 Others

After biofabrication through different methods, large number of spheroids can be collected and then stored in the bio-cartridges (micropipettes) for the subsequent assembly, or stored in cell culture medium or bioreactor for incubation. Preventing undesired fusion of stored tissue spheroids is challenging for scaling-up spheroids (Timothy and Frank 2014).

It has been reported that hypoxia may play a role in regulation of vascularization and production of ECM and growth factors in spheroids (Mineda et al. 2015; Shearier et al. 2016). For some types of cells (e.g. chondrocytes), low-oxygen culture may be beneficial for regeneration, since it increased the expression of cartilage-specific collagen II and aggrecan, stimulated matrix deposition, and improved the quality of chondrospheroids (Shi et al. 2015). Therefore, properly controlled hypoxia culture may provide the environment to obtain the superior regenerative properties of spheroids. However, it should be noted that insufficient oxygen will hurt cells.

The properties of spheroids were also influenced by some other factors such as media additives (Leung et al. 2015), physicochemical characteristics of the culture substrates (Yeh et al. 2014; Lee et al. 2015), and biomaterials directly incorporated in spheroids (Bratt-Leal et al. 2011; Tseng and Hsu 2014). Hence, optimal parameters were utmost important for the successful fabrication of spheroids (Laschke and Menger 2017).

Recently, magnetic fields have been used as a physical force to accelerate the fusion process with active contacts by increasing cell-cell and cell-matrix interactions in cell aggregates. It was demonstrated that paramagnetic cellular spheroids, whose fusion was mediated by magnetic forces, produced a more cohesive and homogenous tissue at earlier time points, when compared to control spheroids without magnetic forces. The use of magnetic forces for accelerating the fusion of paramagnetic cellular spheroids is a critical improvement because those fused tissues can be introduced into post-processing methods for maturation at earlier time point.

4 Bioprinting for Spheroid Fabrication

In the past few years, bioprinting has emerged as one of the most powerful techniques to improve the limitations of cell homogeneity in organoid models. Bioprinting can be defined as a method of biofabrication in which biological materials (e.g. cells, nucleic acids and proteins) can be deposited precisely, and spatial patterns can be pre-defined with cellular-level resolutions (Ozbolat et al. 2016). Moreover, the physiological complexity is able to be more closely imitated by accurately positioning the cells within an organoid. The process parameters which influence bioprinting of organoid, include nozzle diameter, flow rates and so on (Ozbolat and Yu 2013). Organoids are bioprinted using the combination of biomaterials (e.g. hydrogels) and cells. The function of hydrogels is to provide the mechanical support, as well as to act as a carrier for bioactive factors in the spatio-temporal domain. In such cases, it is important to ascertain the toxicity of the biomaterials and their degradation byproducts, and optimize rheology and crosslinking properties to obtain organoid with desired mechanical and structural properties. The most popular bioink for organoid fabrication has been alginate, gelatin-based, collagen, fibrin, polyethylene diacrylate, and natural decellularized ECM (e.g. Matrigel). Comprehensive studies on the properties of different bioinks have been extensively published (Hölzl et al. 2016; Hospodiuk et al. 2017).

Generally, bioprinting are classified into three types, namely the droplet-based bioprinting (DBB), extrusion-based bioprinting (EBB) and laser-based bioprinting (LBB) (Ozbolat and Hospodiuk 2016; Ozbolat et al. 2017). DBB technique has evolved from the inkjet printing technology, and Boland's and Nakamura's groups made pioneering contribution to extending the scope of inkjet printing to bioprinting. In DBB, droplets of bioink are dispensed to form the designed pattern. The nozzle for DBB is actuated by certain mechanism including thermal, pneumatic, piezoelectric, acoustic, electrostatic, or electrohydrodynamic (Gudupati et al. 2016). DBB can achieve considerably high resolutions in the order of 20–100 μm, and is suitable for heterotypic bioprinting. However, DBB has limited ability to bioprint highly viscous bioinks. EBB deposits cell-laden bioinks using pneumatic or piston-driven nozzles. EBB has also been used for a scaffold-free bioprinting, in which only cell aggregates were bioprinted. In this method, the toxicity of biomaterial is not of concern, and high concentrations of cell loading and cell-cell interactions are possible. EBB is also capable of printing heterotypic bioinks with a wide range of viscosities, but limited by a low resolution of 200–400 μm, and shear stress which may cause cell death. Another concern for both EBB and DBB is the solidification mechanism. LBB is based on the forward transfer mechanisms, such as matrix assisted pulsed laser evaporation-direct write (MAPLE-DW), biological laser printing (BioLP). In LBB, a laser absorbing layer is irradiated by laser energy, which evaporates and transfers the bioink onto a collecting substrate. Being an orifice-free technique, LBB does not encounter the clogging-related issues, which usually happen in DBB and EBB. In LBB, solidification is generally achieved by photo-induced reactions. LBB, however, is a costly technique (Ozbolat et al. 2017). Thus, based on the unique capabilities of different bioprinting techniques, several applications for organoid fabrication have been demonstrated.

Recently, deposition of organoids has gained remarkable attention in drug development and testing (Peng et al. 2016, 2017), it was observed that better cell-cell and cell-matrix interactions can be achieved when compared to 2D culture techniques. Although rapid progress is being made in the field of bioprinting, several critical challenges still remain, including high-resolution and accurate bioprinting of tissue spheroids. Chen et al. demonstrated acoustic surface standing waves technique for assembly of organoids in tissue engineering (Chen et al. 2015). Over a few days of culture it was observed that tissue spheroids made from HUVECs tend to merge into a single organoid constructs. The fusion of tissue

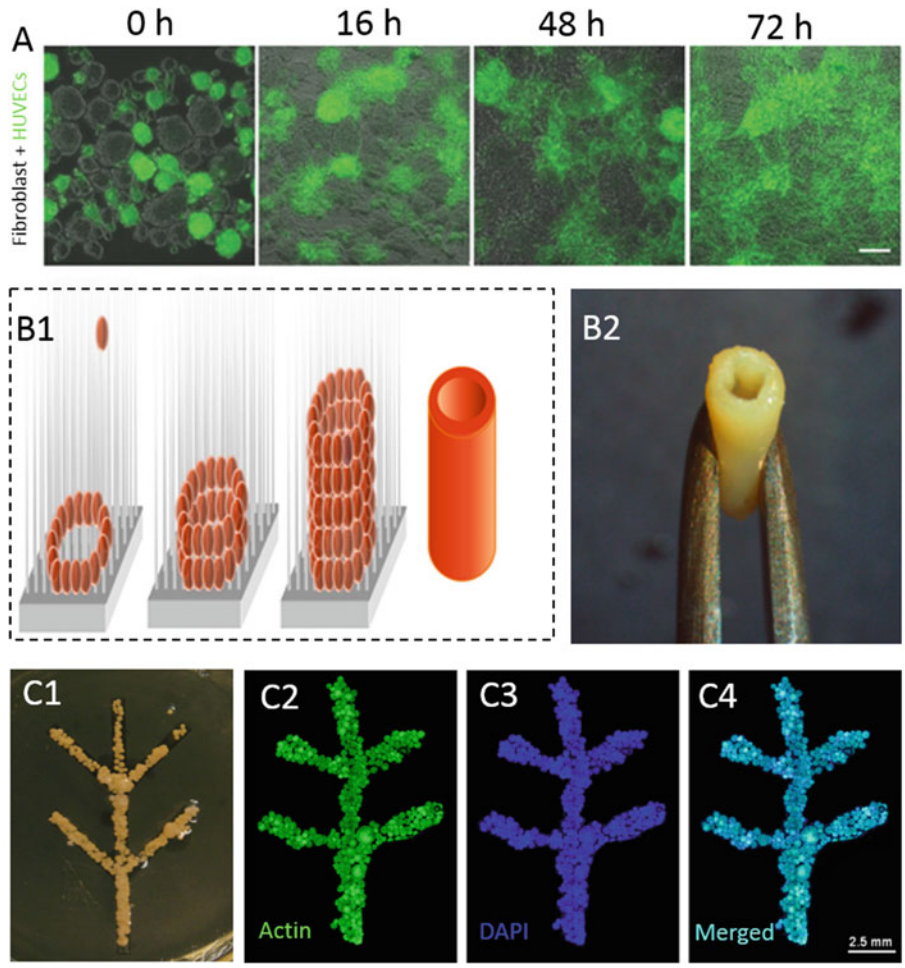

Fig. 3 Bioprinting of tissue spheroids: (**A**) time-lapse images of 1:1 mixture of HUVECs and fibroblast spheroids (reproduced/adapted with permission from Chen et al. 2015); (**B1**) tissue spheroids place along microneedles array in order to fabricate 3D tissues, (**B2**) a matured construct of vascular graft (reproduced/adapted with permission from Itoh et al. 2015); (**C1**) an image showing SPION-loaded endothelial cell spheroids into 3D structure at 48 h, (**C2-C3-C4**) confocal microscopy images of SPION-loaded spheroids: actin (**C2**), DAPI (**C3**) and merged (**C4**) (reproduced/adapted with permission from Whatley et al. 2014)

spheroids were observed day by day (Fig. 3A). This method proved to be very useful in a variety of 3D tissue engineering applications such as stem cell and developmental biology, 3D tumor models for development of personalized medicine and a better, more physiologically-correct platforms for drug screening applications.

Jakab et al. have demonstrated the ability to bioprint using tissue spheroids by loading them into cartridge/micropipette (Jakab et al. 2008). Two piston-based extrusion heads were used in order to deposit the spheroids one by one, while a third pneumatic extruder head deposits embedding hydrogel with collagen gel. In another work, micro-extrusion based bioprinter developed by researchers from the Laboratory of Biotechnical Research in Russia utilizes a conus nozzle design in order to deposit tissue spheroids onto electrospun matrix composed of polyurethane (Mironov et al. 2016). Tissue constructs displayed a high degree of cell viability and ability to spread across the matrix within 7 days after bioprinting. Bioprinting of tissue spheroids offers a complex, more realistic 3D representation of the

tissue when compared to the currently available techniques limited to two-dimensional single cell patterning. Researchers have shown the ability to control the thickness of bioprinted tissue constructs by varying the spacing between the individual spheroids.

Itoh et al. have developed Bio-3D printer system in order to fabricate scaffold-free tubular tissue using multicellular spheroids (MCS) (Itoh et al. 2015). In this bioprinting system, spheroids were picked and placed along thick stainless steel microneedles array (Fig. 3B1–B2). Fusion between the MCS was observed after 4 days period of perfusion within a bioreactor at which time the construct was removed from the needle array and retained its initial configuration of the structure.

Moreover, Blakely et al. demonstrated stacking layers of material to form uniform, large bio-structures. The layers are made by molding the cells to form cell aggregates of various shapes such as toroids and honeycomb sheets. The bioprinter picks up the cell aggregates using vacuum grippers and places them to form large, complex and dense biostructures. This technique can produce large structures rapidly, scaffold-free and do not require the use of many non-cellular materials (Blakely et al. 2015).

In another approach, functionalized superparamagnetic iron oxide nanoparticles (SPIONs) were utilized for fabrication of tissue spheroids which can be assembled in 3D using a magnetic template (Fig. 3C1–C4) (Whatley et al. 2014). The magnetic force between the SPION spheroids and the magnetic template was observed to be sufficient for free-floating magnetic spheroids to self-assemble into predetermined three-dimensional structure within 24 h. Fusion between the spheroids was observed after 10 days of culture within a bioreactor. It was observed that the initial structure defined by the magnetic template was maintained through the bioprinting process and after the fusion between the spheroids have occurred.

5 Future Perspectives

Organoid tissue engineering has emerged as a potential platform to develop *in vitro* models for studying pathophysiological and organogenesis related issues. Several anatomical sub-parts of brain, convoluted tubules of kidney and microvillus structures of intestines have been mimicked in 3D organoid models so far (Xinaris et al. 2015; Clevers 2016; Fatehullah et al. 2016; Yin et al. 2016). However, challenges remain in biofabrication of organoid models with regards to the reproducibility of 3D structures, automation of organoid production, inclusion of perfusion networks, and integration of sensors to collect real time information on cellular status or cell-cell interactions. Biofabrication becomes more challenging as the organoid is more complex in terms of cellular and ECM compositions. Bioprinting techniques have emerged as powerful tools for organoid engineering, which offer different capabilities to process organoids with distinct cell densities and mechanical properties. Some of the successful studies have been discussed in the chapter, from which it can be foreseen that more extensive investigations in bioprinting of organoids will be carried out. These works may probably create the next generation of organoids to cover a wider spectrum of pathological and physiological stages. Till now, bioprinting has been attempted to create simple organoid structures without considering the intricate pathological characterization of the organoids. For example, organoids which are specific for Alzheimer's disease or Parkinson's disease have not been bioprinted. In addition, organoids for personalized medicines or regenerative therapies could be bioprinted by *ex vivo* organ engineering. Apart from expanding the applications of bioprinted organoids, other directions for future studies would be the improvement of the resolution of bioprinting process, especially EBB. At present, EBB produces the most mechanically robust constructs, but is limited by the low resolution. The shear stress induced cell damage at high bioink

viscosities, which would also require to be addressed in future studies. Due to the limitations of current bioprinting for organoids, suitable bioinks are expected to be developed, which are printable and crosslinked under physiologically ambient conditions. More studies are also required to enable efficient integration of bioprinted organoids with organ-on-chip models. Moreover, it would be necessary to develop standardized biomarkers and biosensors allow lab-to-lab reproducibility of bioprinted organoids.

6 Conclusions

This chapter provided an overview of the state-of-the art techniques and challenges for organoid biofabrication. Spheroid fabrication methods, including the hanging-drop method, microfluidic method, liquid overlay method, rotating flask method, spinner flask method, and micro-molding method have been introduced, and their advantages and disadvantages have also been compared. In addition, challenges such as high-throughput, co-culture and vascularization, still exist in 3D spheroid modelling. Bioprinting is expected to make a major contribution to the organoid fabrication due to its flexibility in fabricating multicellular constructs in an automatic high-throughput manner. It is also expected that organoids which are specific to certain physiological and pathological phases or particular patients, would be fabricated with standardized processes for future biological and pharmaceutical investigations.

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Bioengineered Scaffolds for Stem Cell Applications in Tissue Engineering and Regenerative Medicine

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Abstract

Stem cell-based therapies, harnessing the ability of stem cells to regenerate damaged or diseased tissues, are under wide-ranging consideration for regenerative medicine applications. However, limitations concerning poor cell persistence and engraftment upon cell transplantation still remain. During the recent years, several types of biomaterials have been investigated to control the fate of the transplanted stem cells, aiming to increase their therapeutic efficiency. In the present chapter we focus on the general properties of some of these biomaterials, which include polymers, ceramics, and nano-biomaterials. In the first part of the chapter, a brief explanation about stem cell biology, sources, and their microenvironment is provided. The second part of the chapter presents some of the most recent studies investigating different types of biomaterials and approaches that aim to mimic the stem cell microenvironment for a more precise control of the stem cell fate.

Keywords

Biomaterials · Regenerative medicine · Tissue engineering · Stem cells · Microenvironment

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Abbreviations

3D	Three-dimensional
ESCs	Embryonic stem cells
IPSCs	Induced pluripotent stem cells
HSCs	Hematopoietic stem cells
SGZ	Sub-granular zone
ECM	Extracellular matrix
BM-MSCs	Bone marrow mesenchymal stem cells
AFSC	Amniotic fluid derived stem cells
ADSCs	Adipose-derived stem cells
HA	Hyaluronic acid
MMPs	Metalloproteases
GAGs	Glycosaminoglycans
PEG	Poly (ethylene glycol)
PVA	poly (vinyl alcohol)
PLA	poly (lactic acid)
PLGA	poly (lactic-co-glycolic acid)
PLC	Polycaprolactone
BG	Bioactive glass
CNTs	Carbon nanotubes
DLC	Diamond-like carbon
CNFs	Carbon nanofibers

1 Introduction

Regenerative medicine is an interdisciplinary field of study which by combining the principles of engineering and biological sciences seeks for the repair or enhancement of damaged tissues and organs (Mao and Mooney 2015). In the recent years, the restrictions of synthetic implants alongside with the scarcity of organ donors have resulted in a concurrently increasing research in regenerative medicine and biomaterials sciences to provide the patients with better treatment strategies (Bajaj et al. 2014; Londono and Badylak 2015). Generally, in the current regenerative medicine strategies, cells and biomolecules are encapsulated in a three-dimensional (3D) scaffold, where all components play a critical role in neo-tissue formation (Ducheyne 2015). The biomaterial scaffolds mainly act as temporary substitutes, which support the regeneration of damaged tissue by delivery of cells and/or growth factors that have the ability to encourage tissue regeneration (Ducheyne 2015). Current research in biomaterials and regenerative medicine is focused in strategies for optimal harvest of stem cells, enhanced cell survival, and design of novel biomaterials for precise control of the cell microenvironment (Sekuła and Zuba-Surma 2018). ESCs have gained a great attraction owing to their ability to differentiate into any kind of adult cell (Chung et al. 2017). On the other hand, given their minimal immunological and ethical concerns, adult stem cells are also an attractive cell source for regenerative medicine applications (Broughton and Sussman 2016). Moreover, induced pluripotent stem cells (iPSCs) reprogrammed from terminally differentiated cells, which possess similar differentiation ability but less ethical issues as compared to embryonic stem cells, have been also suggested for tissue regeneration (Tabar and Studer 2014; Singh et al. 2015). Stem cell progress is narrowly connected to the biological stem cell niche, which provides essential physicochemical cues that control the intricate signaling pathways regulating the stem cell fate (Sekuła and Zuba-Surma 2018). In recent years, several studies have been done to reveal the molecular pathways which direct stem cell fate to more precisely regulate the uniform differentiation of the cells before transplantation (Almada and Wagers 2016; Rossant and Tam 2017). Hence, the idea of designing biomaterials that could closely mimic the stem cell niches has gained a great attraction among regenerative medicine scientists. A plethora of studies have shown the ability of 3D scaffolds in stimulating encapsulated stem cells to differentiate into different cells and subsequently repairing the damaged tissue (Blanpain and Fuchs 2014; Lane et al. 2014). Biomaterial scientists have currently synthesized a number of biomaterials with various physicochemical modifications, which could mimic the *in vivo* stem cell microenvironment and precisely deliver stem cells and/or growth factors (see Fig. 1). Furthermore, it has been suggested that by manipulating the properties of biomaterials the biological responses to scaffolds

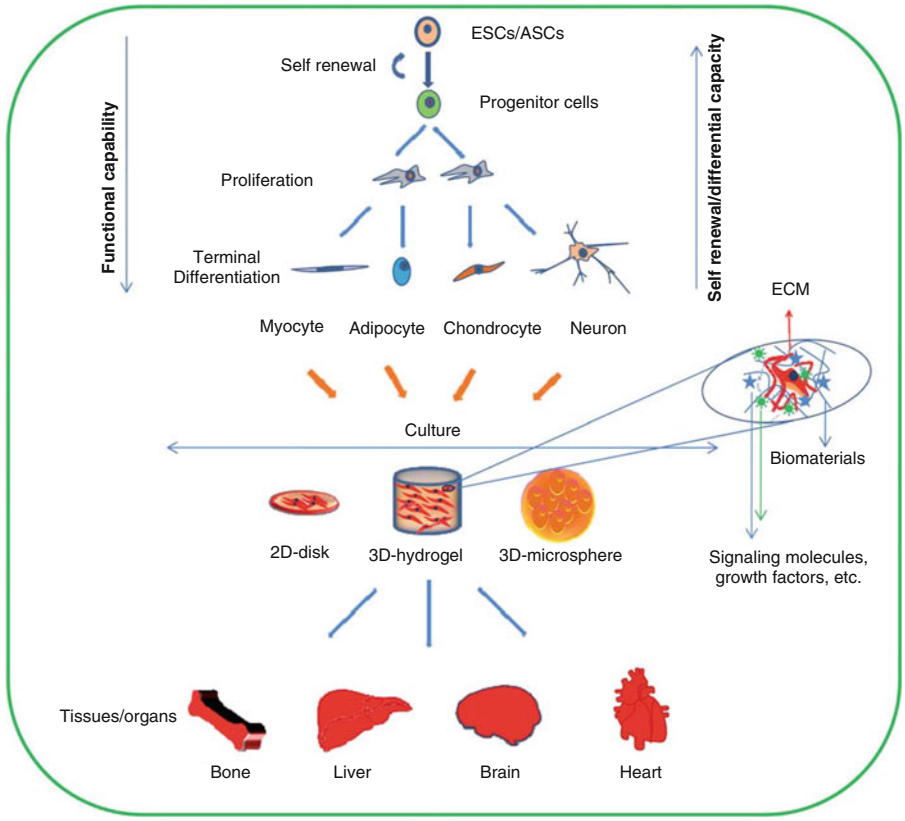

Fig. 1 A schematic representation of stem cell differentiation, followed by cell culture using biomaterials, and their different applications in regenerative medicine. Embryonic stem cells (ESCs) and Adipose derived stem cells (ADSCs) both can self-renew and proliferate. These cells proliferate to progenitor cells, which can differentiate into specific lineage cells. The cells could be cultured into 2D, 3D-scaffold or 3D-microspheres for regenerative medicine applications. Cell cultures can concurrently with using biomaterials include different biochemical signaling molecules and growth factors. (Reprinted from Singh and Elisseeff 2010 with the permission from Royal Society of Chemistry)

and stem cell differentiation could be precisely controlled (Murphy et al. 2014). Although there is a wide range of biomaterials and regenerative medicine strategies, in the present chapter we will primarily focus on the general principles of employing different biomaterials including polymers, ceramics, and nano-biomaterials for guiding stem cells. In the first part of chapter a brief explanation about stem cells biology, sources, and their microenvironment will be given. The second part of chapter will summarize some of the most recent studies using various types of biomaterials to mimic the stem cell microenvironment for a more precise control of the stem cell fate.

2 Stem Cell Biology

Stem cells are generally defined as undifferentiated cells, which possess self-renewal and multipotential differentiation abilities. Stem cell self-renewal is primarily the result of cell division, which occurs in the microenvironment of stem cells known as niche. In the biological conditions, stem cells are in an exceptional microenvironment with dynamic stability, acknowledged as the "niche", which is known to mediate various cellular and molecular signaling pathways controlling the proliferation and differentiation of stem cells (Zhang and Li 2008; Lane et al. 2014; Rana et al. 2017). It has been reported

that the number of stem cells in their niche is kept continuous through a balance between inactive and stimulated cells (Orlacchio et al. 2010). It has been described that stem cell could be divided in a daughter, which stays stem cell, and a progenitor daughter (asymmetric division), or in 2 stem cell daughters (symmetric division) (Yamashita 2009). The asymmetric stem cell division offers accurately daughter stem cells replacement internal and external to the niche, as well as, the progenitor cells replacement which creates a differentiated progeny after exposing to particular molecular signals (Cheng et al. 2008). Some studies have suggested that the self-renewal mechanism of stem cells comprises a mixture of stem cell spindle alignment and cell niche signals. It has been demonstrated that the mitotic spindle, ordered via the accurate arranging of the centrosomes throughout the interphase, is vertical to the cell hub axis and could play a role in asymmetric division (Yamashita 2009; Martino et al. 2012). In the main, the stem cell niche is a definite space in the tissue. Some studies have demonstrated that the osteoblastic and vascular niches support hematopoietic stem cells (HSCs) (Wilson and Trumpp 2006). Stem cell niches, in the brain, are taken in the sub-ventricular zone (SVZ), the lateral wall of the lateral ventricles, and the sub-granular zone (SGZ) of the hippocampal dentate gyrus (Mudo et al. 2009; Rosa et al. 2010). Through linking between stem cells and somatic cell neighbors a particular cyto-architectural association is retained in adult stem cell niches. At this point, stem cells reveal dissimilar scales of structural features including macro, micro, and nanoscale arrangements, which potentially impact on cell functions (Martino et al. 2012). It has been shown that inside the niche, stem cells are potentially uncovered to a mixture of various biomolecules including soluble chemokines, cytokines, growth factors, and insoluble transmembrane receptor ligands and extracellular matrix (ECM) molecules. The ECM molecules influence cell function through controlling the release of GFs and cytokines, sequestering growth factors, and regulating receptor activities (Chen and Jin 2010; Kelleher and Vacanti 2010). Some studies have recognized molecules such as Wnt ligand, notch signaling, and IP3K/Akt, cytokines, which play key roles in the molecular signaling of self-renewal and stem cell proliferation and differentiation (Lapidot and Petit 2002; Fleming et al. 2008; Tian et al. 2015; Mohammed et al. 2016). It has been demonstrated that the Wnt ligand has great effects on the preservation of the HSCs function and inactivity, as well as on the osteoblasts growth and differentiation (Fleming et al. 2008). Additionally, some studies have reported that notch signaling is essential for the assortment of neural progenitors in Drosophila and vertebrates, as well as throughout the arrangement of neural progenitors between various neural subtypes (Louvi and Artavanis-Tsakonas 2006; Chen et al. 2016). Moreover, soluble growth factors and membrane-anchored receptors make up a signaling complex which control gene expression via the organized function of transcription factors (Martino et al. 2012). All of these factors are controlled by epigenetic pathways that arrange variations in cell fate via knock-down of pluripotency genes and initiation of cell differentiation genes. Interestingly, some studies have revealed that cell fate is controlled by a crosstalk between epigenetic alterations and transcription factors such as microRNAs (Guo et al. 2011; Yi and Fuchs 2011).

3 Stem Cells Sources

In regenerative medicine, defining reliable sources of cells is a critical issue (Forbes and Rosenthal 2014; Hao et al. 2017). In general, it is desirable to obtain cells with ability to self-renew, preserved plasticity and repair capacity, which could differentiate into the specific types of cells (Hao et al. 2017). Furthermore, in recent years, there has been an increased amount of evidence indicating that stem cells mediate their regenerative properties through paracrine effects rather than differentiation into the specific tissue types (da Silva Meirelles et al. 2009). While stem cells obtained from the embryonic membrane, placenta, amniotic membrane, and umbilical cord blood can differentiate into several different cell types, cells obtained from adult tissues

can only differentiate into restricted forms (Vallet-Regí and Ruiz-Hernández 2011), indicating that stem cells from various parts of body have dissimilar differentiation abilities. Based on their differentiation capabilities, stem cells have been broadly divided into totipotent, pluripotent, multipotent and unipotent.

Embryonic stem cells are pluripotent, which means that they can differentiate into all lineages of the primary three germ layers. ESCs could potentially allow the fabrication of type-matched tissues for each individual, through stem cell banking or using cloning treatment (Chagastelles and Nardi 2011). Several studies have shown that ESCs could be expanded in culture, which results in large concentrations of cells that could not be directly obtained from a tissue source (Howard et al. 2008; Chagastelles and Nardi 2011). Teratoma development is an evidence of the accurate pluripotent nature of ESCs (Nussbaum et al. 2007). Teratoma indicates the ability of stem cells to form noncancerous tumour when implanted in an immune-deficient animal, which is a major safety concern in the use of ESCs for cell therapy. It also exhibits the significance of using a terminally differentiated cell stock without hiding the stem cell-like properties (Hewitt et al. 2007). Hence, during the implementation of stem cells using a technique for confirming their correct differentiation is crucial (Howard et al. 2008). Using appropriate strategies for preventing teratoma of stem cells and regulating their differentiation are therefore critical issues which should be taken into account before considering them for tissue regeneration applications.

Mesenchymal stem cells are multipotent stem cells that are obtained from embryonic and adult sources. Bone marrow mesenchymal stem cells (BM-MSCs) are the most prevalent applied stem cells for musculoskeletal applications (Ohishi and Schipani 2010). As compared to ESCs, BM-MSCs possess superior accessibility, easier process and reduced risk of tumorigenicity, (Bara et al. 2014). However, there are some limitations in using MSCs including the risk of some phenotypic variations during monolayer culture, and the effects of age of donors and patients on cells functions (Moodley et al. 2017).

Cord blood is another source of stem cells, including ESCs and MSCs, hematopoietic stem cells and endothelial progenitor cells, which is more accessible with the existing of cord blood banks (Sullivan 2008). Due to the wide availability of CB-MSCs and amniotic fluid derived stem cells (AFSC) as well as reduced teratoma risk, some studies have currently suggested using them for tissue regeneration applications (Hao et al. 2017).

ADSCs represent a major source of multipotent stem cells for regenerative medicine applications, too (Zachar et al. 2011). ADSCs could be easily extracted from several human adipose tissues with fewer distress for the donor in comparison with BM aspiration. Because of their ability in differentiation into different cells and their accessibility, several of studies have suggested using ADSCs for tissue regeneration applications (Estes et al. 2010; Tsuji et al. 2014). The therapeutic appeal of ADSC has been demonstrated through several preclinical and clinical trials, which have shown that in addition to the ability to differentiate into different tissue types, ADSCs possess pro-angiogenic, immunosuppressive and pro-wound healing properties.

In addition, amniotic fluid derived stem cells are another source of stem cells exhibiting properties between ESCs and adipose-derived stem cells (ADSCs). These type of stem cells displays several advantages, including a relatively simple culture technique, great differentiation ability, and less immunogenicity and tumorigenicity, with no ethical issues associated with their procurement (Estes et al. 2010; Hsueh et al. 2014).

Additionally, iPSCs, which are obtained by reprogramming terminally differentiated cells have been suggested for use in tissue regeneration therapies (Singh et al. 2015). In the process of iPSCs reprogramming, mature cells from the individual are modified *in vitro* with genes that 'dedifferentiate' them to a pluripotent phase. It has been reported that iPSCs are similar to the natural pluripotent ESCs in several aspects such as the expression of particular genes and proteins, chromatin methylation patterns, culture kinetics, *in vitro* differentiation forms, and teratoma development (Singh et al. 2015; Hao et al. 2017).

4 Scaffold Requirements for Cell delivery

Studies have shown that scaffolds play a key role in directing the fate of encapsulated stem cells, affecting functions such as survival, proliferation, differentiation and migration (Howard et al. 2008; Chang and Wang 2011; Rana et al. 2017). Since in mammalian cells most of these functions are anchorage-dependent, scaffolds are required to promote cell adhesion (Garg et al. 2012), in order to provide a favorable substrate for cell adhesion, proliferation, differentiation, and migration (Mandal and Kundu 2009). In addition, scaffolds should be porous to facilitate the transport of nutrients and biomolecules supporting cell survival. The biodegradability rate of biomaterial should be also carefully taken into account by considering the degradation rate of natural tissues so that it totally disappears when the tissue is regenerated. The degradation products should not be toxic and should be easily eliminated from the organism (Lyons et al. 2008). Biocompatibility, which among other involves the immune response to the implanted biomaterial, is another key property which should be taken into account in the design of scaffolds (Cao and Zhu 2014). If the scaffold is biocompatible and biodegradable, new tissue will finally substitute it, while if it is biocompatible and bioactive, the scaffold will incorporate with the neighboring tissue. If the biomaterial is inert, it will normally become encapsulated by a fibrous capsule. Biomaterials that release toxic degradation products could induce severe immunological reactions, which may lead to cell death and a consequent implant failure (Garg et al. 2012). In addition, encapsulating the essential biomolecules for to control the behavior of the encapsulated stem cells should be also considered (Zhang and Li 2008; Zhang et al. 2012). The mechanical properties of the scaffold should be adequate to protect cells from harmful load-bearing forces without hindering suitable biomechanical agents (Chung and Park 2007). Providing a reproducible micro and macroscopic arrangement with a high surface to volume ratio which highly supports cell attachment is also required (Khang et al. 2006). The scaffold should also promote cell and proteins functions through supporting the interface adherence which is cells and/or proteins attachment to the surface of scaffolds (Chung and Park 2007). Additionally, the porosity arrangement and pores size of scaffolds should be taken into consideration as small pores could inhibit the penetration of cells into the scaffold, whereas large pores inhibit cell attachment. In fact, the percentage of porosity, the pore size distribution, and its interconnectivity are key factors which highly impact on seeded cells in scaffolds (Mooney et al. 1996; Lyons et al. 2008). Besides, the fabrication process of scaffolds in large scale should be easy and cost-effective. Furthermore, the scaffold should have a great loading capacity. The cells should be also homogenously encapsulated into the scaffold. In addition, the physical and chemical stability, as well as biological activity of the encapsulated cells at body conditions need to be carefully evaluated over an elongated period of time (Garg et al. 2012).

5 Biomaterials as Instructive Extracellular Microenvironments for Controlled Differentiation

5.1 Polymers

It has been reported that choosing biomaterials in different tissue regeneration applications is extremely dependent on their physical and chemical surface properties such as their surface roughness (Ranella et al. 2010), architecture (Chang and Wang 2011), charges (Calatayud et al. 2014), energy (Hoefling et al. 2010), and functional groups (Meder et al. 2012). During the recent decades, several polymers have been suggested as suitable substrates for stem cell proliferation and differentiation. Encapsulating stem cells in both natural and synthetic types of polymeric biomaterials presents promising results due to the exceptional properties of polymers including: high surface-to-volume ratio, flexibility of

chemical and physical surface properties, ability to precise control their porosity regarding both size and number, biodegradation, and mechanical property (Ravichandran et al. 2010; Cao and Zhu 2014; He and Benson 2014).

5.1.1 Naturally-Derived Biomaterials

Various natural polymers have been suggested to support the stem cells fate, including collagen, gelatin, alginate, hyaluronic acid (HA), fibrin, chitosan, and acellular tissue matrices (Araña et al. 2014; Das et al. 2015; Kong et al. 2016; Wang et al. 2017). Collagen, the major constituent of the natural ECM, is a fibrous protein containing three polypeptide chains, which are coiled around each other to form a triple-helix structure (Parenteau-Bareil et al. 2010). Collagen is naturally degraded in vivo by metalloproteases (MMPs), representing a very attractive polymer for tissue regeneration applications (Zhu and Marchant 2011). Biodegradable collagen is an attractive material for forming in situ hydrogels because of its capability to quickly form stable gels at physiological temperature (Tan and Marra 2010). As studies have shown, 3D collagen hydrogels provide a suitable environment for stem cell proliferation and differentiation (Zhou et al. 2018). Kim et al. (2015) have recently developed a bio-functional hydrogel by conjugating transforming growth factor-β1 (TGF-β1) to MeGC hydrogels containing type II collagen. The authors examined the efficacy of Col II and TGF-β1 in promoting chondrogenic differentiation of hSMSCs. As it can be seen in Fig. 2, their results showed that incorporating Col II and TGF- β1 into the chitosan hydrogels highly enhanced chondrogenic differentiation of the encapsulated stem cells. In addition, Zhou et al. (2018) have investigated the efficacy of Col II/chondroitin sulfate hydrogel as a promising system for ADSCs delivery. They reported that the delivery system which was cross-linked with 0.02% genipin enhanced the expression of nucleus pulposus-specific genes. After the hydrogel injection, the disc height, water content, ECM synthesis, and arrangement of the deteriorated NP were moderately regenerated.

Gelatin is a partial derivative of collagen, which can simply be achieved by a controlled hydrolysis of collagen (Guillén et al. 2011). This polysaccharide commonly found in nature and is the major component of skin, bones and connective tissues (Ha et al. 2013). It has been shown that gelatin is an very valuable material for stem cell delivery applications as it highly supports stem cell growth and differentiation (Das et al. 2015). Dong and his coworkers have reported designing a hydrogel based on commercially available thiolated gelatin containing a multifunctional PEG for improving the efficiency of stem cell delivery. The in vivo part of study confirmed that the suggested hydrogel significantly promoted cell retention, angiogenesis, and wound closure. The authors suggested that that the hydrogel could be used for modulating stem cell behavior in 3D culture, and delivering them for tissue regeneration applications (Dong et al. 2017). HA is an enzymatically degradable sulphated-glycosaminoglycan (GAG) consisting of numerous alternating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid (Hintze et al. 2009). HA is commonly dispersed throughout the ECM of all connective tissues and especially in the synovial fluid of joints (Necas et al. 2008). This sulphated GAG is a key player in various organic processes such as proteoglycan groups, tissue hydration, nutrient transmission, and cell differentiation (Dicker et al. 2014). Thus, HA and its derivatives have been widely suggested as promising candidates for stem cell delivery due to their innate biocompatibility, biodegradability (naturally degraded by hyaluronidase), and also its exceptional capability to form hydrogels (Snyder et al. 2014; Ansari et al. 2017). For instance, Snyder et al. (2014) have recently synthesized a novel injectable hydrogel based on fibrin/HA encapsulated with BMSCs for OA therapy. The live/dead staining and metabolic tests indicated that the suggested hydrogel provided a favorable 3D environment for BMSC proliferation. Additionally, the quantitative polymerase chain reaction (qPCR) of stem cells encapsulated in the system proved reducing expression of collagen type 1 alpha 1 mRNA with an increase in Sox9 mRNA expression. Another biomaterial

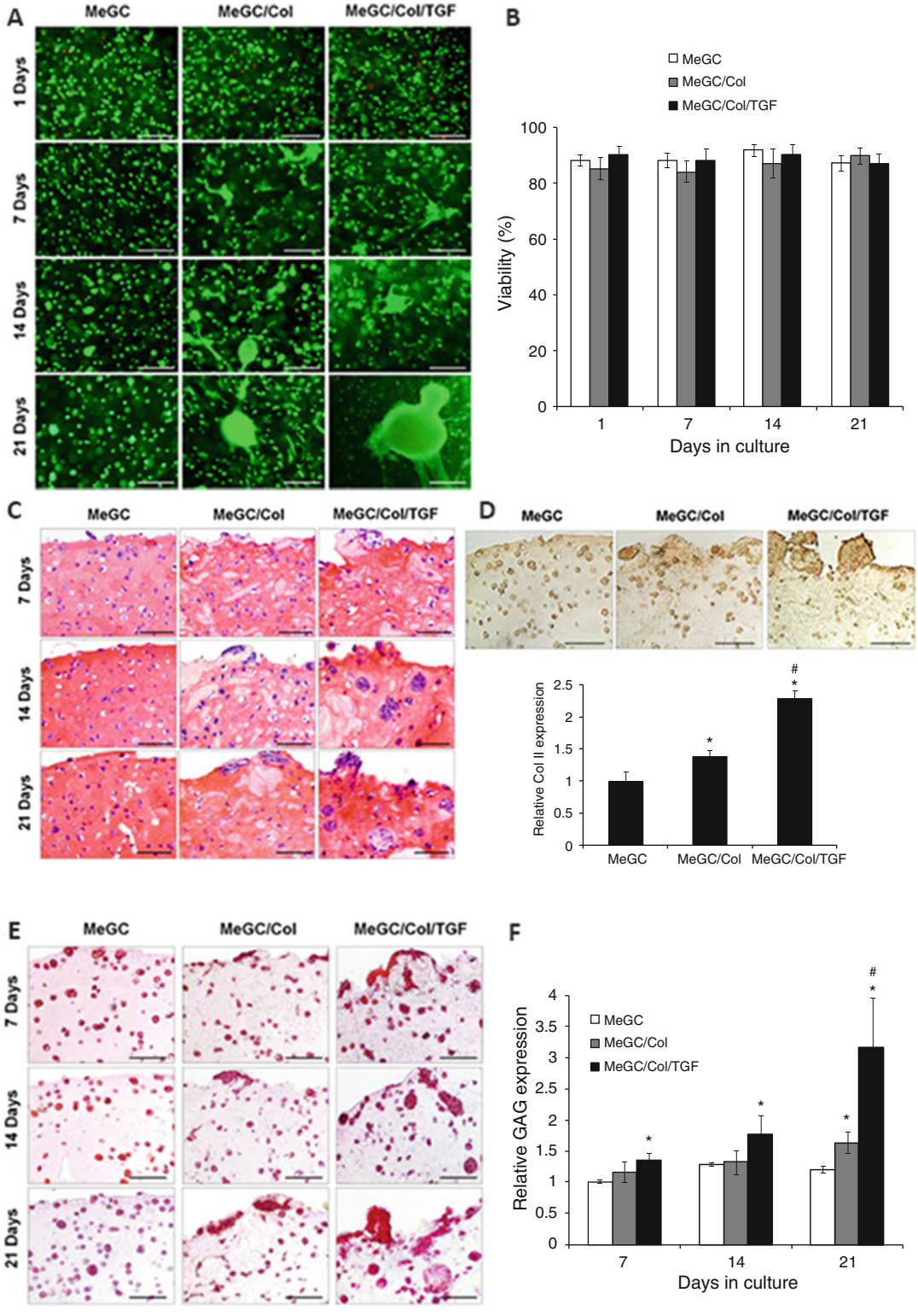

Fig. 2 (**a**) Live/Dead staining of hSMSCs in the hydrogels and (**b**) hSMSCs viability (%) from live/dead image study. (**c**) H & E staining of hSMSCs cultured in hydrogels at days 7, 14, 21 in culture. (**d**) Type II Col staining of hSMSCs cultured in hydrogels at 21 days and quantification of Col II staining by image study. (**e**) Safranin-O staining of hSMSCs cultured in hydrogels and (**f**) quantification of Safranin-O staining by image study. (Reprinted from Kim et al. 2015)

that has been suggested for stem cell differentiation is chitosan. Chitosan is a polycationic polysaccharide includes glucosamine and N-acetyl glucosamine molecules, which is made by deacetylation of N-acetyl-D-glucosamine of chitin to a degree higher than 60% (Rinaudo 2006; Boddohi et al. 2009; Rahmati et al. 2016; Rahmati et al. 2017). Chitin is the second most plentiful naturally derived polymer, which is found in the external skeleton of crustaceans and insects (Sarasam and Madihally 2005). Chitosan is a biocompatible, biodegradable, bio-adhesive, and haemostatic glucosamine polymer, which can successfully support stem cell functions (Singh Dhillon et al. 2013). Additionally, alginate hydrogel is another biocompatible polymer could be used for stem cell delivery applications. Alginate is a linear, hydrophilic, brown algae or bacteria polysaccharide, which includes 1,4-linked β-D-mannuronic and β-L-glucuronic acid units (Tøndervik et al. 2010). The alginate solutions can instantly be a transparent gel by addition of multivalent cations namely Ca_2^+, Mg_2^+, Ba_2^+, or Sr_2^+ that supportively interact with alginate units to create ionic bonds (Paige et al. 1996; Drury and Mooney 2003). The alginate-based hydrogels due to their availability, ability to form gels under physiological conditions, adhesive properties, and non-immunogenicity, have gained great attention in drug delivery, cell encapsulation, and tissue engineering applications (Peer 2012; Sun and Tan 2013; Bidarra et al. 2014; Ho et al. 2016). For instance, Ho et al. (2016) have investigated the effects of contributing adhesive biomaterials such as alginate on the spheroid activity and enhancing the bone-forming potential of MSCs. The researchers encapsulated MSC spheroids into Arg-Gly-Asp (RGD)-modified alginate hydrogels and then examined their efficiency in bone tissue regeneration. The MSC spheroids in RGD-modified hydrogels exhibited meaningfully higher cell survival than spheroids in untreated alginate. After five days in culture, spheroids in RGD-treated hydrogels displayed equivalent levels of apoptosis, but more than a 2-fold rise in VEGF release in comparison with spheroids in untreated gels. In overall their results indicated the ability of alginate to guide the functions of MSC spheroids for bone regeneration. Furthermore, Ansari et al. (2017) have designed a composite hydrogel based on alginate and HA containing TGF-β1 ligand, and Periodontal Ligament Stem Cells (PDLSCs); and then examined the chondrogenic differentiation of encapsulated cells *in vitro* and *in vivo*. Their results demonstrated that PDLSCs, and hBM-MSCs, as the positive control, were marked positive for both toluidine blue and Alician blue staining, whereas demonstrating great amounts of Col II, Aggrecan and Sox-9 expression. In addition, as it can be seen in Fig. 3, it was shown that the chondrogenic differentiation of encapsulated MSCs could be modulated with the modulus elasticity of the suggested system, revealing the crucial role of the microenvironment on stem cell fate. The histological and immunofluorescence staining also established ectopic cartilage-like tissue repair inside the injected hydrogels. Interestingly, PDLSCs demonstrated superior ability for chondrogenic differentiation than hBM-MSCs.

In the recent years, acellular tissue matrices or decellularized tissues have also shown a potential support for stem cells delivery and growth (Nie et al. 2015; Jang et al. 2017). Nie and his coworkers have currently used acellular dermal matrix scaffold as a promising carrier for the delivery of ADSCs and demonstrated that the system increased the diabetic wound healing via a paracrine mechanism, with improved granulation tissue development and enhanced re-epithelialization and neovascularization (Nie et al. 2015). Generally, natural polymers have shown effective cell attachment and potentially support cell proliferation and differentiation. However, scaffolds made of pure natural polymers possess some inherent limitations that have limited their widespread use, such as their poor mechanical properties, rapid biodegradation and high batch-to-batch variability.

5.1.2 Synthetic and Biosynthetic Biomaterials

Synthetic polymers are attractive materials in regenerative medicine applications specifically in designing scaffolds for stem cell delivery, because their physical and chemical properties

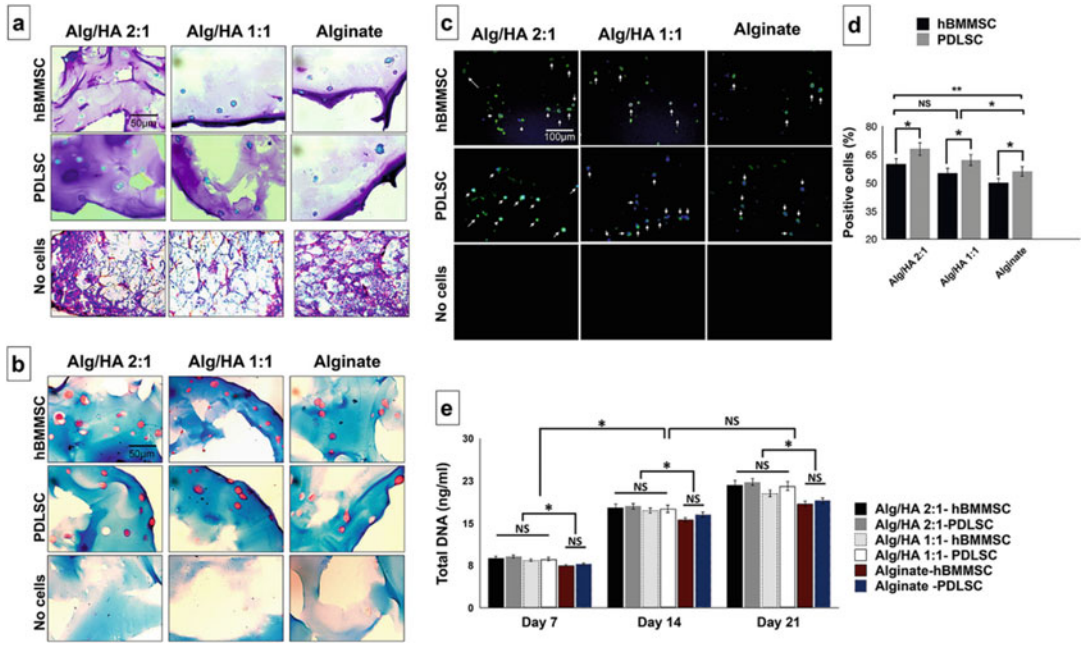

Fig. 3 *In vitro* chondrogenic differentiation of seeded MSCs. The histochemical test established spread-out cell morphology for both alginate/HA and alginate hydrogel with (**a**) positive toluidine blue (**b**) and Alcian blue staining presenting the development of chondrogenic tissue-related ECM. (**c**) Positive staining in the immunofluorescence labeling approving the fabrication of type-II collagen by seeded PDLSCs. (**d**) The number of cells positive for antibodies against Col II was counted and superior Col II expression amounts in PDLSCs in comparison with hBMMSCs was detected. Both alginate/HA hydrogels demonstrated statistically superior concentration of Col II staining than the group with alginate hydrogel. (**e**) DNA content for MSCs encapsulated in various systems after 3 weeks of chondrogenic initiation. (Reprinted from Ansari et al. 2017 with Elsevier permission)

are more controllable and reproducible than those of natural-based polymers (Patenaude and Hoare 2012). This type of polymers are proper for simply forming into a range of different 3D shapes, which could be designed with particular block structures, degradable chemistries, and narrow molecular weight distribution (Tan and Marra 2010). It has been shown that the degradation rate of synthetic polymers could be regulated by variable monomer properties, arrangements, and crosslinking concentrations. Since the degradation of these kind of polymers mainly occurs via hydrolysis, some studies have suggested biomimetic scaffolds with enzyme-mediated degradation positions such as MMP degradable peptides (Yu et al. 2016). Poly(ethylene glycol) (PEG), poly(vinyl alcohol) (PVA), poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone (PLC) are among the most frequently used synthetic polymers for stem cell applications (Amer et al. 2015; Yao et al. 2017; Richardson et al. 2018). Some studies have shown the ability of these synthetic polymers in promoting stem cells adhesion and directing their differentiation toward a desired lineage. Amer et al. (2015) have designed a PEG-based hydrogel having collagen type I, and peptide crosslinkers for 3D culture and release of hESC-derived pancreatic progenitors. The authors reported that the hydrogel could promote viable aggregates, and aggregate size, as well as provided facile support of aggregates, without adversely affecting on stem cells differentiation. In addition, the differentiation efficacy of ADSCs to chondrocytes has been recently developed by designing a composite scaffold containing both synthetic and natural polymers. The scaffold synthesized by incorporating TGF-β1-loaded gelatin microspheres into PLGA structure. The results

of work supported the hypothesis that the suggested scaffold could highly enhance cartilage regeneration by encouraging ADSCs differentiation to chondrocytes.

5.2 Ceramics

In comparison with the metals and polymers, bio-ceramics have been widely suggested as candidates for orthopedic and dentistry applications due to their exceptional properties such as increased density, wear resistance, and biocompatibility (Best et al. 2008; Dorozhkin 2015). Bio-ceramics have been generally classified into three basic kinds, which comprise bio-inert high strength ceramics (such as alumina (Al2O3), zirconia (ZrO2) and carbon), bioactive ceramics (such as bioglass and glass ceramics) and bio-resorbable ones (Best et al. 2008). Among bio-ceramics, zirconia-based bio-ceramic has attracted a great attention due to its superior mechanical strength and fracture toughness, biocompatibility, and aesthetic properties (Komine et al. 2010). Several studies have investigated the ability of zirconia implants in supporting stem cell differentiation. Kitagawa et al. (2012) have reported the ability of zirconia microwell scaffolds in promoting chondrogenic differentiation of hMSCs. Their results indicated that hMSCs for a short time adhered to the scaffold prior to releasing and entering the microwells. Additionally, the physical limitations forced by the microwells allowed hMSC groups to evenly differentiate into hyaline chondrocyte-like cells. Chondrogenic aggregates in microwells upregulated Col II, ACAN, and COMP genes. In contrast, chondrogenesis in pellet cultures was varied with the expression of CD105, Col X, and Col I genes. Besides, it has been acknowledged that alumina has high hardness and abrasion resistance properties which could be potentially useful for skeletal regeneration applications. Studies have shown that by manipulating the physicochemical properties of alumina substrates the behavior of stem cells in physiological conditions changed (Bauer et al. 2009; Kitagawa et al. 2012). In addition, bioactive glasses (BG) which have a great ability of forming HA-like layer in both *in vitro* and *in vivo* conditions have gained a considerable attention among scientists and surgeons in the recent years. These materials are fabricated from glass precursors including silica (SiO_2), boric acid (B_2O_3), and phosphoric oxide (P_2O_5), network modifiers, and intermediate oxides. Some studies have incorporated stem cells in bioactive glass ceramics for investigating their proliferation and differentiation activities after implantation (Bosetti and Cannas 2005; Houreh et al. 2017). In addition, Houreh et al. (2017) have currently demonstrated that the release of different ions in bioactive glasses could effectively affect stem cell fate.

5.3 Nano-biomaterials

It is a well-known fact that the natural ECM is an intricate system comprised of a several components in both micro and nano scale dimensions. However, it has been reported that hydrogels, porous scaffolds, microspheres and microparticles have stimulated stem cells differentiation, they still fail to totally bio-mimic the nano scale dimensions of ECM. Several studies have in fact shown that topographical cues at the nanoscale, such as nanofibers, nanopits and nanopillars, effectively control stem cell functions, including proliferation, migration and differentiation (Singh and Elisseeff 2010). Furthermore, it appears that cell behavior is controlled by the combined contribution of micro- and nano-topographical cues from the substrate (Dolatshahi-Pirouz et al. 2015). In the recent decades, several studies have shown the feasibility of using carbon-based nanomaterials such as carbon nanotubes (CNTs), graphene, fullerene, quantum dots (QDs), diamond-like carbon (DLC), and carbon nanofibers (CNFs) as potential carriers for stem cells (Gizzatov et al. 2015; Onoshima et al. 2015; Ahadian et al. 2016; Kim et al. 2016). Yao et al. (2017) have designed some nanofibrous scaffolds based on PCL/PLA nanofibers by using thermally induced nanofiber self-agglomeration. The 3D scaffolds had high

porosity of 95.8 % as well as interconnected and hierarchically structured pores. As it can be seen in Fig. 4, the authors reported that the incorporation of PLA into PCL nanofibers could significantly enhance hMSCs osteogenic differentiation *in vitro* and bone development *in vivo*. Furthermore, Bauer et al. (2009) have investigated the effects of nanotopography on the stem cell differentiation on ZrO_2 and TiO_2 nanotubes. Their results indicated that the pure geometric diameter in the range between 15 and 100 nm governs over other properties of a biomaterial which could influence on stem cell behavior. However, some studies have exhibited that nanomaterials could inhibit stem cell differentiations. For instance, Park et al. (2009) have demonstrated that silica nanoparticles could potentially inhibit ESCs proliferation and differentiation.

6 Concluding Remarks and Future Perspectives

The discovery and development of efficient biomaterials that could precisely control the stem cell functions is a crucial issue in regenerative medicine. In the recent years, various types of

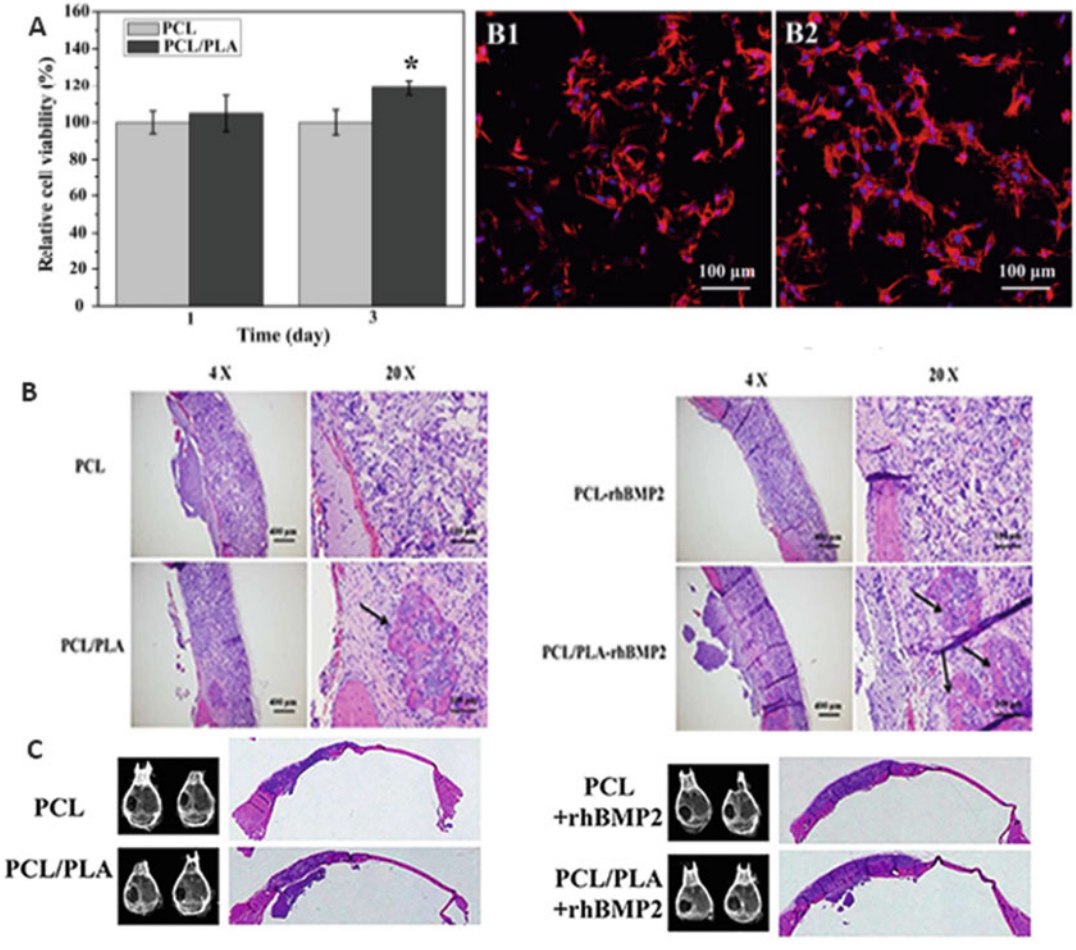

Fig. 4 (**a**) hMSCs viabilities on PCL-3D and PCL/PLA-3D scaffolds after culturing for one and three days. hMSCs morphologies on (B1) PCL-3D and (B2) PCL/PLA-3D scaffolds after culturing for 16 h. (**b**) Radiographic test and macro-view of the histological scaffolds of PCL, PCL/PLA, PCL-rhBMP2, and PCL/PAL-rhBMP2 samples after six weeks of implantation. (**c**) H&E staining of the regenerated bones six weeks after *in vivo* implantation. (Reproduced from Yao et al. 2017 with the permission from Elsevier)

scaffolds that could potentially help the discovery of biochemical and biophysical regulators of stem cell fate have been investigated. However, there is still a lack of sufficient evidence concerning the specific micro environmental cues that critically control the stem cell fate. Synthesizing suitable scaffolds with adhesive binding sites is crucial for improving the incorporation efficacy of peptides, ligands, and growth factors. In addition, a plethora of smart biomaterials have been currently suggested as promising candidates for monitoring stem cells fate after transplantation. However, there is still a crucial need of suggesting active live-cell markers that would allow observing gene expression alterations of stem cells after transplantation in real time. Designing more reliable tests for investigating the cell functions after transplantation could potentially enhance our understanding of stem cell biology. Furthermore, the identification of particular mechanisms involved in tissue repair could be helpful in designing more suitable stem cell delivery systems. In addition, it should be taken into account that the physicochemical properties of each biomaterial could significantly affect stem cell functions. Hence, in order to design suitable microenvironments for stem cells, the precise investigation of the influence of each property of biomaterials on the cells fate prior to *in vivo* implantation is crucial. Overall, effective regenerative medicine strategies will demand a more intense collaboration between biologists and biomaterials scientists in the future.

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Mesenchymal Stem Cells and Calcium Phosphate Bioceramics: Implications in Periodontal Bone Regeneration

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Abstract

In orthopedic medicine, a feasible reconstruction of bone structures remains one of the main challenges both for healthcare and for improvement of patients' quality of life. There is a growing interest in mesenchymal stem cells (MSCs) medical application, due to their multilineage differentiation potential, and tissue engineering integration to improve bone repair and regeneration. In this review we will describe the main characteristics of MSCs, such as osteogenesis, immunomodulation and antibacterial properties, key parameters to consider during bone repair strategies. Moreover, we describe the properties of calcium phosphate (CaP) bioceramics, which demonstrate to be useful tools in combination with MSCs, due to their biocompatibility, osseointegration and osteoconduction for bone repair and regeneration. Also, we overview the main characteristics of dental cavity MSCs, which are promising candidates, in combination with CaP bioceramics, for bone regeneration and tissue engineering. The understanding of MSCs biology and their interaction with CaP bioceramics and other biomaterials is critical for orthopedic surgical bone replacement, reconstruction and regeneration, which is an integrative and dynamic medical, scientific and bioengineering field of research and biotechnology.

Keywords

Bioceramics · Bone regeneration · Calcium phosphate · Dental · Mesenchymal stem cells · Tissue engineering

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Abbreviations

ALP	Alkaline phosphatase
BM	Bone marrow

BMPs	Bone morphogenetic proteins
CaP	calcium phosphate
CD	Cluster of differentiation
DFPCs	Dental follicle progenitor cells
DPSCs	Dental pulp
GMSCs	Gingival mesenchymal stem cells
HA	Hydroxyapatite
HUVEC	Human umbilical vein endothelial cells
IDO	Indoleamine 2, 3-dioxygenase
IFN-γ	Interferon-γ
IL-	Interleukin-
MSCs	Mesenchymal stem cells
PD	programmed death
PDGF	Platelet-derived growth factor
PDLSCs	Periodontal ligament
PGE2	Prostaglandin E2
PLC-BCP	Poly-ε-caprolacton coated-biphasic calcium phosphate
Runx2	Runt-related transcription factor 2
SCAP	Apical papilla derived stem cells
SDF-1	Stromal cell-derived factor 1
SHED	Exfoliated deciduous teeth
TGF-β1	Transforming growth factor-β1
Th	T helper
TNF-α	Tumor necrosis factor-α
Tregs	T Regulatory
B-TCP	beta-tricalcium phosphate

1 Introduction

According to different health predictions, the increasing amount of elderly people in the world will suffer an increase in bone defects in the near future. In the last decade, 86% of adults over 70 suffered periodontal diseases, with restoration of teeth becoming a growing challenge (Zhang et al. 2013). Bone defects arising after tooth loss normally result in bone loss, which difficults posterior dental implants. Autologous bone grafts were commonly used to treat bone defects but they usually resulted in limitations and varied side effects (Egusa et al. 2012). This posts the necessity to identify an optimal strategy to treat these requirements, minimizing the risks and the costs for the patient and allowing for an efficient recovery, offering patients a solid and long term outcome (Zorin et al. 2014).

In the last decades, tissue engineering has experienced a significant growth due to the combination of approaches coming from multidisciplinary fields such as biology, material science and bioengineering. There has been an increase in the development and application of different biomaterials that can help achieving osteogenic regeneration, solving the difficulties encountered by previous approaches based on the use of grafts. These biological substitutes are able to restore and maintain the normal function of bone, which is a major need in maxillofacial surgery due to either the absence of bone or to the low quality of the available one (Yousefi et al. 2016).

Among biomaterials, Calcium Phosphate (CaP) bioceramics are one of the most commonly used in the field of osteogenic regeneration, since they have a similar composition to the bone mineral (Kim et al. 2017; Vivanco et al. 2011; Vivanco et al. 2012; Eliaz and Metoki, 2017; Raghavendra et al. 2017) and are biocompatible, biodegradable, osteoinductive and osteoconductive, among others. Moreover, CaP salts have the ability to form mineralized tissues, and therefore constitute an ideal candidate for endodontic therapies (Zhang et al. 2013).

Mesenchymal Stem Cells (MSCs) constitute a heterogeneous multipotent population that can be harvested from several adult tissues such as bone marrow, adipose tissue, and umbilical cord Wharton's Jelly dental tissues (Čamernik et al. 2018). MSCs show the potential to differentiate into several specialized cell types, including adipocytes, chondrocytes and osteocytes, among others. MSCs have been shown to mobilize and recruit to damaged tissues, then collaborating to tissue or organ repair and regeneration. Thus, MSCs play key roles in the healing and homeostasis of every organ and tissue via their self-renewal and differentiation capacity (Hu et al. 2018).

There is a great expectation from dental MSCs for periodontal regenerative therapies, since they are capable of differentiating into cementoblast-like cells as well as of developing *in vivo* alveolar

bone, cementum and periodontal ligaments tissues. These properties indicate the feasibility and the potential of MSCs for periodontal and tooth regeneration in combination with biomaterials (Shi et al. 2015; Ercal et al. 2018).

In this review we will describe the main characteristics of MSCs, such as osteogenesis, immunomodulation and antibacterial properties, which we believe are key parameters to consider during their usage in tissue repair and regeneration. Moreover, we describe the properties of CaP bioceramics, which demonstrate to be useful, due to their biocompatibility and chemical resemblance, for bone repair. Also, we overview some features of MSCs from dental cavity, which seem to be promising candidates for bone regeneration and tissue engineering in combination with CaP bioceramics.

2 Mesenchymal Stem Cells

Stem cells are defined as cells without differentiation that are capable of self-renewal and can differentiate into multiple cell types (Fuchs and Segre 2000; Blau et al. 2001; Fortier 2005). They can be embryonic or adult depending on their source of origin (Blau et al. 2001). In adults, cells with these properties are known as mesenchymal stem cells, due to their capability of differentiation into mesenchymal lineages such as osteogenic, chondrogenic, adipogenic and myogenic (Bruder et al. 1994; Yoo et al. 1998; Wakitani et al. 1995; Pittenger et al. 1999). The first evidence of this finding was made by Friedenstein in the 1970s, when his group of investigation described some stromal cells that derived from marrow, which, when put into culture, had a spindle shape and formed colonies from which they multiplied (Friedenstein et al. 1976; Digirolamo et al. 1999). Later experiments demonstrated *in vivo* that these cells can form bone and cartilaginous tissue (Horwitz et al. 2002). On the other hand, MSCs can be induced *in vivo* to non-mesenchymal cells such as nervous tissue cells. The most classic source of MSCs has been the bone marrow (Devine 2000). Nevertheless, currently diverse studies have been able to isolate MSCs from different sources, such as peripheral blood, adipose tissue, lipoaspirate, periodontal ligament, dental pulp, and gingival tissue, among others (Zuk et al. 2002; Sakaguchi et al. 2005). *In vitro*, MSCs can be expanded and their properties will remain until several passages (Lange et al. 2007; Lawson et al. 2017). This feature positions them as a good target for researchers in the field of tissue regeneration, although there is controversy about their use once cultured and expanded *in vitro* (Gupta et al. 2016). One relevant consideration at the time of regeneration therapy is the source of origin of the MSCs, which will influence the efficiency of their differentiation (De Bari and Roelofs 2018).

In vitro, these cells are capable of attaching to plastic, expressing specific surface antigens and showing multipotent differentiation potential, features that define MSCs (Shahdadfar et al. 2005; Salzig et al. 2015). Nowadays, there is still controversy about their denomination; recently the name of Medicinal Signaling Cell was suggested, alluding to its capability to signal cellular respiration molecules. Caplan (2017) says literally: "*Because the function of MSCs in vivo is secretory and primarily functional at sites of injury, disease or inflammation, now favor this terminology*".

On the other hand, the specific surface markers of MSCs vary depending on their source of origin. However, there is consensus that the positive expression markers analyzed by flow cytometry should be positive for CD105, CD73 and CD90. As additional criteria for MSCs, these cells should lack expressions of: CD45 (pan-leukocyte marker), CD34 (marker of primitive hematopoietic progenitors and endothelial cells), CD14 and CD11b (expressed in monocytes and macrophages), CD79a and CD19 (cell B markers) and HLA-DR (they are not expressed in MSCs unless they are stimulated). The purity level of MSCs that is suggested is 95% with expression of CD105, CD73, CD90 being 2–5% maximum for the hematopoietic antigens expressions (Lee 2008; Ghaneialvar et al. 2018).

MSCs are responsible for tissue regeneration, while their therapeutic ability, depending on

whether MSCs are supplied endogenously or exogenously, depends on "when, where and how" they are distributed or presented at the site of injury (Caplan 2015).

As a result of previous studies, there is a robust body of evidence that supports the potential use of these cells in tissue engineering due to their high proliferation rate, their immunomodulatory capability and their ability to reach out sites of damaged tissue (Lee 2008; Ghaneialvar et al. 2018; Parton and Mason 2012; Yubo et al. 2017). Therefore, there is a growing interest in the complete understanding of the functioning of molecular and cellular bases of MSCs due to their great potential use in regenerative medicine, which is still faced with challenges when applying cell therapies, especially in orthopedic, traumatological and musculoskeletal diseases. Since the research from the 70s, in which the MSCs concept was generated, a growing number of scientific papers has developed, reaching 60.000 works in 2018 only related to MSCs. Areas of biochemistry, genetics, and molecular biology are presented as the most relevant in the number of papers, followed by Medicine, which is an indicator of the need to determine the cellular and molecular basis for their right therapeutic use (Scopus, 2018). On the other hand, at the beginning of 2018 there were more than 600 clinical trials with MSCs on human patients, with 175 completed to date (Clinicaltrials, 2018). All this information and date show the importance of having a consensus about the proper use of MSCs in different types of diseases.

2.1 Osteogenic Differentiation of MSCs

With regard to tissue repair and regeneration, MSCs, under appropriate stimuli, act by direct differentiation into more specialized cells. One of the most important roles of MSCs in bone regeneration is their strong capacity to differentiate into osteoprogenitors, playing a critical role in the formation and maintenance and healing of this tissue (Dimitriou et al. 2005).

MSCs role in bone remodeling may involve both endochondral ossification, which includes first differentiation into chondrocytes and subsequent calcification, and intra-membranous ossification, that involves a direct osteoblasts MSCs differentiation (Dimitriou et al. 2005; Thompson et al. 2002). MSCs differentiation into osteoblasts is a complex interaction between paracrine and autocrine signals that trigger several cellular and molecular mechanisms to achieve full osteogenic differentiation (Garg et al. 2017). Actually, osteogenic differentiation involves a timely orchestrated activation of specific transcription factors, which regulate gene expression and further define osteoblast phenotype. Activation of two transcription factors, runt-related transcription factor 2 (Runx2) and downstream osterix is crucial for osteoblast differentiation, and impaired activity of each of these two transcription factors results in complete absence of mineralized skeleton (Stains and Civitelli 2003). The early osteogenic marker-protein, expressed in committed osteo-progenitors, is alkaline phosphatase (ALP), while more mature osteoblasts express osteocalcin, osteonectin and osteopontin (Frith and Genever 2008). Runx2 is activated through many signaling pathways, including bone morphogenetic proteins (BMPs) and Transforming growth factor-β1 (TGF-β1) among others (James 2013). BMPs are involved in the MSCs and/or osteoblast differentiation towards chondrocytes and osteoblasts (Garg et al. 2017). The abundance of different types of BMPs varies in relation to skeletal elements. BMP-2, −4, −6, −7 and − 9 are of special importance in bone formation, and they act trough Runx2 and osterix activation, while BMP-3 and BMP-13 present exceptions in the subfamily, and act as inhibitors of osteogenic differentiation (Xiao et al. 2007; Shen et al. 2009).

BMP-2, −6 and −9, among 14 BMPs from a comprehensive study, seem to be the most potent factors to induce osteoblastic MSC differentiation. Interestingly, BMP-2 is expressed on Day 1 of fracture healing to stimulate MSCs differentiation, while BMP-6 and -9 are expressed at later stages in the animal model (Cheng et al. 2003).

BMP-2 is being intensively investigated in tissue engineering and bone regeneration, with means to develop the most suitable delivery system, such as BMP-2 and dexamethasone incorporated in nanoparticles, or BMP-2 and platelet-derived growth factor (PDGF) incorporated into macroporous beta-tricalcium phosphate (β-TCP) system or plasmids encoding BMP-2 complex with polyethylenimine to transfect human adipose derived MSCs (Zhou et al. 2015; Del Rosario et al. 2015; Atluri et al. 2015). Researchers have also identified BMP-9 as one of the most potent osteogenic inducers in MSCs, and also demonstrated that osteogenic differentiation induced by BMP-9 can be mediated by MAPKs in periodontal ligament-derived MSCs (Ye et al. 2014), as well as by canonical Wnt signaling which includes both beta-catenin and Runx2 recruitment to osteocalcin promoter in mesenchymal C3H10T1/2 cells (Tang et al. 2009). As recently demonstrated by Li et al. (2015) BMP-6, in cross talk with vascular endothelial growth factor, induces osteogenic differentiation of human adipose tissue-derived MSCs via p38 MAPK, suggesting that combined application of these cells and factors to the fracture site might be useful for bone reparation. BMPs play other roles in the healing process, such as stimulating the synthesis and secretion of other bone and angiogenic growth factors, direct endothelial cells activation for angiogenesis, and regulating callus formation (Dimitriou et al. 2005; Garg et al. 2017). In addition, BMP heterodimers, such as BMP-4/−7 and BMP-2/−7, increase MSCs proliferation and osteoblastic differentiation both *in vitro* and *in vivo*, showing enhanced osteoinductive activity (Dimitriou et al. 2005).

Therefore, BMPs increase bone differentiation and regeneration via induction of a cascade of events including osteoprogenitors chemotaxis, cell proliferation and differentiation, angiogenesis, and an increased controlled synthesis of extracellular matrix production (Bessa et al. 2008).

TGF-β is a potent chemotactic stimulator of MSCs, enhancing proliferation of MSCs, pre-osteoblasts, chondrocytes, and osteoblasts. TGF-β initiates signaling for BMPs synthesis in osteoprogenitor cells, inhibits osteoclast activation and stimulates osteoclast apoptosis. TGF-β and PDGF that are released by activated platelets in early stages of fracture healing induce MSCs migration, activation, and proliferation, along with angiogenesis and inflammatory reactions. TGF-β's osteoinductive potential, however, is limited and has shown various side effects, thus limiting its clinical use for bone regeneration aside from enhancing proliferation (Dimitriou et al. 2005; Pelissier et al. 2004). Furthermore, TGF-β did not induce osteogenic differentiation per se, but further induced osteoclast recruitment that provides a setting for bone formation and maintenance (Crane and Cao 2014). Namely, TGF-β and BMP-2 are required for normal fracture healing. Both TGF-β and BMPs receptors expression rise up early during bone repair to decrease as the callus cells differentiate to the start of bone formation. Without these two factors, MSCs osteogenic differentiation is disabling, thus inhibiting bone healing (Simmons et al. 2004; Ho et al. 2015).

Other factors also contribute to bone remodeling process by stimulating osteoblastic differentiation of MSCs, such as Insulin-like growth factor type 1 that plays a role in regulating early differentiation of MSCS into osteoblasts (Koch et al. 2005; Crane et al. 2013) and Stromal cell-derived factor 1 (SDF-1) which enhances, in a BMPs-mediated way, MSCs differentiation into osteoblasts (Ito 2011). SDF-1 is also a potent chemoattractant for MSCs migration to the site of injury. Moreover, MSCs over expressing SDF-1 lead to increased MSCs migration and transplanting these cells to the site of bone injury increased bone mineral density and new bone formation (Kitaori et al. 2009; Ho et al. 2015).

Finally, the wound-healing capacity of MSCs has led to studies in tissue engineering and regenerative medicine, such as seeding MSCs onto scaffolds to repair fractures and critical-sized bony defects (Bateman et al. 2017).

2.2 Immunomodulatory Properties of MSCs

Due to their tropism to inflammatory sites, the chemotactic responses of MSCs are generally considered to resemble those of immune cells. Consistent with this, inflammatory cytokines are strongly involved in modulating the mobilization of the bone marrow (BM)-MSCs in the bone marrow niche and the further trafficking and homing of those cells to damaged tissues (Barcellos-de-Souza et al. 2013). Because of their trophic and immunomodulatory functions, MSCs are generally considered to possess greater advantages in cell-based regenerative medicine (Samsonraj et al. 2017).

MSCs can migrate to the sites of inflammation and show potent immunomodulatory and anti-inflammatory effects through cell-cell interactions with lymphocytes or through the production of soluble factors (Poggi et al. 2018). It is generally accepted that MSCs: (i) suppress T-cell proliferation, cytokine secretion and cytotoxicity and regulate the Thelper balance of Th1/Th2. MSCS are able to suppress T cell activation and proliferation and decrease their response by shifting them from a Th1 to a Th2 immune phenotype (Matthay et al., 2017); (ii) regulate the functions of; (iii) increase B-cell viability but may also inhibit their proliferation and arrest the cell cycle; in addition, MSCs affect the secretion of antibodies and production of co-stimulatory molecules of B cells; (iv) inhibit maturation, activation and antigen presentation of dendritic cells; and (v) adult MSCs also inhibit interleukin-2-induced natural killer cell activation (Volarevic et al. 2017).

There are several key immunomodulatory factors and cytokines expressed by MSCs. One of the most important is TGF-β, which suppresses T-cell response through numerous TGF-β signaling pathways. In terms of activation and function, TGF-β cytokines may bind to TGF-β receptors on T cells and inhibit IL-2 production, cytotoxic T lymphocyte activation, clonal expansion of memory CD8+ T cells, and expression of perforin, an essential mediator for CD8+ T cell killing of tumor cells (Wu et al. 2015). Indoleamine 2, 3-dioxygenase (IDO), a critical rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway, produces tryptophan depletion that halts the growth of various cells. Moreover, IDO inhibits effector T cell proliferation, DC maturation, B cell proliferation, IgG secretion, and natural killer cell activity (Mellor et al. 2017). Prostaglandin E2 (PGE2), produced by Cyclooxygenase-2, has a multifunctional role in pathological processes and regulates inflammation. Production of PGE2 by MSCs is increased following tumor necrosis factor-α (TNF-α) or Interferon-γ (IFN-γ) stimulation. Furthermore, PGE2 increases the expression level of anti-inflammatory cytokine IL-10 and decreases expression of TNF-α, IFN-γ, and IL-12 in dendritic cells and macrophages. PGE2 also dampens secretion of IFN-γ and IL-4 in Th1 and Th2 cells, respectively, and promotes proliferation of T-regulatory (Treg) cells. Nitric oxide, produced by inducible nitric oxide synthase after stimulation by inflammatory factors, has been shown as one of the major mediators of T-cell suppression by MSCs. MSCs-secreted IL-6 inhibits monocytes differentiation toward DCs and subsequently induce a decrease in the stimulatory ability of DCs on T cells (Volarevic et al. 2017; Mellor et al. 2017).

Interestingly, the immunosuppressive function of MSCs licensed by IFN-γ and TNF-α produced by T cells can be further amplified by IL-17 through enhancing inducible nitric oxide synthase mRNA stability (Volarevic et al. 2017). Moreover, IL-17 enhances BM-MSCs T-cell immunosuppression by inhibiting surface CD25 expression and suppressing the synthesis of Th1 cytokines, IFN-γ, TNF-α, and IL-2. Furthermore, T cell suppression correlates with increased expression of IL-6 and increased levels of inducible-Tregs (Sivanathan et al. 2015).

In addition to the soluble factors production, MSCs, by cell-cell interaction, may suppress T-cell activation via induction of T-cell apoptosis through interaction of programmed death (PD)-1 molecule with its cognate ligands PD-L1 and PD-L2. Furthermore, direct contact between MSCs and purified T cells is required for Treg

induction (Davies et al. 2017). MSCs-to-T cell contact induces IL-10 secretion, which attenuates T cell proliferation, and stimulates HLA-G5 secretion which in turn inhibits activated T cells and natural killer-cell cytotoxicity (Selmani et al. 2008).

Although MSCs express low quantities of IL-10 themselves, they can indirectly enhance local IL-10 by promoting macrophage repolarization from pro-inflammatory type 1 phenotype towards anti-inflammatory type 2 phenotype, which is characterized by expressing high IL-10 levels. Moreover, IL-10 from MSCs reprogrammed type 2 macrophages may inhibit neutrophil influx into damaged tissue, thus preventing further excessive damage (Kim and Hematti 2009). In addition, MSCs modulate natural killer cells activity impairing their cytotoxic activity, cytokine production and granzyme B release (Matthay et al. 2017). Also, MSCs block T-cell differentiation towards Th17, and promote Th17 phenotype shift into FoxP3 T-regulatory cells (Matthay et al. 2017; Ghannam et al. 2017).

In vivo studies indicate that systemic administration of MSCs contributes to the immunosuppression in graft-versus-host-disease models, multiple sclerosis, inflammatory bowel disease, diabetes as well as cardiomyopathies. An increased number of clinical trials shows the feasibility of MSCs use in cellular therapies, in acute graft-versus-host-disease, severe osteogenesis imperfecta by allogenic BM transplantation, acute myocardial infarction, aplastic anemia, osteoarthritis, diabetes, among other conditions (reviewed in Squillaro et al. 2016; Samsonraj et al. 2017).

Regardless of the fact that MSCs from different sources may differ in their mechanisms and capacities for immunomodulation (Samsonraj et al. 2017), the characterization of MSCs immunosuppressive functions can provide an important functional parameter to predict *in vivo* the efficacy of MSCs (Miteva et al. 2016; Kalluri 2016). Moreover, MSCs may produce an immune tolerant microenvironment thus reducing the risk of the rejection of biomaterial-based implants. These functions can be a key aspect to consider in the use of MSCs in tissue bioengineering to control for the potential excessive immune response to bio-implants for organs and tissue regeneration.

2.3 MSCs Antimicrobial Properties

As abovementioned, MSCs-based therapy appears to be a promise but one of the main concerns related to the unwanted risk of infection when they are used in combination with bio-materials. Remarkable, several studies suggest that MSCs possess the capacity to exert antimicrobial effects, either directly by producing anti-bacterial factors or indirectly by regulating host immune response against pathogens (Alcayaga-Miranda et al. 2017).

MSCs can secrete soluble antibacterial proteins and peptides such as lipocalin-2, which has a bacteriostatic effect by sequestering bacterial iron chelator sidephores to impede iron transfer to bacteria (Alcayaga-Miranda et al. 2017). MSCs also produce hepcidin that exerts a broad spectrum of antimicrobial activity against fungal species and clinical relevant bacteria such as Escherichia coli, S. epidermidis, S. aureus, and group B streptococci (Alcayaga-Miranda et al. 2015, 2017). Furthermore, MSCs secrete considerable amounts of human cathelicidin hCAP-18/LL-37 that possess a broad spectrum of antimicrobial activity, which participates in bacterial clearance both *in vitro* and *in vivo*, moreover LL-37 exhibits several immunomodulatory effects, and chemotactic and pro-angiogenic functions (Krasnodembskaya et al. 2010).

IDO also contributes to MSCs antibacterial functions. MSCs-expressing IDO exhibit a broad spectrum of antimicrobial direct effects against bacteria and protozoal parasites (Meisel et al. 2011).

Also, MSCs increase macrophage pathogen phagocytosis by promoting type 2 phenotype, which results in the reduction in the number of colony forming units of blood P. aeruginosa in a mouse model of bacterial peritonitis (Matthay et al. 2017; Krasnodembskaya et al. 2012).

Interestingly, MSCs seem to have anti-viral properties in an IDO dependant fashion, since a

reduced CMV and HSV-1 replication in human MSCs was observed, and IDO inhibitor disables MSCs resistance to virus replication (Meisel et al. 2011; Thanunchai et al. 2015; Sharpe 2016).

In tissue engineering using biomaterials one of the main concerns is the risk of infection, which is predominantly caused by infection around the implant leading to the loss of supporting circumferential bone that causes the failure of insert (Eliaz and Metoki 2017). In this aspect, *in vivo* studies demonstrated that, in preclinical models, MSCs possess the ability to diminish pathogen burdens, which seem to be independent on the way of administration, doses or number of injections (Alcayaga-Miranda et al. 2017 and references therein). Thus, MSCs mainly via its intrinsic antimicrobial properties, may contribute to the safe use combined with bio-materials for tissue engineering.

3 Dental-Derived MSCs

A vast variety of adult stem cells has been identified within the oral cavity, including teeth and their supporting structures. These stem cell populations are collectively named dental MSCs and share many phenotypical and functional properties (Sharpe 2016). Namely, dental MSCs are neural crest-derived ecto-mesenchymal cells located in deciduous and permanent adult teeth pulp and periodontal ligaments (Sharpe 2016; Hernández-Monjaraz et al. 2018).

Dental MSCs are denominated according to its origin: dental pulp (DPSCS); periodontal ligament (PDLSCs); Gingival mesenchymal stem cells (GMSCs); exfoliated deciduous teeth (SHED); dental follicle progenitor cells (DFPCs); and apical papilla derived stem cells (SCAP) (Sharpe 2016; Ercal et al. 2018; Hernández-Monjaraz et al. 2018). Although, these dental MSCs share similarities to BM-MSCs such us multi-potent differentiation, immunoregulatory capacities, they possesses some advantages over BM-MSCs like less invasive procedure for their isolation and better ex vivo expansion (Hernández-Monjaraz et al. 2018). Namely, dental MSCs play key roles in tooth homeostasis, repair and regeneration. For instance, DPSCs remain active and generate odontoblast to repair dentine damage (Shamir et al. 2015; Bakopoulou and About 2016). Similar to BM-MSCs, dental MSCs can be immunophenotyping by their expression of the surface markers CD73, CD90 and CD105 and the lack of hematopoietic markers such as CD14, CD45, CD34, CD25, and CD28 (Chalisserry et al. 2017).

3.1 DPSCS

DPSCS were the first type of dental MSCs enzymatically isolated from the pulp chamber of the third molar, and also included SHED and DPSC. These cells demonstrated to have clonogenic capacities and high proliferative rates, and they exhibit typical fibroblast morphology. Moreover, they possess dentinogenic, osteogenic, adipogenic, neurogenic, chondrogenic, and myogenic differentiation potential. DPSCS also have the greatest potential to produce a high volume of mineralized matrix, which positions these cells as promising candidates for regenerative dental therapies (Bakopoulou and About 2016; Hernández-Monjaraz et al. 2018).

3.2 PDLSCs

The human periodontal ligament is a specialized fibrous connective tissue located between the cementum and the alveolar bone and is implicated in the maintaining and supporting the teeth. PDLSCs demonstrate fibroblast-like phenotype, high proliferation rate and clonogenicity. Also, PDLSCs possess multilineage differentiation capacities such as osteogenic, adipogenic and chondrogenic under specific inductive medium. Moreover, they can regenerate cementum, alveolar bone and periodontal ligament tissues

(Bakopoulou and About 2016; Chalisserry et al. 2017; Seo et al. 2014).

3.3 GMSCs

GMSCs are relatively easy to isolate from gingival lamina propria and present a faster proliferation rate (Zhao et al. 2015). GMSCs retain a stable phenotype, maintain normal karyotype and telomerase activity at higher passages, and are not tumorigenic, despite their origin from healthy or hyperplastic/inflamed gingival tissue (Venkatesh et al. 2017). Moreover, GMSCs present anti-inflammatory and antimicrobial properties and a high osteogenic regeneration potential both *in vitro* and *in vivo* (Zhao et al. 2015). Furthermore, GMSCs transplantation may form connective tissue-like structures (Venkatesh et al. 2017). All these features have positioned them as a promising cell source in the field of regenerative medicine and more specifically in the area of bone tissue engineering.

3.4 SCAP and DFPCs

SCAP and DFPCs are located only in the developing tooth germ before they erupt into the oral cavity. Dental follicle is ecto-mesenchymal in origin and surrounds the enamel organ and dental papilla while SCAP are at the tip of growing tooth (Hernández-Monjaraz et al. 2018). DFPCs isolated from follicle of human third molars displayed fibroblast-like morphology and expressed various biomarkers such as Notch-1, STRO-1, and nestin (Morsczeck et al. 2005). Meanwhile, SCAP express the early mesenchymal surface markers especially CD24, which could be a unique marker for this cell population (Sonoyama et al. 2006). Both kinds of cells form adherent colonies and can differentiate into odontoblast or osteoblast, cementoblast and periodontal ligaments (Handa et al. 2002; Bakopoulou and About 2016; Hernández-Monjaraz et al. 2018).

4 Calcium Phosphate Bioceramics

Most common bone diseases, such as osteoporosis, periodontitis, arthritis, tumor-induced osteolysis, etc., lead to no or poor healing of fractured bone. These problems related to bone remodeling can be addressed by using porous scaffolds that can help bone regeneration using principles of tissue engineering. Bone scaffolds are structural elements needed to fill bony defects, support load, and provide a guide for new bone formation. More generally, tissue engineered scaffolds are required to meet several criteria, which can be classified under: architectural, structural mechanics, mass transport, surface properties, product degradation and cell-material interaction properties; and the changes of these factors with the time both *in vitro* and/or *in vivo* (Hutmacher et al. 2004; Hollister 2005. Scaffolds must also provide mechanical stability during cell differentiation and tissue regeneration. The main parameters controlling scaffold mechanical and mass transport properties at the macro scale are the elastic modulus of the base material, mean pore size, roughness and amount of porosity. The mean pore size should promote cell movement and bone growth (Hutmacher 2000) whereas porosity should ensure not only migration, attachment and differentiation of cells in the scaffold, but also flow for nutrient transport and waste evacuation (Hutmacher 2000; Karageorgiou & Kaplan, 2005). It has been established that bone scaffolds must be manufactured from base materials that promote cell proliferation and differentiation, thereby allowing complete integration. Biomaterials used in bone repair can be made of ceramics, natural polymers, synthetic polymers, and composites (Marquis et al. 2009). Table 1 shows a summary of the most common biomaterials used for bone tissue engineering (Marquis et al. 2009). The requirements of bone scaffold materials are: biocompatibility, osteoconductivity, osteoinductivity, bioactivity, porosity, biodegradability, and mechanical properties (Van Gaalen et al. 2008).

Table 1 Biomaterials used in tissue engineered scaffolds

Materials	Advantages	Disadvantages	Types
Inorganic materials	Biocompatible Osteoconduction Osteointegration similar to bone	Osteoinduction	HA
	Resorbable or non-resorbable affinity with BMP's	Brittle	TCP
		Difficult to mold in 3D	Porous coralline
		Exothermic	CP cement
			Bioactive glass
			Ti
			Hyaluric acid
Natural polymers	BiocompatibleOsteoconductionOsteointegrationAffinity for growth factors	Osteoinduction	Alginate
		Pathogen agents transmission	Collagen
		Difficult sterilization	Starch
			Chitosan
			PEG
Synthetic polymers	OsteoconductionOsteointegration	Breakdown products	Poloxamer
	Reproducible manufactureReadily tailored controlled release properties	Cell recognition	Poly(alpha-hydroxy acids)
	Easy sterilization	Osteoinduction	
		Possibility of protein denaturation by solvents or crosslinker	PLA
			PGA
			Poly(ortho ester)
			Polyanhydride
			Poliphosphazene
			Polyphosphonate
			Collagen-bioactive glass
Composite materials	Use a variety of materials	Complex manufacturing process	Collagen-HA-alginate
			Starch-bioactive glass
			PLA-chitosan
			PLA-PEG-HA
			PLGA
			PLGA-bioactive glass
			PLGA-PEG

HA hydroxyapatite, *PEG* poly(ethylene glycol), *PGA* poly(glycolide), *PLA* poly(lactide), *PLGA* poly(DL-lactide-co-glycolid), *TCP* tricalcium phosphate. Adapted from Marquis et al. (2009).

Bioceramics are excellent candidates for bone replacement due to their quantified biocompatibility and chemical similarity to the mineral phase of bone (Dorozhkin 2010). CaP based bioceramics are commonly used in bone scaffolds because of their inherent biocompatibility, osteoconductivity, osteogenecity, and osteointegrity (Dorozhkin 2010; Eliaz and Metoki 2017). Table 2 shows the most common CaP bioceramics used in tissue engineering. Among CaP based bioceramics, hydroxyapatite (HA) and tricalcium phosphate (TCP) are the most commonly used in clinical applications. CaP properties vary significantly with their crystallinity, grain size, porosity, and composition. High crystallinity, low porosity and small grain size tend to give higher stiffness, compressiveness, strength and toughness. Some *in vivo* studies have shown that 95% of these calcium phosphates are resorbed in 26–86 weeks (Knaack et al. 1998; Wiltfang et al. 2002). In addition, their degradation depends on

Table 2 Synthetic calcium phosphate bioceramics used in bone scaffolds (Huang and Best 2007)

Calcium Phosphate	Formula	Ca/P (ratio)
Tetracalcium phosphate, TTCP	$Ca_4O(PO_4)_2$	2.0
Hydroxyapatite, HA	$Ca_{10}(PO_4)_6(OH)_2$	1.67
Tricalcium phosphate (α, β, γ), -TCP	$Ca_3(PO_4)_2$	1.5
Octacalcium phosphate, OCP	$Ca_8H_2(PO_4)_6 \bullet 5H_2O$	1.33
Dicalcium phosphate dehydrate, DCPD, brushite	$CaHPO_4 \bullet 2H_2O$	1.0
Dicalcium phosphate, DCP, montite	$CaHPO_4$	1.0

their phases, with crystalline TCP having a higher degradation rate than crystalline HA (Vicente et al. 1996; Ahmed 2004). Their natural brittleness, low strength and toughness may limit the use of bioceramics in load bearing structures; however, continuous research efforts are resulting in bioceramics with the required mechanical and bioresorbable properties for tissue engineering scaffolds.

4.1 Biological Requirements of CaP Bioceramics Scaffolds

4.1.1 Biocompatibility

Biocompatibility is the property of a material to be compatible with tissues. More important, biocompatible materials do not provoke toxicity when implanted in the organism (Williams 2008; Xu et al. 2017). Actually, this is a critical and essential requirement for bio-materials in order to achieve full tissue regeneration by supporting cellular activities and avoiding host undesirable local or systemic responses (Xu et al. 2017). Governed by the bulk and surface composition of the scaffolds, biocompatibility is the ability of a material "to perform its intended function, including an appropriate degradation profile" (van Blitterswijk et al. 2008). Namely, CaP based biomaterials and its end products demonstrated short-term and long-term biocompatible properties (Habraken et al. 2016). Naturally occurring biomaterials offer the greatest potential in terms of biocompatibility, however some shortcomings such as large batch-to-batch variations and poor mechanical properties, have encouraged the use of synthetic biomaterials such as polymers and bioceramics.

4.1.2 Osteoconductivity

Osteoconductivity is the ability of the scaffold to support the attachment, proliferation and migration of bone cells, essential for successful bone substitution. CaP-based scaffolds offer good osteoconductivity because of their chemical similarity to the inorganic phase of natural bone. However, it has been shown that osteoconductivity of bioceramic scaffolds can be improved by increasing microporosity (Hing et al. 2005).

4.1.3 Osteoinductivity

Osteoinductivity is the ability of biomaterials to recruit and stimulate progenitor cells osteogenesis (Albrektsson and Johansson 2001). Although osteoinductivity is not one of the main abilities of CaP based bioceramics, some can induce *in vivo* bone formation without presence of exogenous osteogenic factors, and these biomaterials are describe as having "intrinsic" osteoinductivity (LeGeros 2008). Furthermore, the osteoinductive ability can be due to combined topography, composition, and micro and macroporosity effects of bioceramics, which permits the in vivo entrapment of osteoprogenitor cells and BMPs (LeGeros 2008). Interestingly, CaP based bioceramics osteoinductivity can be improved by the addition of 5–10 wt% magnesium that enhances BM-MSC- adhesion and osteogenic differentiation (Zhang et al. 2015). Nonetheless, strategies to enhance osteoinductivity including the incorporation of osteoprogenitor cells, growth factor and bioactive proteins/peptides have been demonstrated to exhibit favorable effects on bone regeneration (Xu et al. 2017 and references therein).

4.1.4 Bioactivity

Bioactivity is defined as the ability of bone scaffolds to bind directly to the surrounding bone without the formation of fibrous tissue and is one of the main properties of CaP based bioceramics (Xu et al. 2017). Usually, biocompatibility is evaluated by determining apatite production in a simulated body fluids that contain ion concentrations similar to human blood plasma, therefore a bioactive material is the one that, in a supersaturated solution, accelerates apatite crystallization using HA as control (Yuan et al. 2000). Bioactive CaP based bioceramics can be further improved by combining with bioactive glass via calcium and phosphate ion release (Sadiasa et al. 2014).

4.1.5 Biodegradability

Biodegradability of a scaffold material is its ability to gradually degrade *in vivo*. Although non degradable biomaterials are generally stronger than biodegradable biomaterials, the latter are preferred for bone tissue regeneration (Bueno and Glowacki 2011). While scaffold material degrades, new tissue replaces the scaffold material and mechanical load is gradually transferred from the scaffold to the new bone and surrounding natural bone. The *in situ* degradation rate of a scaffold depends on design parameters, for example chemical composition and structure; and, characteristics of the environment, for example, vasculature, mechanical loading, tissue ingrowth, enzymatic activity, acidity, temperature, and ionic strength. Bioceramic scaffolds degrade relatively slowly by physiochemical, cell mediated, or mechanical degradation mechanisms. In the case of CaP-based scaffolds, HA resorbs a negligible amount and therefore is considered practically nondegradable, whereas TCP degrades relatively fast (Bueno and Glowacki 2011).

4.1.6 Porosity

Porosity is an important requirement for neovascularization, osteogenic cell infiltration, and bone ingrowth into the defect site (Karageorgiou and Kaplan 2005). Ideally, a scaffold should exhibit different levels of porosity in order to mimic the hierarchical pore size distribution present in natural bone tissue (Sánchez-Salcedo et al. 2008). While it is accepted that pore size is an important variable affecting the ability of bioceramic scaffolds to stimulate cell ingrowth and new bone formation (Hutmacher 2000; Karageorgiou and Kaplan 2005), research data on an optimal scaffold pore size for efficient bone regeneration remain inconclusive. In general, a minimal pore diameter of 100 μm has been claimed to facilitate cell ingrowth (Karageorgiou and Kaplan 2005) and pore diameters larger than 200 μm have been accepted to support new bone formation (Gauthier et al. 1998; Flautre et al. 2001; Galois and Mainard 2004). Some studies have concluded that a pore size of 300–400 μm was optimal to promote bone formation in periodic microstructure scaffolds made of HA (Kuboki et al. 2001). Some studies have suggested that a minimum pore size of 75 μm to 300 μm enhances bone in-growth, while other investigations have found that an optimal size is in the range of 100–500 μm (Karageorgiou and Kaplan 2005; Bobyn et al. 1980; Eggli et al. 1988; Cheung et al. 2007). Some *in vivo* studies on scaffolds with controlled and homogeneous pore distributions have found that there was no significant difference in bone regeneration for pore sizes in the range of 400–1200 μm (Hollister et al. 2005; Schek et al. 2006). In addition, pore size has been observed to influence not only osteoconduction, but also the vascularization of a bone scaffold. More recently, researchers have found bone formation in interconnected micropores less than 10 μm in size from scaffolds fabricated with both macro- (>100 μm) and micro-porosity (<10 μm) (Lan Levengood et al. 2010a, b). Thus, it has been shown that microporosity also promotes bone growth into scaffolds (Eurell et al. 2006).

4.1.7 Mechanical Properties

Mechanical properties of a bone scaffold are required to be similar to the native tissue it replaces (Hollister 2009). With a comparable stiffness to the surrounding natural tissue, scaffolds can provide temporary mechanical

stability while promoting load transfer from mechanical stimuli to the healing site. When scaffolds are weaker than the host bone, they may fail to support physiological loads following implantation. Conversely, scaffolds that are stiffer than the surrounding host bone may lead to stress shielding and osteolysis of the surrounding tissue (Khan et al. 2008). Stress shielding takes place when mechanical stresses are mainly absorbed by the stiff scaffold structure while the surrounding native tissue does not receive the mechanical stimulation which is needed for bone health. Hence bone resorption results and bone formation is compromised, resulting in lysis around the implant with subsequently failure (Hollister 2009).

Beyond the biological requirements, 11 sequential steps have been described, which are collectively necessary for the gradual bioceramic incorporation into the new bone tissue formation, and which take place in the bioceramics and the surrounding biological environment interface: 1) bioceramic dissolution; 2) precipitation from solution onto the bioceramic; 3) ion exchange and structural rearrangement at the bioceramic/tissue interface; 4) interdiffusion from the surface boundary layer into the bioceramic; (5) solution-mediated effects on cellular activity; 6) deposition of either the mineral phase or the organic phase without integration into the bioceramic surface; 7) deposition with integration into the bioceramic; 8) cell recruitment toward bioceramic surface; 9) cell attachment and proliferation; 10) cell differentiation; and 11) extracellular matrix generation (Eliaz and Metoki 2017).

5 Dental MSCs in Bone Engineering

CaP based bioceramics became a commonly used approach in the last years in combination with stem cells which have become the future of regenerative medicine due to their extraordinary rates of cell proliferation and differentiation (Lobo et al. 2015; Xu et a., 2017).

In the last decades tissue engineering is opening new opportunities for tissue and organs regeneration. Tissue engineering is a specialized area of science and bio-engineering combining principles of cell biology, tissue physiology and development with biomaterials with the aim to fabricate, repair and replace tissues in damaged organs. The success of tissue engineering depends in part of an appropriate three-dimensional extracellular matrix or scaffold containing regulatory signals that can instruct progenitor cells for correct tissue differentiation, which is subsequently implanted in target or defect tissues. The combination of MSCs and biomaterials may reduce or eliminate the limitations observed in traditional regenerative strategies by reducing the lag phase of progenitor cells recruitment to the tissue or organ to repair and regenerate (reviewed in Han et al. 2014 and references therein).

In general, MSCs cultured onto biomaterials for osteo-regeneration are analyzed by several differentiation markers at different culturing intervals, including RUNX2, osterix, ALP, collagen type-I, osteocalcin, bone sialoprotein and osteopontin (Sun et al. 2018), which allow to finely determine the feasibility of bone formation in the MSCs/bioceramics implants.

CaP bioceramics are widely used for bone regeneration, both in orthopedics and in dentistry, due to their good biocompatibility, osseointegration and osteoconduction (Eliaz and Metoki 2017), and have probed to easily combine with cells previous to the implant to the injury site. In this sense, MSCs have been probed to enhance bone regeneration in CaP bioceramics with or without combination with other biomaterials, for instance biphasic calcium phosphate ceramics, β-TCP and HA; HA and mineralized COL, β-TCP and HUVEC-derived extracellular matrix (Zhou et al. 2011; Seol et al. 2014; Tang et al., 2015; Xu et al. 2016). Moreover, an *in vitro* study, comparing biphasic CaP, TCP and HA, indicates that biphasic CaP possess better osteoinduction, suggesting that this composite may have higher prospects for healing bone defects *in vivo* (Li et al. 2017). Recently, new strategies combine MSCs encapsulation in biodegradable materials, such as

chitosan/β-glycerophosphate hydrogel, to protect cells during bioceramic scaffold formation without reduction of osteogenic differentiation of MSCs (Liu et al. 2018). Similarly, MSCs encapsulation in alginate-fibrin fibers in combination with TCP/dicalcium phosphate based bioceramics, increased the potential of MSCs in bone tissue engineering (Wang et al. 2016).

Among all the different types of stem cells, MSCs, including those from oral cavity, are the most used population due to their proliferation, differentiation and self-renewal potential. When combining stem cells and bioceramics, one must choose the most optimal cell type based on obtaining a sufficient number of cells, their regenerative potential, nutrient delivery and vascular ingrowth (Zhang et al. 2013), but there are also some relevant bioceramic parameters, such as its surface (roughness), sterilization of the bioceramic to avoid infection as well as pore size and interconnectivity, which have been shown to play a crucial role in cell adhesion, nutrient exchange, cellular proliferation, osteogenic differentiation and vascular ingrowth (Habibovic and de Groot 2007; Zhang et al. 2013). Nevertheless, there is still a lack of agreement in the literature so far, mainly due to the fact that previous studies *in vitro* and *in vivo* revealed different findings and so a deeper and more comprehensive understanding of the morphological and microstructural properties of the bioceramic remains an essential feature in the field. So, the combination of CaP bioceramics and MSCs has demonstrated to potentiate the process of cell proliferation and osteogenic differentiation and can be further applied to different areas of dental health, such as osteogenic regeneration, periodontal regeneration and post implantitis, among others (Sakaguchi et al. 2005; LeGeros 2008; Holzapfel et al. 2013; Masaoka et al. 2016).

Due to their immunoregulatory, anti-inflammatory, angiogenic and high mineralization characteristic dental MSCs are good candidates for bone regeneration (Sharpe 2016; Chalisserry et al. 2017). For instance, DPSCs in combination with biomaterials, such as Bio-Oss, promoted cementum, bone, and PDL regeneration (Khorsand et al. 2013). Also, DFPCs have shown to improve bone regeneration on titanium implants by expressing spontaneous osteogenic differentiation capacities (Lucaciu et al. 2015). DPSCs and PDLSCs seeded into collagen-chitosan scaffold demonstrated to have odontogenic differentiation without the addition of differentiation factors (Ravindran et al. 2014). When DPSCs are seeded on a porous poly-D-L-lactide-glycolide scaffold, they differentiate into odontoblast-producing dentine, and regenerate pulp-like tissue as well (Huang et al. 2010). Furthermore, DPSCs from exfoliated teeth may also contribute to the new bone formation acting as osteoinductive for osteoblast or osteo-progenitors from host (Miura et al. 2003), which can enhance biomaterials with low osteoinductive properties in bone tissue engineering.

Specifically, dental-derived MSCs probe to have excellent osteo-regeneration capacities when combined with CaP bioceramics with or without combination with other biomaterials. In this aspect, porosity of CaP based bioceramics seems to be critical for odontogenic differentiation of DPSCs. Three different HA to beta TCP ratios of biphasic CaP, with a 300 μM mean pore size and 65% porosity, where analyzed, and a ratio of 20% shows to have the best potential of DPSCs odontoblast differentiation, as they expressed high ALP levels and higher bone sialoprotein, dental matrix protein-1, and dentin sialophosphoprotein gene expression (AbdulQader et al. 2015). Similarly, the same group in another study corroborated that 20 biphasic CaP scaffolds/ 80 HA to beta-TCP ratio, with 300 μm mean pore size and 65%, porosity lead to better odontogenic properties for DPSCs (AbdulQader et al. 2016). Moreover, this composition does no exhibit genotoxic effects on DPSCs, suggesting the safety of this biomaterial for DPSCs use in dental and orthopedics applications (Wahab et al. 2018).

Interestingly, the combination of CaP bioceramics with Calcium silicate potently induces the ALP and osteocalcin expression of DPSCs, showing high biointeractivity as release of Ca and OH ions, thus indicating Ca-silicate/ CaP bioceramic as useful biomaterials for dental pulp and dentin therapy by providing better

regenerative odontogenic induction (Gandolfi et al. 2015). Poly-ε-caprolacton coated-biphasic calcium phosphate (PLC-BCP) with the modified melt stretching and multilayer deposition technique demonstrated to increase both *in vitro* and *in vivo* DPSCs osteo differentiation. In rabbit calvaria defects model DPSCs PLC-BCP bio-engineered implants increased the newly formed bone compared to the empty defect and scaffold control groups (Wongsupa et al. 2017a, b).

DPSCs encapsulated in alginate-fibrin fibers combined with TCP/dicalcium phosphate based bioceramic, with 62% porosity, proliferate and differentiate towards osteogenic lineage, as is observed by higher ALP activity, RUNX2, osteocalcin and collagen-type I gene expressions, as well as increased mineralization rates, thus potentiating their use for dental orthopedics tissue engineering (Wang et al. 2016).

Gold and iron nanoparticles incorporation in CaP bioceramics greatly enhances DPSCs osteogenic differentiation and bone matrix mineral synthesis, suggesting nanoparticles as bioactive additives to enhance osteoinduction and bone and dental regeneration bioceramic properties (Xia et al. 2018a, b).

PDLSCs have been also used in combination with biphasic calcium phosphate scaffolds for Osseo regeneration. Yi et al. (2016) transduced PDLSCs to express BMP-2 that reduces bone healing in a critical-size rat calvarial bone defect model. In vitro analysis indicated that PDLSCs inhibit the capacity of BMP-2-induced osteogenesis, which corroborated in vivo results. The authors indicated that the use BMP-2-overexpressing PDLSCs needs to be reconsidered in combination with CaP scaffolds in critical-size rat calvarial bone defect model.

CaP phosphate coating of polycrystalline titanium surface seems to enhance osteoblastic response of PDLSCs. Titanium surface was coated with Ca/P ratio of 1.74 and cells were cultured in absence of osteogenic inductors, and PDLSCs show elevated ALP levels than uncoated titanium surfaces. These data indicate that CaP coating of titanium surfaces provides better bioactive conditions for PDLSCs osteoblastic differentiation, which may enhance osseointegration of PDLSCs containing implants *in vivo* (Winning et al. 2017).

Interestingly, KHPO4 addition in cultured medium of SCAP enhances their osteo/odontogenic potential both in vitro and vivo (Wang et al. 2013), which can be in the line of CaP bioceramics dissolution and ion release and increasing MSC bone differentiation. Moreover, calcium-enriched mixture, mainly composed by calcium oxide, sulfur trioxide, phosphorous pentoxide and silicon dioxide, demonstrates the least cytotoxicity compared with mineral trioxide aggregate, tricalcium silicate and dicalcium silicate, calcium-enriched mixture, Biodentine and octacalcium phosphate, without significant differences in biocompatibility (Saberi et al. 2016). Nevertheless, further studies are necessary to determine the *in vivo* feasibility use of SCAP in combination with CaP bioceramics for periodontal tissue engineering applications.

Therefore, as dental MSCs possess the capacity to regenerate dental tissues, including cementum, alveolar bone and periodontal ligament tissues, they are considered as useful candidates for periodontal tissue engineering (Bakopoulou and About 2016; Chalisserry et al. 2017). Besides, MSCs derived from dental tissues, adipose tissue-derived stroma cells, umbilical cord, and induced pluripotent stem cell-derived mesenchymal stem cell, among others, also showed promising results for the regeneration of mineralized tissues in combination with CaP bioceramics (Wang et al. 2013; Wang et al. 2014; Saberi et al. 2016; Wang et al. 2018).

6 Concluding Remarks

Adult MSCs have opened great expectations due to their versatile functions in the body, where their multipotent differentiation capacity is one of the most important. Besides, adult MSCs reside in all tissues and organs, participating in tissue homeostasis, in part due to their anti-inflammatory, immunomodulatory and antimicrobial capabilities, among others. In dental current regenerative procedures MSCs derived from

oral cavity demonstrated to have tremendous potential to develop new feasible therapies to periodontal regeneration and reconstructive therapies (Sharpe 2016; Ercal et al. 2018; Hernández-Monjaraz et al. 2018). Periodontal regeneration requires many aspects to be considered, including appropriate progenitors cells, different signaling molecules, and last but not least important a matrix scaffolds that serve as guidance for 3D tissue reconstruction. In this sense, tissue engineering may provide scaffolds for bone and dental regeneration, such as the main subject of this review; CaP bioceramics, which present high malleability and good biocompatibility, osseointegration and osteoconduction. Moreover, they can be combined with other biomaterials in order to increase their success when implanted in combination with MSCs. To that purpose, prosthodontists must become familiar with the basic aspects of stem cells as well as with the cellular mechanisms underlying the regenerative process; scientists and clinicians must work together performing *in vitro* and *in vivo* studies first, isolating and adjusting the most relevant parameters in order to obtain the safest and most effective regenerative strategy for the clinic (Egusa et al. 2012). In line with this, there is a need to develop a solid agreement that can be commonly used as a generalized protocol for stem cells based therapies. An optimal therapy suitable for humans can only be achieved if clinicians, bioengineers and scientists work together, combining their expertise and sharing their knowledge towards a safe and effective therapy to periodontal regeneration. Despite of the great evolution experienced in the field of regenerative medicine in the last decades, there is still a lack of knowledge about its *in vivo* biology and so it is essential to become aware of the risks involved in the use of stem cells and the negative effects that they may produce in the host. Therefore, many cellular, biological, and molecular aspects as well as technical and clinical issues are implicated in the healing processes and need to be understood in order to increase the success of dental MSCs and CaP bioceramics applications.

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Dental Stem Cells in Bone Tissue Engineering: Current Overview and Challenges

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Abstract

The treatment of bone that is impaired due to disease, trauma or tumor resection creates a challenge for both clinicians and researchers. Critical size bone defects are conventionally treated with autografts which are associated with risks such as donor site morbidity and limitations like donor shortage. Bone tissue engineering has become a promising area for the management of critical size bone defects by the employment of biocompatible materials and the discovery of novel stem cell sources. Mesenchymal stem cells (MSCs) can be isolated with ease from various dental tissues including dental pulp stem cells, stem cells from apical papilla, dental follicle stem cells, stem cells from human exfoliated deciduous teeth, periodontal ligament stem cells, gingival stem cells and tooth germ derived stem cells. Outcomes of dental MSC mediated bone tissue engineering is explored in various *in vivo* and *in vitro* preclinical studies. However, there are still obscurities regarding the mechanisms underlying in MSC mediated bone regeneration and challenges in applications of dental stem cells. In this review, we summarized dental stem cell sources and their characterizations, along with currently used biomaterials for cell delivery and future perspectives for dental MSCs in the field of bone tissue engineering. Further efforts are necessary before moving to clinical trials for future applications.

Keywords

Bone regeneration · Bone tissue engineering · Dental stem cells

Abbreviations

ALP:	Alkaline phosphatase
bFGF:	Basic fibroblast growth factor
BMMSCs:	Bone marrow derived mesenchymal stem cells
BMP:	Bone morphogenic protein
CDM:	Chemically defined media
Col I:	Collagen I
DFSCs:	Dental follicle stem cells
DPSCs:	Dental pulp stem cells
ECM:	Extracellular matrix
FBS:	Fetal bovine serum
GFP:	Green fluorescence protein
GMSCs:	Gingival mesenchymal stem cells

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HA/TCP: Hydroxyapatite/tricalcium phosphate
HOX: Homeobox
IGF-1: Insulin-like growth factor 1
MSCs: Mesenchymal stem cells
OPC: Osteopontin
OPN: Osteonectin
PDLSCs: Periodontal ligament stem cells
SCAP: Stem cells from apical papilla
SHED: Stem cells from exfoliated human deciduous teeth
TGF-β1: Transforming growth factor-beta 1
TGSCs: Tooth germ mesenchymal stem cells
TNF-α: Tumor necrosis factor α

1 Introduction

Bone tissue is the major supportive structure in human physiology that enables body movement, protection of organs and plays important roles in metabolic and synthetic functions such as calcium-phosphate homeostasis and production of progenitor cells. Various reasons including trauma, necrosis, or tumors might result in large bone defects and failure in bone healing. These defects that will not completely heal over the lifetime of a person or an animal are called critical size defects (Spicer et al. 2012). Critical size bone defects generally require surgeries to be reconstructed with autogenous bone grafts or bone graft substitutes. However, there are still challenges encountered in the healing of critical size bone defects caused by insufficient number of progenitor cells and their lack of migration into the defect site and failure to differentiate into osteoblasts (Oryan et al. 2017). Thus, the approach to deliver stem cells to the defect site on scaffolds with osteoinductive properties supplemented with or without various growth factors to enhance bone healing has attracted great attention.

An ideal cell source for tissue engineering should be easily accessible and highly available. It should require non-invasive or minimally invasive collection procedures, cause minimal donor site morbidity, have capacity to generate sufficient number of cells after expansion and the capacity to differentiate into cell lineages of interest as a response to differentiation cues. Also it needs to manipulate the native environment to promote tissue regeneration and integration, and should lack immunogenicity, tumorigenicity and the risk of disease transmission (Marolt 2015).

Mesenchymal stem cells (MSCs) are present in adult tissues and play critical roles in local tissue healing and regeneration. Upon injury, MSCs are recruited from their homes and migrate to the injury site along the blood vessels by the help of receptors they express such as P-selectin and vascular cell adhesion molecules. Since they can sense and respond to factors and cytokines secreted in an injury environment, it makes sense to use MSCs in tissue engineered constructs for regeneration of defects (Sundelacruz and Kaplan 2009). First isolated from bone marrow, MSCs were able to form colonies and differentiate towards osteogenic, adipogenic and chondrogenic lineages (Friedenstein et al. 1970; Bianco et al. 2008). Although bone marrow derived MSCs (BMMSCs) show high stability in culture and high affinity to differentiate into osteoblastic lineage, there are disadvantages including invasive harvesting process, low cell yield and risk of infection in donor site (Oryan et al. 2017).

Epithelial–mesenchymal interactions control tooth organogenesis. Mammalian tooth development begins in the jaws before erupting into the mouth and occurs via the interactions between oral epithelial cells of ectoderm origin and neural crest cells forming the mesenchyme through their coordinated division, growth and differentiation events. During odontoblast differentiation, stem cells in dental germ go through a series of asymmetric division where one of the daughter cells gives rise to terminally differentiated odontoblasts and the other daughter cell remains as stem cell (Yildirim 2013). Even after tooth organogenesis is complete, these stem cells reside in the dental pulp which gives human dental pulp a limited ability to repair itself in case of caries or trauma (Ramazanoglu et al. 2013). When severe tooth damage occurs involving both enamel and dentin tissues penetrating through the pulp, it

stimulates new odontoblasts to form and produce dentin to repair the lesion (Volponi et al. 2010). This situation led the researchers to think that dental pulp can be a good source for the isolation of stem cells of ectomesenchymal origin. Dental tissues have gained attention since their first isolation from dental pulp in 2000 (Gronthos et al. 2000) due to their high accessibility and multilineage differentiation capacity (Yalvac et al. 2010a). Since then stem cells of dental origin have been isolated from tissues at an earlier developmental stage such as tooth germs, dental follicle and primary exfoliated deciduous teeth or adult dental tissues such as adult dental pulp, apical papilla, periodontal ligament and gingiva. As these cells are derived from neural crest, which gives rise to craniofacial cartilage and bone, they can be used for craniofacial bone reconstruction in humans using autologous therapeutic treatments (Chalissery et al. 2017). Stem cell rich dental tissues are obtained during standard dental procedures and discarded as surgical waste products; hence harvesting cells from these tissues do not raise ethical concerns. Studies revealed dental stem cells as a MSC population with the capacity for self-renewal and potential for multilineage differentiation.

2 Dental Stem Cells

2.1 Dental Pulp Stem Cells

Dental pulp stem cells (DPSCs) are the first identified dental stem source with mesenchymal characteristics and osteogenic potential. The dental pulp develops from the dental papilla and originates from ectomesenchyme. DPSCs can be harvested from adult tooth pulp tissues through enzyme treatment (Gronthos et al. 2000). Compared to BMMSC, DPSCs exhibited higher CFU-F and proliferation rates along with a similar gene expression profile for genes related to mineralization (Shi et al. 2001). However, DPSCs transplants formed dentin-pulp like structure by *ex vivo* expansion whereas ectopic bone formation was present for BMSC transplants *in vivo* (Gronthos et al. 2002). Osteogenic induction of DPSCs and their combinations with various scaffolds were investigated comprehensively and resulted in positive outcomes. A living autologous bone tissue was generated *in vitro* from DPSCs, which was followed by a successful lamellar bone formation upon subcutaneous implantation of this newly formed tissue *in vivo* (Laino et al. 2005). Osteogenic induction is commonly achieved by the addition of ascorbic acid, dexamethasone and β-glycerophosphate to the growth medium. When DPSCs are cultured in osteogenic medium for 21 days, *in vitro* osteogenic differentiation is demonstrated by positive staining for mineralized matrix nodules, significantly increased alkaline phosphatase (ALP) levels and up-regulation of osteogenic markers such as Runx2, osteopontin (OPN), osteocalcin (OCN) (Alge et al. 2010; Mori et al. 2010). After 40 days of culture, human DPSCs are shown to form a 3D structure similar to a woven fibrous bone with physical qualities of bone. When transplanted in rats, these structures also displayed areas of vascularization (Paino et al. 2017).

A combination of distinct growth factors as supplements to osteogenic medium can be used for the facilitation of osteogenic differentiation. DPSC cultures with the exposure to vascular endothelial growth factor (VEGF)-A165 peptides showed enhanced differentiation towards osteoblasts which may be critical in clinical conditions that require the promotion of bone regeneration and vascularization (D'Alimonte et al. 2011). DPSCs, in the presence of basic fibroblast growth factor (bFGF), exhibited osteogenic differentiation *in vitro* and when transplanted into mice, showed bone and cartilage formation (Morito et al. 2009). However, a recent study displayed opposing results as bFGF was shown to have an inhibitory effect on osteogenic differentiation of DPSCs. In the same study, another factor that plays critical role in extracellular matrix (ECM) formation, epidermal growth factor, was shown to induce calcium deposit production and increase ALP and OCN expression (Del Angel-Mosqueda et al. 2015). Along with the proper selection of growth factors for better osteogenic differentiation, promotion of cell differentiation towards osteoblasts can be dose dependent and

can differ through culture conditions. It was shown that a low dose of tumor necrosis factor α (TNF-α) treatment promoted mineralization of DPSCs whereas a high dose suppressed osteogenic differentiation via suppression of Wnt/β-catenin signaling (Qin et al. 2015). The effect and mechanisms of growth factors and regulatory pathways for osteogenic differentiation need to be elucidated before regular use of such supplements for bone regeneration.

The use of DPSCs seeded scaffolds has yielded successful outcomes for *in vivo* bone regeneration when transplanted subcutaneously or in bone defects. Although woven bone samples obtained by *in vitro* conditions without scaffolding can lead to *in vivo* bone formation (Paino et al. 2017), a suitable scaffold is still necessary in the treatment of critical size bone defects. Various biomaterials have been investigated including hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic (Otaki et al. 2007; Zhang et al. 2008; Asutay et al. 2015), poly-ε-caprolactone-biphasic calcium phosphate (Wongsupa et al. 2017), calcium phosphate (Ling et al. 2015; Wang et al. 2016), biodegradable polyesters (Kwon et al. 2015), hydrogels (Paduano et al. 2017; Tsukamoto et al. 2017), collagen (Pisciotta et al. 2012; Maraldi et al. 2013; Niu et al. 2014; Chamieh et al. 2016) and gelatin scaffolds (Li et al. 2011).

Despite promising results of DPSCs' differentiation potential and advancements in biomaterials field, a few studies showed unsuccessful results in bone formation. Two studies by Annibali et al. showed that the transplantation of DPSCs with deproteinized bovine bone and β-TCP to calvarial bone defects did not significantly increase bone mineral density compared to scaffolds alone (Annibali et al. 2013; Annibali et al. 2014). Hence, recent investigations on properties of biomaterials aim to increase cell survival and proliferation in the defect area to induce osteogenesis. In this regard, electrospun composites are fabricated by combination of biocompatible polymers and bioactive ceramics as scaffolds for DPSCs to increase cell attachment and osteoconductivity. High cell attachment and viability was observed as well as good *in vitro* osteogenic differentiation and *in vivo* ectopic bone formation (Prabha et al. 2017). Encapsulation of DPSCs in non-toxic hydrogels also yielded high cell viability and proliferation (Cavalcanti et al. 2013). With the progression of tissue engineering constructs, more studies with successful results will be noted in the literature.

Although there is paucity regarding clinical studies on dental stem cells, DPSCs are the first dental stem cell source for its application in the treatment of bone resorption. DPSCs seeded onto a collagen sponge was used to fill in the extraction sites resulted in complete bone restoration and a follow-up of 3 years exhibited a fully compact regenerated bone with a higher matrix density than control alveolar trabecular bone (D'Aquino et al. 2009; Giuliani et al. 2013). Present applications are insufficient to evaluate bone formation capacity of DPSCs as advanced and more methodological investigations are obligatory.

2.2 Stem Cells from Human Exfoliated Deciduous Teeth (SHED)

A distinct population of stem cells can be isolated from the remnant crown pulp tissue of exfoliated human deciduous teeth, SHED, which is highly proliferative and can induce bone formation *in vivo*. Studies investigating immunomodulatory properties showed that SHED highly express MSC markers, including CD105, CD146, STRO-1, and CD29, but are negative for CD31 and CD34 (Yamaza et al. 2010; Yasui et al. 2016). Earlier studies indicated that SHED could not differentiate into osteoblasts directly but induced new bone formation by creating a template for murine host osteogenic cells (Miura et al. 2003). Osteoinductive capacity of SHED was also shown by their ability to repair critical-size calvarial defects in immunocompromised mice (Seo et al. 2008). However, green fluorescence protein (GFP) labeled SHED in β-TCP scaffolds was shown to differentiate directly into new bone in a critical size defect in swine mandible whereas non-specific staining with GFP was detected in the local bone tissue, suggesting that SHED was engrafted at the defect site and contributed to

bone regeneration (Zheng et al. 2009). A through-through mandibular defect of 9 mm in dog model was successfully treated with 5 year cryopreserved SHED without immune response indicating a competent capacity of proliferation and osteogenesis (Behnia et al. 2014).

SHED differ from DPSCs since deciduous teeth are different than permanent teeth due to their developmental process and function. SHED demonstrate a higher cell proliferation rate compared to DPSCs which is explained by the high expression of distinct growth factors such as FGF and TGF-β1 by SHED (Nakamura et al. 2009). OCT-4 expression, an indicator of stemness, is also significantly higher in SHED compared to BMMSCs (Aghajani et al. 2016). A comparison for osteogenic capacity revealed that SHED exhibit more enhanced osteogenic properties than DPSCs as indicated by higher ALP activity and expression of OCN and Col I (Koyama et al. 2009; Ching et al. 2017). As DPSC transplants form mainly connective tissue with small amounts of mineralization, SHED transplants display bone-like structures without hematopoietic marrow elements. These findings might suggest DPSCs as a suitable tool for soft tissue regeneration whereas SHED might be used for engineering mineralized tissues (Wang et al. 2012b).

2.3 Stem Cells from Apical Papilla (SCAP)

Dental papilla is the soft tissue at the apex of a developing permanent tooth, which contributes to tooth formation and evolves into the dental pulp. The apical papilla contains fewer blood vessels and less cellular components compared to pulp tissue and it is separated from the pulp by an apical cell rich zone (Sonoyama et al. 2006). As the apical papilla turns into pulp tissue, it is indistinct whether the SCAP converts into DPSCs (Huang et al. 2009). Nonetheless, a comparison between SCAP and DPSCs revealed significantly higher mineralization and proliferation rates for SCAP, which might be an advantage for future dental tissue engineering applications (Sonoyama et al. 2008; Bakopoulou et al. 2011). SCAP can be isolated from extracted wisdom teeth through enzyme treatment since human apical papilla tissues of other permanent teeth at the developing stage are not appropriate for stem cell isolation clinically. Among various positive MSC markers for SCAP including STRO-1, CD29, CD73, CD90, CD105, CD146, CD166; surface marker CD24 is suggested as a distinct marker of SCAP and the high CD24 expressing group of a SCAP population is shown to demonstrate a higher capacity for osteogenic differentiation than the low CD24 expressing group (Sonoyama et al. 2006; Aquilar and Lertchirakarn 2016). A comprehensive characterization of dental stem cells might shed light to distinct subpopulations of heterogeneous cell populations which might show better differentiation towards desired lineages.

Osteo/odontogenic induction of *ex vivo* expanded SCAP on HA or HA/TCP scaffolds when transplanted into immunocompromised mice revealed mineralized tissue consistent with dentin formation (Sonoyama et al. 2006; Abe et al. 2008). After 42 days of culture in osteogenic inductive medium, human SCAP displayed osteogenic differentiation shown by mineralized nodule formation and expression of osteogenic markers (Park et al. 2009).

Only a few studies investigated the influence of growth factor treatment on bone formation of SCAP. Insulin-like growth factor 1 (IGF-1) treatment on SCAP was shown to enhance osteogenic differentiation but reduce odontogenic differentiation capacity which might indicate a distinct potential for bone tissue engineering (Wang et al. 2012a). Treatment with bFGF was shown to significantly increase cell proliferation and CFU-F formation efficiency along with the expression of stem cell gene markers such as STRO-1, Nanog, Oct 4 and Rex1, in addition to suppression of osteogenic differentiation. However, when SCAP were exposed to bFGF during cell passaging before osteogenic induction, enhanced differentiation was indicated (Wu et al. 2012). Different timing of a factor exposition can significantly alter differentiation potential of SCAP by acting at a specific stage of cell development.

Another effective method of enhancing osteogenesis is gene transfection of mesenchymal stem cells. Lentiviral-mediated co-transfection of BMP2 (bone morphogenic protein 2) and VEGF gene in SCAP was shown to promote osteogenic differentiation. In addition, BMP9 transfected immortalized SCAP was demonstrated to induce osteo/odontogenic differentiation (Wang et al. 2014; Zhang et al. 2016).

Recent studies have investigated molecular mechanisms underlying osteogenic differentiation of SCAP since the clarification of these pathways would provide insights to preceding studies. Homeobox (HOX) genes play important roles in the differentiation regulation of MSCs. Results of investigations indicate that the depletion of HOXA5 gene inhibited osteogenic differentiation and proliferation whereas depletion of HOXC10 promoted osteogenic differentiation in SCAP *in vitro* (Li et al. 2017a, b). Another investigation of a homeobox gene, MEIS2, revealed that its knockdown in SCAP decreased ALP activity and mineralization while inhibiting mRNA expression of ALP, bone sialoprotein and OCN (Wu et al. 2015).

2.4 Dental Follicle Stem Cells (DFSCs)

Dental follicle is the loose connective tissue surrounding the enamel organ and the dental papilla of the developing tooth germ prior to eruption (Morsczeck et al. 2005). During tooth development, dental follicle has key functions in bone resorption and development of the periodontium. Stem cells can be isolated from teeth follicles of impacted human third molars. When transplanted in critical size defects in calvaria, DFSCs harvested at the early stage of crown formation showed newly formed bone and vascularization after 4 weeks similar to that of BMMSCs (Tsuchiya et al. 2010). Expression of stem cell markers such as STRO-1 and Notch-1 were found to be similar between human BMMSCs and DFSCs (Aonuma et al. 2012). A recent study has demonstrated that DFSCs expressed Oct4, a human embryonic stem cell marker whereas Nanog, a pluripotent cell marker, was weakly expressed and down-regulated throughout the following passages (Lima et al. 2017). In addition, DFSCs reduced their osteogenic capability *in vitro* with increasing cell passages, as complete loss of ability was seen around passage 11 (Yao et al. 2013). These findings might indicate that dental follicle tissue contains a heterogeneous stem cell population.

Stem cells from skin, bone marrow, and dental follicle were compared for their osteogenic potential *in vitro* on demineralized bone matrix and fibrin glue without using osteogenic induction medium and *in vivo* by implanting subcutaneously to athymic mice. All cell types are shown to express osteoblast related genes including Runx2, osterix, OCN and osteonectin and they were mineralized *in vitro*. However, DFSC group showed the highest OCN expression and calcium content *in vivo* (Park et al. 2012) revealing the potential of stem cells isolated from dental tissues in bone tissue engineering. DFSCs seeded on gelatin sponges containing β-tricalcium phosphate cultured in osteogenic medium without dexamethasone confirmed new bone formation *in vivo* (Takahashi et al. 2015). Interestingly, polycaprolactone scaffolds seeded with DFSCs achieved 50% bone regeneration at 8 weeks in rat calvarial critical size defects (Rezai-Rad et al. 2015).

Microarray analysis of growth factors revealed BMP6, which is important in both early and late osteogenic differentiation, to be up-regulated in DFSCs during differentiation. Addition of BMP6 to cell culture resulted in the elevation of transcription factors that are critical for bone formation such as Dlx-5, Runx2 and Osterix (Takahashi et al. 2013). Another study showed that addition of BMP6 to induction medium of late passage of DFSCs increase osteogenesis whereas no further increase was observed with early passage DFSCs (Yao et al. 2013). Transfection of rat DFSCs with another member of growth factor family, BMP9, was shown to promote osteogenesis after 14 days of incubation (Li et al. 2012).

2.5 Tooth Germ Stem Cells (TGSCs)

Researchers generally have cultured stem cells derived from immature tooth tissues by dissecting dental follicle and apical papilla separately and established either DFSCs or SCAP cultures. However, stem cells of dental origin, that are responsible for tooth development, are derived from both epithelial cells and the underlying mesenchyme comprised of neural crest cells. Thus, reciprocal signaling pathways between these cell groups should be considered in designing a culture system. The use of whole tooth germ including dental follicle and its surrounding tissues is an alternative strategy which makes use of reciprocal interactions between stem cells of ectoderm and mesoderm origin in order to preserve their stemness (Yalvac et al. 2010a, b; Ramazanoglu et al. 2013).

First isolated from the dental mesenchyme of human third molar tooth germ during late bell stage, TGSCs demonstrate capability to differentiate into osteoblasts, neural cells and hepatocytes *in vitro* (Ikeda et al. 2008). As tooth germ tissues of third molars in humans undergo organogenesis unlike any other permanent teeth, around age 6, embryonic tissues remain undifferentiated providing a unique primitive tissue that can be harvested during childhood. Further investigations of pluripotency have shown that TGSCs expressed developmentally important transcription factors including Oct-4, Nanog, sox2, c-myc mRNA and klf4 mRNA (Ikeda et al. 2008; Yalvac et al. 2010a).

According to studies that use pig as a model animal for isolation of tooth germ stem cells from third molars, TGSCs seeded on fibronectin modified polybutylene succinate foam scaffolds demonstrated differentiation towards osteogenic cells as indicated by high ALP activity and von Kossa staining (Abay et al. 2016). STRO-1 sorted and unsorted TGSCs isolated from pig (Gurel Pekozer et al. 2017) and human sources (Ercal et al. 2017) exhibited mineral accumulation, high ALP activity and up regulation of osteogenic markers *in vitro*. Transfection of TGSCs with BMP2 and BMP7 promoted osteogenic differentiation (Taşlı et al. 2014). As there is a paucity of studies regarding TGSCs, following studies are necessary to establish tooth germs as one of the primary sources for bone regeneration.

2.6 Periodontal Ligament Stem Cells (PDLSCs)

Periodontal ligament is an extensively investigated dental stem cell source along with the dental pulp. First isolated from human third molars, PDLSCs could differentiate into cementoblast-like cells, adipocytes and collagen forming cells *in vitro* and showed the capacity to contribute periodontal tissue repair by generating a cementum/PDL structure *in vivo* (Seo et al. 2004). Subsequent investigations demonstrated that PDLSCs promote osteoblastic differentiation and form mineralized tissue *in vitro* under osteogenic induction conditions and have the ability to regenerate bone *in vivo* (Gay et al. 2007; Grimm et al. 2011). Immunomodulatory properties of PDLSC were also reported revealing PDLSCs as candidates for allogeneic stem cell-based therapies (Wada et al. 2009). Various scaffolds including HA/TCP, HA/ECM, nano-HA-collagen-polylactic acid, bovine bone grafts and gelatin sponges are shown to maintain the viability and osteogenic capacity of PDLSCs and demonstrate bone formation *in vivo* (Kim et al. 2009; He et al. 2011; Tour et al. 2012; Yu et al. 2013b, 2014). However, when compared to BMMSCs in the treatment of calvarial bone defects, PDLSCs showed slow differentiation into osteoblasts *in vivo* but finally reconstructed the defect, which was justified by microenvironmental induction and *in situ* signals on PDLSCs in calvarium. Hence PDLSCs can be more effective in the regeneration of periodontium considering their origin (Kadkhoda et al. 2016).

Investigations on the effects of growth factors on the proliferation and differentiation of PDLSCs revealed that while VEGF increased calcium nodule formation and ALP activity, FGF-2 promoted proliferation but down-regulated the expression of Runx2, ALP and collagen type I (Lee et al. 2012). IGF-I could stimulate proliferation and enhance

mineralization of PDLSCs both *in vitro* and *in vivo* by mediating osteogenic differentiation via activation of Mitogen Activated Protein Kinase signaling pathway (Yu et al. 2012). Stimulation of PDLSCs with recombinant human insulin-like growth factor binding protein 5 resulted in enhancement of the migration, proliferation, chemotaxis and osteogenic differentiation in an inflammatory niche revealing a potential application in clinical conditions such as periodontal diseases (Han et al. 2017).

Transfection of PDLSCs using recombinant adenovirus encoding BMP2 formed more and better quality bone than only PDLSCs groups. When used as combination with HA particles and collagen gel in the regeneration of peri-implant bone defects, BMP2 transfected PDLSCs exhibited new bone formation along with re-osseointegration, thus demonstrating an attractive therapeutic approach (Jung et al. 2014; Park et al. 2015).

As harvesting periodontal tissue necessitates extraction of the tooth, inflamed periodontal ligaments of periodontally compromised teeth with extraction indications might be an alternative source for obtaining PDLSCs. A comparison between inflamed (I)-PDLSCs and healthy (H)-PDLSCs demonstrated that I-PDLSCs have an increased proliferation capacity and migration potential but a decreased capacity for osteogenic differentiation. Although both cell types exhibited MSC characteristics, I-PDLSCs showed impaired osteogenic differentiation and tissue regeneration (Tang et al. 2016). However, as inflamed periodontal ligament is abundant in daily dental practice, these cells can be further explored with additional methods to reduce inflammation for their better use in tissue regeneration.

PDLSCs are the only dental stem cell source studied with a randomized clinical trial for its use in the treatment of periodontal intrabony defects. Results indicate that the use of autologous PDLSCs combined with bovine bone grafts significantly increase alveolar bone height, but there was no significant difference between the cell group and the control group (Chen et al. 2016). No clinical safety issues were encountered during the trial; nevertheless more studies with increased sample sizes are necessary for further evaluations.

2.7 Gingival Mesenchymal Stem Cells (GMSCs)

GMSCs offer an alternative dental stem cell source that is relatively easier to access as they can be obtained as a byproduct from resected gingival tissues. The gingiva is composed of epithelium and connective tissue that is an important component of human periodontium surrounding the necks of the teeth and covering alveolar bone. The connective tissue component is separated from de-epithelized gingival tissue, minced and digested for cell isolation (Fawzy El-Sayed and Dörfer 2016). Considered as a novel postnatal stem cell source, GMSCs display stable morphology along with high proliferation rates compared to BMMSC, sustain MSC characteristics at high passages and maintain karyotype and telomerase activity during prolonged culture time (Srivastava et al. 2010). GMSCs also exhibited immunomodulatory properties by eliciting a potent inhibitory effect on T cell proliferation in response to mitogen stimulation (Zhang et al. 2009). Although GMSCs showed osteogenic differentiation under osteogenic induction conditions as indicated by OCN expression and positive Alizarin Red S staining *in vitro*, transplantation of GMSCs mixed with HA/TCP in dorsal pouches of immunocompromised mice resulted in connective-tissue like formations without osteogenic differentiation (Zhang et al. 2009). Interestingly, when transplanted into Class III furcation defects in alveolar bone, GMSCs enhanced new bone formation and differentiated into osteoblasts, cementoblasts and PDL fibroblasts (Yu et al. 2013a). However, endogenous stem cells were present in the newly formed tissue indicating the importance of local microenvironment on the regeneration capacity.

Studies also demonstrated that GMSCs could repair mandibular wounds and critical size calvarial defects in rats after 2 months (Wang et al. 2011). Systemically transplanted GMSCs

could home the mandibular defects and promote bone formation in mice after 3 weeks (Xu et al. 2014). A recent study demonstrated the encapsulated GMSC in silver lactate containing arginyl-glycyl-aspartic acid coupled alginate hydrogel scaffolds as an alternative for peri-implantitis treatment due to its antimicrobial properties and osteogenesis potential (Diniz et al. 2016). Although it is relatively a novel stem cell source, incoming positive results might render GMSCs as a valuable source for bone tissue engineering due to their easy accessibility in the oral cavity.

3 Future Perspectives and Challenges

For 17 years since the first isolation of them, we have learned so much about the stem cells of dental origin while there is still a lot to discover. Their proliferative and multilineage capacity as well as the accessibility of the sources makes them attractive for therapeutic purposes. However, preclinical evaluations of dental stem cells on especially large animal models followed by randomized clinical trials are required to be performed.

One of the main challenges faced in clinical applications of stem cells is the limited cell availability. The majority of clinical studies for bone regeneration focus on bone marrow and adipose derived mesenchymal stem cells (Padial-Molina et al. 2015). Although various studies investigate regenerative properties of dental stem cells in comparison to other sources, the optimal cell source and expansion protocols are not comprehensively identified.

Donor age is also another factor that should be taken into consideration since it affects regenerative properties of stem cells. Stem cells from adult dental tissues such as dental pulp, periodontal ligament or gingiva can be harvested from patients of different ages. However, the proliferation and migration capacity and differentiation ability of these cells decrease as donor age increases. A study on PDLSCs revealed that cells from donors whose age is 62.6 ± 6.8 expressed less MSC markers than younger donors and failed to form PDL structures *in vivo* (Zhang et al. 2012). Another study on donor age showed that DPSCs from patients <22 years exhibited a significantly faster doubling time than the cells from patients $\geq$22 years (Kellner et al. 2014). Changes on the MSC properties due to age need to be considered whenever they are aimed to be used for tissue regeneration.

The presence of animal proteins in fetal bovine serum (FBS), which is frequently used in growth media, may pose a risk in terms of immune reaction in possible clinical applications (Ramamoorthi et al. 2015). Furthermore, the composition of FBS is unknown and varies between batches, thus hampering the reproducibility of experiments (Ledesma-Martínez et al. 2016). When used as an alternative to FBS, human platelet lysate was shown to increase proliferation rates of DPSCs and SCAP and enhanced osteo/odontogenic differentiation (Abuarqoub et al. 2015). According to Wen et al. (2016) 2% modified platelet-rich plasma was an optimal substitute for 10% FBS in differentiation and proliferation of SHED. Human serum can be a substitute for FBS and may accelerate the transition of *in vitro* studies to clinical trials (Khanna-Jain et al. 2012; Pisciotta et al. 2012). However, there is still immunogenicity problem when allogeneic human serum is used. Autologous serum usage can eliminate the risk of immune reaction whereas it is also problematic due to insufficient amounts of human serum to generate clinically relevant number of stem cells from a patient. Besides, serum from elderly patients may not support cell growth (Jung et al. 2012). Regardless of human or xeno source, there is a risk of contamination with pathogens such as viruses, prions, mycoplasma, endotoxins or other immunogenic agents. Chemically defined media (CDM), in which all the constituents and their concentrations are known, is also an alternative for media formulations in therapeutic applications. DPSCs were previously cultured in

a CDM from a producer and shown to have a slower growth rate compared to FBS containing media group. However, CDM allowed the maintenance of stem cell like characteristics shown by the ability to differentiate towards odontoblastic lineage *in vitro* and to form dentine-like structures when transplanted into immunodeficient mice (Takeda-Kawaguchi et al. 2014).

MSCs can be contaminated with other cell types such as hematopoietic cells, fibroblasts, endothelial cells during isolation. Besides, MSC populations are inherently a heterogeneous mixture of cells with varying proliferation and differentiation potentials. Thus, developing cell-surface antigen profile of stem cells is required for the better purification and identification of MSCs (Kolf et al. 2007). Sorting of a subpopulation of MSCs, based on a cell-surface antigen profile, which has a superior differential potential towards a distinct lineage might provide a better source of cells for therapeutic purposes. A recent study revealed that CD271 (Low+)/CD90(High+) DPSCs promoted new bone formation in critical size calvarial defects (Yasui et al. 2016). Another study showed that CD271 positive subpopulation of dental stem cells exhibited the most effective odontogenic differentiation (Alvarez et al. 2015). STRO-1 positive fraction of rat dental pulp stem cells were shown to differentiate towards odontoblastic lineage whereas STRO-1 negative fraction could not. However, heterogeneous group of cells could also differentiate into odontoblasts rendering sorting procedure not worthy (Yang et al. 2007). Gurel Pekozer et al. (2017) and Ercal et al. (2017) also obtained similar results for osteogenic differentiation of STRO-1 positive, STRO-1 negative and heterogenous TGSCs of porcine and human origin, respectively. Thus, cell isolation based on surface characteristics result in different outcomes; however investigations with properly selected surface markers and their combinations may provide better answers for differentiation potentials.

Preservation of these cells while stemness properties are maintained is essential for future applications in clinical scenarios. For example it was investigated that DPSC isolation is possible for the next 5 days of post-extraction and cryopreservation of intact teeth allows the recovery of viable stem cells (Perry et al. 2008). Cryopreservation of intact exfoliated deciduous teeth is shown to be a useful method for preserving SHED as these cells exhibited no difference in proliferation and differentiation characteristics compared to fresh SHED (Lee et al. 2015). Improvements in cryopreservation of tissues without cell expansion is beneficial for culturing stem cells only when needed for clinical use avoiding the risks of contamination during cell isolation and increasing the feasibility of stem cell banking

Despite tremendous amount of preclinical studies, very few clinical trials evaluated dental stem cells for bone tissue engineering. Only DPSCs and PDLSCs were evaluated for their clinical applications. Although more comprehensive experimental and clinical studies about the therapeutic efficacy of dental stem cells are necessary, their value and potential are undeniable. Thus, it is likely that in near future dentists will be able to isolate and store stem cells using the ready to use dental stem cell kits as part of their routine dental practice.

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Graphene Based Materials in Neural Tissue Regeneration

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Abstract

Due to its extraordinary features such as large surface area, high electrical conductivity, chemical stability and mechanical properties, graphene attracts great interest in various fields of biomedical sciences including biosensors, cancer therapy, diagnosis and regenerative medicine. The use of graphene-based materials has been of great interest for the design of scaffolds that can promote neural tissue regeneration. Recent studies published over the last few years clearly show that graphene and graphene based materials promote adhesion, proliferation and differentiation of various cells including embryonic stem cells (ESC), neural stem cells (NSC), mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC). Therefore graphene based materials are one of the promising nanoplatforms in regenerative medicine for neural tissue injury. With its unique topographic and chemical properties, graphene is used as a scaffold that could provide a bridge between regenerating nerves. More importantly, as a conductive substrate, graphene allows the continuation of electrical conduction between damaged nerve ends. The integration of supportive cells such as glial, neural precursor or stem cells in such a scaffold shows higher regeneration when compared to currently used neural autografts and nerve conduits. This review discusses the details of such studies involving graphene based materials with a special interest on neural stem cells, mesenchymal stem cells or pluripotent stem cells.

Keywords

Graphene oxide · Mesenchymal stem cells · Neural stem cells · Pluripotent stem cells

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Abbreviations

2D	Two dimentional
3D	Three dimentional
1 step-G	One-step growth
2 step-G	Two-step growth
BDNF	Brain-derived neurotrophic factor
b-FGF	Basic fibroblast growth factor
CNS	Central nervous system
Cu	Copper
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELF-EMF	Extremely low frequency electromagnetic fields
ESCs	Embryonic stem cells
FGF-2	Fibroblast growth factor 2
G	Graphene
GO	Graphene oxide
hADMSCs	Human adipose-derived mesenchymal stem cells
hMSCs	Human mesenchymal stem cells
hNPCs	Human neural progenitor cells
hNSCs	Human neural stem cells
IFNγ	Interferon-γ
iPSCs	Induced pluripotent stem cells
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
MSCs	Mesenchymal stem cells
NGLC	Nanocrystalline glass-like carbon film
NGF	Nerve growth factor
NGO	Nanosized graphene oxide
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PADM	Porcine acellular dermal matrix
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PDMS	Polydimethylsiloxane
PEDOT	Poly (3,4-ethylenedioxythiophene)
PEG	Poly (ethylene glycol)
PN	Peripheral nerve
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
PU	Polyurethane
rGO	Reduced graphene oxide
SCI	Spinal cord injury
SCs	Schwann cells
SDIA	Stromal cell-derived inducing activity
siNPs	Silica nanoparticles
TBI	Traumatic brain injury
TCPS	Tissue culture polystyrene
TiO_2	Titanium dioxide

1 Introduction

The mammalian brain is an extraordinary organic machine that has fascinated scientists and clinicians for hundreds of years. A complex network of chemical and biochemical components of more than a dozen million neurons leads to the emergence of thought, emotion, memory and life. In contrast, fine imbalances or damage to this system can cause serious complications in physical, motor, psychological, and cognitive functions. Furthermore, the loss of inevitable nerve tissue due to degenerative diseases and traumatic injuries is destructive due to the limited regenerative ability of the central nervous system (Shah et al. 2016).

Currently, there are nanotechnology-based approaches developed to direct neural differentiation and regeneration. Among these approaches, stem cell based regenerative medicine shows the greatest hope for repairing and regenerating damaged nerve tissue. However, the establishment of controlled and reliable methodologies (eg, neurons and oligodendrocytes) that direct stem cell differentiation to specialized cells has been a major problem in the field.

2 Graphene and Its Properties

Graphene is a two-dimensional crystal formed by sp2 hybridization of carbon atoms arranged in the form of a honeycomb and composed of single atomic layers of graphite (Bitounis et al. 2013; de Lázaro et al. 2014; Novoselov 2011). Even though the existence of single grafic plates is theoretically debated (Slonczewski and Weiss 1958), the presence of two-dimensional

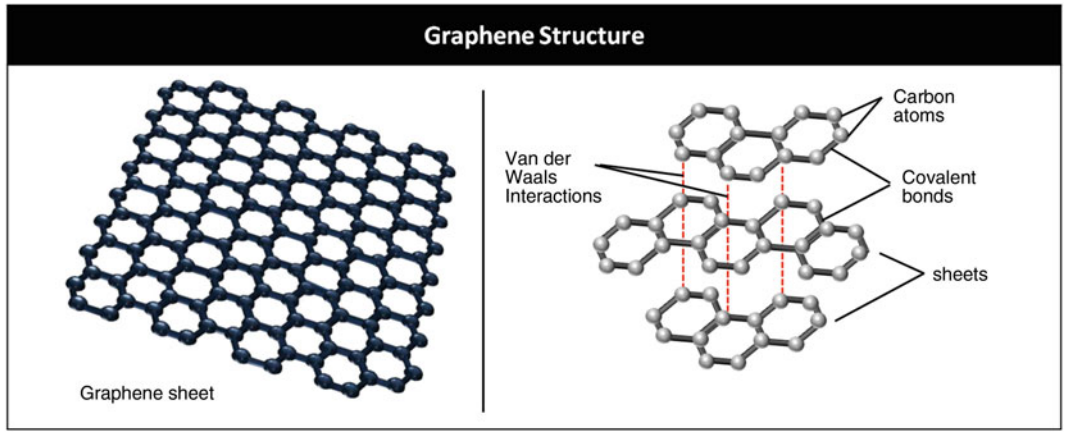


Fig. 1 Graphene structure: Graphene is an allotrope of carbon consisting of a single layer of carbon atoms arranged in a hexagonal lattice. Graphite is composed of stacked layers of graphene sheets, which are held together by the weak Van der Waals interactions

atomically fine crystalline materials was considered physically impossible (Venables et al. 1984). For the first time in 2004, Novoselov and Geim isolated and characterized a single graphene layer by 'Scotch Tape' method (Novoselov et al. 2004). Novoselov and Geim were awarded the Nobel Prize in 2010 for their discovery of this new carbon allotrope (Geim 2011; Novoselov 2011). The simple planar arrangement of the carbon atoms within the single layer of graphene and the covalent bonding between these carbon atoms give graphene unique properties (Fig. 1).

The generation of novel or improved graphene based biomaterials has had a major impact on nanotechnology. Due to its two-dimensional structure, high surface area, high electrical conductivity, chemical stability and bendable properties, graphene attracts great interest in various fields of biomedical sciences including biosensors, cancer therapy, diagnosis and regenerative medicine (Okan et al. 2016; Saner et al. 2010). All these features provide the ability to immobilize many substances such as metals, drugs, biomolecules, fluorescent probes and cells on graphene surface (Reina et al. 2014).

The application of graphene based materials in cell biology and physiology allows targeted interactions at the basic molecular level. In neuroscience, for example, it requires specific interactions with neurons, glial cells or other neuronal cells. Exemplary investigations include development of advanced molecular imaging technologies, engineering hybrid materials used in neural regeneration and developing technologies designed for targeted delivery of drugs and small molecules to blood-brain barrier and neuroprotection (Silva 2006).

2.1 Biocompatibility of Graphene Based Materials

Numerous studies have been conducted to improve the use of graphene in the biomedical field and to understand the toxicity profile in preclinical studies. By engineering surface chemistry, colloidal properties, water solubility and size, graphene-based biomaterials can become more biocompatible (Bussy et al. 2013). The toxicity profile of graphene based materials has been studied extensively in both in vitro and in vivo systems and there are promising results suggesting that these materials will be able to translated into clinical settings in the future (Seabra et al. 2014). In vitro studies have shown that when oxidised (referred to as graphene oxide-GO), or surface functionalized (with biodegradable polymers), toxicity related to graphene could

be abolished (Ali-Boucetta et al. 2013). Supporting cell culture studies, animal models have also shown that graphene-based materials which are small and single layer or conjugated to polymers such as PEG can be eliminated from living systems (Bussy et al. 2013). Therefore, considering such studies, it is possible to produce biocompatible biomaterials which will not cause toxicity.

3 The Nervous System

The nervous system is a network of signals that allows the brain and other parts of the body to function simultaneously. Neurons are electrically excitable cells which are using a concentration gradient of various ions (sodium, potassium, calcium, chloride etc.) and provide electrical conduction through the release neurotransmitters such as acetylcholine. Since neurons do not have division capabilities (suspended in G0 phase), it is of great importance to establish new treatment options for neural diseases by using the ability of stem cells to differentiate into neuronal cells (Fraczek-Szczypta 2014; Shin et al. 2016).

Glial cells are the most abundant cell type in the nervous system. Virchow first explained that there were cells present in neural tissue other than neurons. The first characterization of glial cells is a result of microscopic studies and metallic impregnation techniques developed by Ramon y Cajal and Rio Hortega in particular. Using gold impregnation, astrocytes; a few years later, oligodendrocytes and microglia, using silver carbonate impregnation were named (del Río-Hortega 1921, 1928).

Figure 2 summarizes the different types of cells present in CNS and PNS (Amoh et al. 2005; Frostick et al. 1998; Gardin et al. 2016; Liu et al. 2000; Nedergaard 1994; Ramírez-Jarquín et al. 2014; Scholz and Woolf 2007; Sedaghati et al. 2011; Spassky et al. 2005; Zhou et al. 2010)

The most common injuries affecting the nervous system are peripheral nerve injuries (CNS), spinal cord injury (SCI), and traumatic brain injury (TBI) (Gardin et al. 2016). The incidence of peripheral nerve injury (PNI) is estimated to be between 13 and 23 per 100,000 people per year in developed countries and causes partial or complete loss of motor, sensory and autonomic function in the relevant segments of the body. On the other hand, spinal cord injuries with an incidence of 750 out of every 1,000,000 people in the world are seriously threatening life (Wyndaele and Wyndaele 2006).

Most of the peripheral nerve injuries are repaired using nerve autografts, but they are limited to the source of donor nerves and may cause morbidity of the donor site (Zhou et al. 2010). Typically, axons in the micro perimeter of the peripheral nerve (PN) may be self-regulating at a relatively short distance (not greater than 5 mm). Regeneration of PN begins with the separation of Schwann cells (SCs) from axons, occurs as a result of incision of myelin sheaths and is phagocytozed by glial cells. SCs that break from the axons can multiply and enhance axonal guidance (Terenghi 1999). On the other hand, the cell loss during spianl cord injury can not be replaced by the body itself, and thus the spinal cord function is permanently lost. Due to the limited internal regenerative abilities, experts from different fields are encouraged to seek new ways to regenerate damaged or diseased nervous system, such as many pharmacological approaches, stem cell treatments, delivery of neurotrophic factors and biomaterial use (Lee-Kubli and Lu 2015).

4 Graphene in Regenerative Medicine

Ding and colleagues stated that a Web of Science search for “graphene” and “tissue engineering” showed that the majority of studies with graphene-based materials were based on bone and neural tissue regeneration (Ding et al. 2015). This trend clearly demonstrates the global importance of the graphene and the increase in interest of scientists in this field. In short, novel biomaterial platforms that can be used in regenerative medicine.

As confirmed by the increase in the number of publications, it is not surprising that the graphene has shown great interest in nanomedicine and

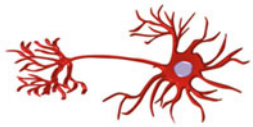
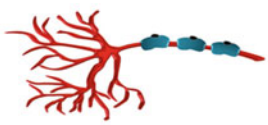
Types of cells in nervous system	Functions
Astrocytes 	• have functional neurotransmitter receptors, modulating the conduction properties of neighbouring neurons (Nedergaard 1994) • feature inhibition mechanisms to the regenerating neurites (Zhou et al. 2010) • have important roles in the homeostasis of the CNS (Gardin et al. 2016)
Ependymal cells 	• Provide the transport of cerebrospinal fluid and in brain homeostasis (Spassky et al. 2005)
Microglial cells 	• are macrophages of the CNS and permanent cells in the brain's immune system (Gardin et al. 2016)
Neurons 	• are the most common cell type in the CNS and PNS (Ramírez-Jarquín et al. 2014) • manipulate information to communicate neural networks (Gardin et al. 2016)
Oligodendrocytes 	• produce myelin and myelinated host axons in the central nervous system (CNS) (Liu et al. 2000) • provide axonal regeneration in CNS.
Satellite cells 	• encompass the cell bodies of dorsal root ganglia neurons (Scholz and Woolf 2007) • enhance the expression of glial fibrillary acidic protein (Scholz and Woolf 2007)
Schwann cells 	• support neuron regrowth (Amoh et al. 2005) • form myelin sheaths surrounding axons (Amoh et al. 2005) • provide regeneration of peripheral nerve (Frostick et al. 1998) • are important in axonal regeneration because they secrete neurotrophic factors (Sedaghati et al. 2011)

Fig. 2 **Cells in the nervous system**: Neurons and various types glial cells are found in the nervous system

biomedical applications. Recent studies published over the last few years clearly show that graphene and graphene based materials promote adhesion, proliferation and differentiation of various cells such as embryonic stem cells (ESC), neural stem cells (NSC), mesenchymal stem cells (MSC) and induced pluripotent stem cells (iPSC) and thus graphene based materials are known to be one of the promising nanoplatforms in regenerative medicine (Bressan et al. 2014).

Currently, there are various preclinical applications of graphene-based biomaterials showing bone tissues regeneration (Lee et al. 2011; Nayak et al. 2011), partial repair of muscle mass and loss of function (Kenry et al. 2018), usage in cardiac therapies (Park et al. 2014), regeneration of adipose tissue (Gomillion and Burg 2006). Due to the complexity of the anatomy and physiology of the nervous system compared to the other tissues, the repair and regeneration of injured and malfunctioning neural tissue via graphene based materials still need to be investigated further (Fraczek-Szczypta 2014).

5 Graphene, Neurons and Glia Cells

Recently, the use of graphene-based materials has been of great interest for the design of scaffolds that can promote neuron regeneration. With its unique topographic and chemical properties, graphene is a promising scaffold that could provide a bridge between regenerating nerves. More importantly, as a conductive substrate, graphene allow the continuation of electrical conduction between damaged nerve ends. The integration of supportive cells such as glial and neural precursor cells in such a scaffold can enhanec regeneration when compared to currently used neural autografts and nerve conduits.

Differentiation of stem cells to neurons or neuronal cells for neural regeneration is a critical step while developing stem cell based therapies. Several types of graphene scaffolds have been studied which supported significant stem cell differentiation.

Park et al. used a graphene scaffold as an inducer to differentiate human neural stem cells (NSCs) into neurons (Park et al. 2011). Wang et al. showed that human MCS underwent more neuronal differentiation on fluorine-functional graphene sheets compared to pristine graphene material (Wang et al. 2012). Selective differentiation of stem cells to neurons or oligodendrocytes for regeneration of central nervous system is a highly preferred situation and Shah et al. showed that even in the absence of growth factors, NSCs can differentiate into oligodendrocytes by using only GO-coated cell culture surfaces (Shah et al. 2014).

One of the greatest advantages of using graphene in nerve tissue damage, which also makes it more preferred than other conventional biomaterials, is the ability to create functional neural network connectivity. For, example, when embryonic neural progenitor cells were cultured on three dimentional GO-layers, it was observed that there were not only neurons and glial cells differentiated on these layers, but also a neural network rich in dendrites, axons and synaptic connections (Serrano et al. 2014).

6 Graphene and Neural Tissue Injuries

Currently, there is no cure for spinal cord injury (SCI), however, various scientific studies have recently begun to investigate the potential use of stem cells for SCI. In these cases, stem cell therapies focus on the introduction of neurons and oligodendroglia cells at the injury site in order to create a good microenvironment for the regenerating cells (Salewski et al. 2010). In this regard, mesenchymal stem cells, Schwan cells, glia cells and neurons from olfactory mucosa, neural stem cell and progenitor cells and embryonic stem cells have been used (Schroeder et al. 2016).

In spinal cord injury, there are two potential approaches in which stem cells are used: transplantation of stem cells into the injury site or use of nerural precursor cells at the damaged spinal cord. Transplantation of stem cells is a risky task and

requires a precise surgical procedure, and there is a possiblity that the immune system may reject new cells (in cases of embryonic and neural stem cell use). The enhancement of the treatment potential with the help of biomaterials such as graphene has been suggested in various studies. According to these, when graphene-based materials are applied together with stem cells or neural precursor cell, they induce the differentiation towards neurons, oligodendroglia and astrocytes (Barnabé-Heider and Frisén 2008; Nayak et al. 2011; Park et al. 2011; Wang et al. 2012).

In addition to the two dimentional stem cell culture system, there are studies involving three dimentional graphene based materials which better mimic the in vivo microenvironment. Li et al. has shown that they can achieve proliferation and differentiation of neural stem cells using a 3D graphene scaffold (Li et al. 2013; Nayak et al. 2011). In another study, 3D graphene oxide scaffolds were tested in spinal cord injury-induced rats, and an increase in tissue repair was observed (López-Dolado et al. 2015). However, in this study even though no local or systemic toxicity was observed, the use of diisocyanate-containing cross-linkers which was involved during the long-term production processes graphene, increases the toxicity risks of this application. In addition, the researchers have only investigated one type of graphene and the effects of different surface chemistry and derivatives with topography have not been investigated and the mechanism of tissue repair has not been explained.

7 Graphene and Stem Cells

With the purpose of neural tissue regeneration, graphene based materials have been tested with various cell sources and mostly with neural stem/progenitor cells and other stem cells including mesenchymal, embryonic or induced pluripotent stem cells. Various graphene based materials have been combined not only with polymers or differentiation factors but also under electrical or magnetic field. This review will discuss the details of studies performed with neural stem cells, mesenchymal stem cells or pluripotent stem cells.

7.1 Neural Stem Cells

Neural stem cells are multipotent in origin and they are present in the adult central nervous system. They can renew themselves and give rise to new neurons and supporting neuronal cells. Activation of NSCs or their transplantation into areas of central nervous system injury can lead to regeneration in animal models (Barkho and Zhao 2011). Various graphene based materials and hybrid systems have been studied with neural stem cells (Table 1). In 2011, Park et al. generated laminin coated graphene substrates and following NSC seeding, authors observed enhanced neural differentiation (Park et al. 2011). Laminin coatings have been combined with graphene based materials in various studies. It has been suggested that it improves cell seeding and survival on the biomaterial substrates. In addition to extracellular proteins such as laminin, researchers have included differentiation factors such as b-FGF, EGF, PDGF or NGF to improve the differentiation protocol (Park et al. 2011; Solanki et al. 2013).

In hybrid systems, graphene has been combined with nanofibers or other nanoparticles. For examples, graphene and Silica based nanoparticle hybrid structures successfully aligned axons, the differentiation and growth of adult hNSCs (Solanki et al. 2013). In another study, titanium oxide combined reduced GO (rGO) substrates were produced and their flash photo stimulation resulted in a $\sim$ 23-fold increase in the neural to glial cell ratio (Akhavan and Ghaderi 2013).

Another study used GO sheets coated with PCL nanofibers which were generated by electrospinning. When NSCs were seeded on these hybrid srufaces oligodendrocyte differentiation was improved (Shah et al. 2014). Weaver et al. showed that GO-poly (3,4-ethylenedioxythiophene) nanocomposite films can also induce oligondendrocyte differentiation (Weaver and Cui 2015). In another microfiber study, nanostructured rGO poly-D-lysine microfibers were demonstrated to be more successful in adhesion and proliferation of NSCs than 2D graphene film and tissue culture plate (Guo et al. 2017).

Table 1 Graphene based hybrid systems have been studied with neural stem cells

Types of graphene	Additional materials or factors	Main findings	Reference
Graphene substrates	+ Laminin + b-FGF + EGF	Long term culturing of hNSCs on laminin coated graphene films enhanced neural differentiation	Park et al. (2011)
GO nanosheets	+ ECM protein patterning + 300 nm SiNPs + Laminin + b-FGF + EGF	The graphene-nanoparticle hybrid structures successfully aligned axons, the differentiation and growth of adult hNSCs	Solanki et al. (2013)
3D graphene foams	+ EGF + FGF-2	Foams improved proliferation of NSCs and enhanced the NSCs differentiation towards astrocytes and especially neurons	Li et al. (2013)
rGO	+ rGO/TiO2 heterojunction film + Flash photo stimulator	Flash photo stimulation of human neural stem cells on graphene/TiO2 heterojunction resulted in a ~ 23-fold increase in the neural to glial cell ratio	Akhavan and Ghaderi (2013)
2D- and 3D-graphene materials	+ LPS + TCPS + EGF + FGF-2	3D graphene causes less neuroinflammation in microglia than 2D graphene For neural repair and neurogenesis, the studies on the 3D graphene topic should be increased	Song et al. (2014)
GO	+ PCL nanofibers	NSCs were seeded on GO sheets coated with electrospunned PCL nanofibers GO-nanofiber hybrid scaffolds showed enhanced differentiation into oligodendrocyte lineage cells	Shah et al. (2014)
GO/PEDOT nanocomposite films	+ PEDOT + IFNγ + PDGF	PDGF and IFNγ supported neuronal and oligodendrocyte lineage differentiation GO/PEDOT can be customized to develop therapeutic potential	Weaver and Cui (2015)
Nanostructured rGO microfibers	+ poly-D-lysine + EGF + b-FGF	Nanostructured rGO microfibers were demonstrated to be more successful in adhesion and proliferation of NSCs than 2D graphene film and tissue culture plate	Guo et al. (2017)
3D printted graphene scaffolds	+ PU + 3D printing	Graphene–polyurethane composite hydrogel enhanced the oxygen metabolism and neural differentiation of NSCs	Huang et al. (2017)
3D Graphene scaffolds	+ monophasic current stimulation + Neurobasal media	iPSC-derived hNPCs were used Electrical stimulation to enhance NPCs neurogenesis was promising and suggested further investigation of the therapy	Nguyen et al. (2018)

2D two dimensional, *3D* three dimensional, *b-FGF* basic fibroblast-growth factor, *ECM* extracellular matrix, *EGF* epidermal growth factor, *FGF-2* fibroblast growth factor 2, *GO* graphene oxide, *hNPCs* human neural progenitor cells, *hNSCs* human neural stem cells, *IFNγ* interferon-γ, *iPSCs* induced pluripotent stem cell, *LPS* lipopolysaccharide, *NSCs* neural stem cells, *NPCs* neural progenitor cells, *PCL* polycaprolactone, *PDGF* platelet-derived growth factor, *PEDOT* poly (3,4-ethylenedioxythiophene), *PU* polyurethane, *rGO* reduced graphene oxide, *siNPs* silica nanoparticles, *TCPS* tissue culture polystyrene, TiO_2 titanium dioxide

In addition to the above reports with 2-dimentional (2D) graphene based materials, there are also studies performed in 3-dimention (3D). One of the first studies showed that, in the presence of FGF-2 and EGF, 3D graphene foams improved the proliferation of NSC and enhanced their differentiation towards astrocytes and especially neurons (Li et al. 2013). In another comparison study between 2D and 3D graphene based materials, Song et al. suggested that 3D graphene causes less neuroinflammation in microglia than 2D graphene (Song et al. 2014). Furthermore, monophasic electrical stimulation was also shown to enhance NPCs neurogenesis on 3D graphene scaffolds (Nguyen et al. 2018). Recently, 3D printing technologies have been also combined with graphene based substrates and Huang et al. reported that 3D printed graphene–polyurethane composite hydrogel enhanced the oxygen metabolism and neural differentiation of NSC (Huang et al. 2017).

7.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) which are having multipotent differentiation potential, can be obtained from bone marrow or fat tissue. MSCs are currently being investigated preclinically for the treatment of various diseases and are being tested in clinical trials (Ullah et al. 2015). Similar to NSCs, MSCs have been also investigated with different graphene based materials in order to test their efficacies for neural tissue regeneration (Table 2). When GO was combined with porcine acellular dermal matrix, human adipose derived mesenchymal stem cells (hADMSCs) showed differentiation to ectodermal cells, especially neurons in the presence of differentiation factors (Kim et al. 2015b). On the other hand, there were studies which reported enhanced differentiation even in the presence of such factors. For examples, cell alignment using printed PDMS channel arrays on fluorinated graphene enhanced the neuro-induction of hMSCs in the absence of growth or differentiation factors (Wang et al. 2012).

Similar to NSC studies, 3D printing was also combined with MSCs. In 2015, when MSCs were incubated on 3D printed graphene polylactide-co-glycolide scaffolds, in vitro studies showed neurogenic differentiation with significant upregulation of glial and neuronal genes in the absence of differentiation factors. In vivo studies suggested that 3D printed scaffolds showed promising biocompatibility over the course of at least 30 days (Jakus et al. 2015). In another recent study, 1 step- and 2- step growth graphene were shown to be effective at neuronal differentiation of bone marrow-derived human mesenchymal stem cells (Lee et al. 2018)

Electrical stimulation also improves the MSCs differenation on graphene based substrates. For examples, Lee et al. reported that extremely low frequency electromagnetic fields exposure synergistically increased biological efficacy of neuronal differentiation of hMSCs grown on graphene-coated substrates (Lee et al. 2015). Later, in 2016, electric pulses generated by the triboelectric nanogenerator were shown to induce neural differentiation of MSCs when cells were grown on rGO- Poly (3,4-ethylenedioxythiophene) hybrid microfibers (Guo et al. 2016b).

7.3 Pluripotent Stem Cells

Pluripotent stem cells are classified in two categories, embryonic and induced pluripotent. Embryonic stem cells (ESCs) are derived from the inner cell mass of a blastocyst whereas induced pluripotent stem cells (iPSCs) are generated from somatic cells following forced expression of reprogramming factors (Chin et al. 2009; Takahashi and Yamanaka 2006; Thomson et al. 1998). In one of the first studies involving graphene and pluripotent stem cells (Table 3), mouse iPSCs were cultured on both graphene or GO surfaces spontaneously differentiated into ectodermal and mesodermal lineages, and authors reported that these materials could be a promising strategy to use in differentiation protocols in pluripotent stem cell cultures (Chen et al. 2012).

Table 2 Graphene based hybrid systems have been studied with mesenchymal stem cells

Types of graphene	Additional materials or factors	Main findings	Reference
GO	+ PDMS arrays	Cell alignment using printed PDMS channel arrays on fluorinated graphene enhanced the neuro-induction of hMSCs even in the absence of growth or differentiation factors	Wang et al. (2012)
NGO (100 nm)	+ 3D PADM + b-FGF + BDNF + NGF	hADMSCs were differentiated to ectodermal cells (neuron) on NGO grid patterns	Kim et al. (2015b)
3D printable graphene	+ polylactide-co-glycolide	In vitro studies showed neurogenic differentiation with significant upregulation of glial and neuronal genes in the absence of differentiation factors In vivo studies suggested that 3D printed scaffolds showed promising biocompatibility over the course of at least 30 days	Jakus et al. (2015)
Graphene substrate	+ ELF-EMF + Hydrocortisone + Forskolin + Valproic acid + Insulin	ELF-EMF exposure synergistically increased biological efficacy of neuronal differentiation of hMSCs grown on graphene-coated substrate	Lee et al. (2015)
Graphene monolayers	+ 3D spheroid cultures of hMSCs	Graphene monolayers regulated the interactions at cell-substrate or cell–cell interfaces, consequently promoting the neurogenesis of hMSCs as well as the outgrowth of neurites	Kim et al. (2015a)
rGO microfibers rGO-PEDOT hybrid microfibers	+ PEDOT + Electrical stimulation + b-FGF	By inducing electric pulses generated by the triboelectric. Nanogenerator, neural differentiation of MSCs was dramatically improved	Guo et al. (2016b)
rGO nanosheets	+ PADM scaffold	rGO-assembled PADM scaffold enhanced the differentiation of MSCs into neuronal cells 7 days after seeding	Guo et al. (2016a)
1 step-G and 2 step-G Graphene	+ 35-mm-thick Cu foils	1 step- and 2- step growth graphene were effective at neuronal differentiation of bone marrow-derived human mesenchymal stem cells	Lee et al. (2018)

1 step-G one-step growth, *2 step-G* two-step growth, *3D* three dimensional, *ADSCs* adipose derived stem cells, *BDNF* brain-derived neurotrophic factor, *b-FGF* basic fibroblast growth factor, *Cu* copper, *ELF-EMF* extremely low frequency electromagnetic fields, *GO* graphene oxide, *hADMSCs* human adipose-derived mesenchymal stem cells, *hMSCs* human mesenchymal stem cells, *MSCs* mesenchymal stem cells, *NGF* nerve growth factor, *NGO* nanosized graphene oxide, *PADM* porcine acellular dermal matrix, *PDMS* polydimethylsiloxane, *PEDOT* poly (3,4-ethylenedioxythiophene), *rGO* reduced graphene oxide

Later in 2012, when mouse embryonic stem cells were seeded on graphene or graphene oxide surfaces under stromal cell-derived inducing activity, only GO effectively promoted mouse embryonic stem cell differentiation towards dopamine neurons compared to only graphene treated cells or negative control groups (Yang et al. 2014). In a recent study, transgenic mouse embryos were used to derive substantia nigra dopaminergic cells which were then cultured on graphene flakes containing nanocrystalline glass-like carbon films. The results demonstrated neuronal capability and there was a direct relationship between the thickness of the films and cell maturation (Rodriguez-Losada et al. 2017).

Table 3 Graphene based hybrid systems have been studied with pluripotent stem cells

Types of graphene	Additional materials or factors	Main findings	Reference
Graphene and graphene oxide substrates	+LIF	Mouse iPSCs cultured on both G and GO surfaces spontaneously differentiated into ectodermal and mesodermal lineages	Chen et al. (2012)
Graphene and graphene oxide	+SDIA	Only GO effectively promoted mouse embryonic stem cell differentiation towards dopamine neurons compared to graphene treated cells or control group	Yang et al. (2014)
Graphene flakes	+ NGLC composed of curved graphene flakes joined by an amorphous carbon matrix	Substantia nigra dopaminergic cells were derived from transgenic mouse embryos Culturing on NGLC demonstrated neuronal capability to a certain extent There was a direct relationship between the thickness of the films and cell maturation	Rodriguez-Losada et al. (2017)

ESCs embryonic stem cells, *G* graphene, *GO* graphene oxide, *iPSCs* induced pluripotent stem cells, *LIF* leukemia inhibitory factor, *NGLC* nanocrystalline glass-like carbon film, *SDIA* stromal cell-derived inducing activity

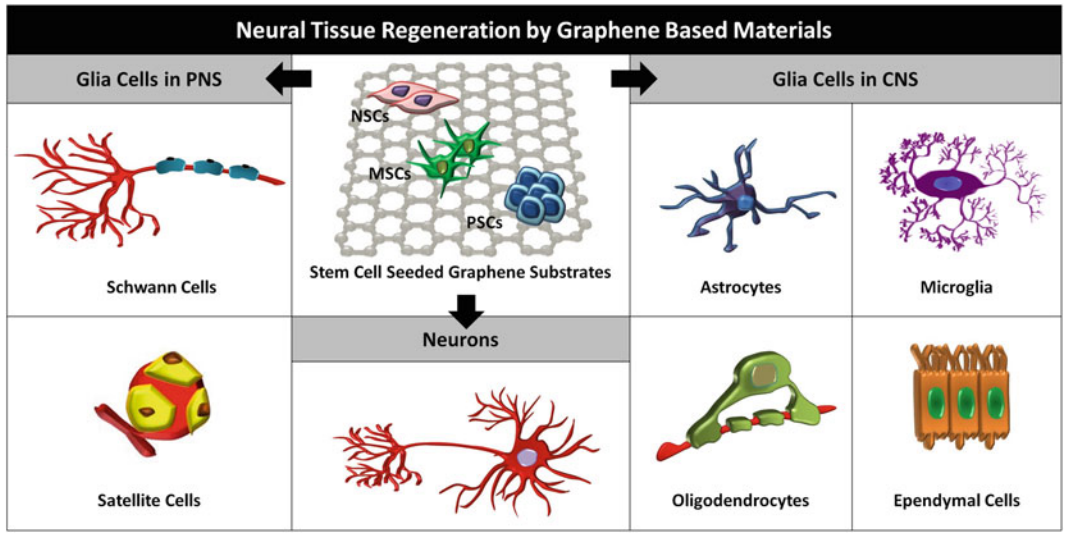


Fig. 3 Neural tissue regeneration by graphene based materials: Seeding neural stem cells, mesenchymal stem cells or pluripotent stem cells on graphene based scaffolds results in the differentiation towards neurons and glial cells

8 Conclusion an Future Perspectives

Graphene has a tremendous interface and a very conductive path for the conduction of electricity (Chen et al. 2011). Neurons in this count are electro-active and electrical stimulation can affect the behavior of stem cells (Chang et al. 2011; Ghasemi-Mobarakeh et al. 2011). For this reason, graphene has been used in various neural tissue regeneration studies which are summarized in Fig. 3. With the development of graphene derivatives, more specific needs for injury repair can be met. Most of the studies have examined the differentiation potential of Schwann cells, oligondendrocytes or neurons, however more detailed experiments are needed to understand the effect of graphene based materials on differentiation of other glial cells such as astrocytes, microglia or ependymal cells, since a healthy microenvironment would be needed for efficient neural tissue repair.

In addition to the development of novel graphene derivatives, 3D printing technologies are getting improved. As seen from the above Tables, there are already reports in literature which involves printing of graphene with additional polymers and growth factors. Most of the time, following the production of these 3D printed scaffolds, cells are seeded on their surface to evaluate differentiation potential. With the help of advanced bioprinting technologies, researchers will be able to print cells and materials together which can anatomically and histologically mimic the healthy neural tissue.

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Tissue Engineered Skin Substitutes

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Abstract

The fundamental skin role is to supply a supportive barrier to protect body against harmful agents and injuries. Three layers of skin including epidermis, dermis and hypodermis form a sophisticated tissue composed of extracellular matrix (ECM) mainly made of collagens and glycosaminoglycans (GAGs) as a scaffold, different cell types such as keratinocytes, fibroblasts and functional cells embedded in the ECM. When the skin is injured, depends on its severity, the majority of mentioned components are recruited to wound regeneration. Additionally, different growth factors like fibroblast growth factor (FGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) are needed to orchestrated wound healing process. In case of large surface area wounds, natural wound repair seems inefficient. Inspired by nature, scientists in tissue engineering field attempt to engineered constructs mimicking natural healing process to promote skin restoration in untreatable injuries. There are three main types of commercially available engineered skin substitutes including epidermal, dermal, and dermoepidermal. Each of them could be composed of scaffold, desired cell types or growth factors. These substitutes could have autologous, allogeneic, or xenogeneic origin. Moreover, they may be cellular or acellular. They are used to accelerate wound healing and recover normal skin functions with pain relief. Although there are a wide variety of commercially available skin substitutes, almost none of them considered as an ideal equivalents required for proper wound healing.

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Abbreviations

3D	3 Dimensional
LOEX	Laboratoire d'Or- ganogenese Experimentale
AATB	American Association of Tissue Banks
MHC	Major histocompatibility complex
AMSCs	Adipose-derived MSCs
MSCs	Mesenchymal stem cells
BMSCs	Bone marrow-derived MSCs
NK	Natural killer
CEA	Cultured epithelial autograft
PCL	Poly-ε-caprolactone
CSS	Cultured skin substitutes
PDGF	Platelet-derived growth factor
CTGF	Connective tissue growth factor
PDLA	Poly-D-lactic acid
EB	Epidermolysis bullosa
PDLLA	Poly-DL-lactic acid
ECM	Extracellular matrix
PEG	Polyethylene glycol
EGF	Epidermal growth factor
PGA	Polyglycolic acid
ESCs	Embryonic stem cells
PHB	Poly-β-hydroxybutyrate
FDA	US Food and Drug Administration
PLA	Polylactic acid
FGF	Fibroblast growth factor
PLCL	Poly L-lactide-co- ε –caprolactone
FGF-1	Fibroblast growth factor-1
PLGA	Polylactic-co-glycolic acid
FGF-2	Fibroblast growth factor-2
PLLA	Poly-L-lactic acid
FTSG	Full-thickness skin grafting
POE	Polyhydroxyortho esters
GAG	Glycosaminoglycan
PU	Polyurethane
HA	Hyaluronic acid
PVA	Poly vinyl alcohol
HIV	Human immunodeficiency virus
SAPs	Self-assembling peptides
IL-1	Interleukin-1
TBSA	Total body surface area
IL-6	Interleukin-6
STSG	Split-thickness skin grafting
IL-8	Interleukin-8
TGFα	Transforming growth factor-α
IPSCs	Induced pluripotent stem cells
TGFβ	Transforming growth factor-β
KGF	keratinocyte growth factor
UMSCs	Umbilical cord-derived MSCs
KGF-1	keratinocyte growth factor-1
UV	Ultraviolet
LCs	Langerhans cells
VEGF	Vascular endothelial growth factor

1 Introduction

Skin is a protective coverage of body against Ultraviolet (UV) radiation and harmful mechanical, chemical, and microbial agents. Moreover, it plays a leading role in thermoregulation and fluid balance, in our body (Böttcher-Haberzeth et al. 2010, Groeber et al. 2011a, b, Biedermann et al. 2013). Skin traumas caused by different factors such as burns, leg ulcers, diabetic foot ulcers, as well as congenital giant nevi result in skin disintegration and cell necrosis which emphasize the constant need for appropriate treatment in order to either heal or regenerate the lesion in a way that resemble the function and structure of natural skin (Tannous et al. 2005; Arneja and Gosain 2007; Schiestl et al. 2010).

Normal skin has the ability to regenerate epidermis injury by a population of highly proliferative stem cells with self-renewal property present in basal layer of skin (Catalano et al. 2013; Vig et al. 2017). However, limited self-renewal ability of skin in severe deep wounds necessitates the use of different skin grafting methods like split-thickness skin grafting (STSG) consist of the whole epidermis and a part of dermis and full-thickness skin grafting (FTSG) including the whole dermis and epidermis. These methods usually could be adopted only in small areas of wounded skin (MacNeil 2008; Biedermann et al. 2013). Autotransplantation with STSG which leads to

scar and hypertrophic keloid formation has been considered as the preferred strategy for coverage of excised burn wounds. Compared with STSG, FTSG usually results in less scarring and could be performed in limited area of total body surface area (TBSA). Donor site shortage causes insufficient coverage with autologous skin grafting in patients undergoing high TBSA burn. Complications associated with surgical processes and scaring are serious limitations of this conventional method for wound healing (Shakespeare 2001; Supp and Boyce 2005a, b; Böttcher-Haberzeth et al. 2010; Damanhuri et al. 2011; Blais et al. 2013; Sheikholeslam et al. 2017). Additionally, more sever and deeper wounds also fail to achieve proper wound care that contribute to chronic wounds formation as a consequence of wound healing process dysregulation (Catalano et al. 2013). From a clinical point of view, a variety of chronic wounds are treatment resistant and one of the main reason for amputations and mortality (Vig et al. 2017). Despite the fact that an effective solution for lack of donor availability seems allogeneic or xenogeneic skin grafts, this methods have their own drawbacks including recipient's immune response or even immune rejection and the risk of infectious disease transmission. Uncertainty regarding their safety and short-term survival restrict their clinical applications (Erdag and Morgan 2004; Sheikholeslam et al. 2017; Vig et al. 2017). Bioengineered skin substitutes, developed over recent decades, seem as a promising alternative for conventional skin grafts and demonstrate a dramatic increase in variety and efficacy. These skin replacements try, to some degree, to eliminate technical constraints arising from common transplantation methods to regenerate injured skin areas (Alonso and Fuchs 2003; Clark et al. 2007; de Mel et al. 2012; Dixit et al. 2017a, b). They are available in various forms mainly classified into epidermal, dermal, and dermoepidermal or composite skin analogs which could be composed of cell-based or cell-free scaffolds applying to skin defects (Damanhuri et al. 2011; Nicholas et al. 2016a, b; Nicholas and Yeung 2017a, b; Sheikholeslam et al. 2017). They are used to accelerate the wound healing process and recover the skin functions with relieving the pain. Achieving a sufficient number of well differentiated cells with normal phenotypes to make a functional coverage, efficient vascularization in order to increase the durability and viability, biocompatibility, biodegradability, non-carcinogenic crosslinks, cost-effectiveness, lack of transmissible disease risk, and prevention of recipient's immune system stimulation are key factors that should be considered in order to produce a safe and high quality engineered skin requirements (Shakespeare 2001; MacNeil 2007; Böttcher-Haberzeth et al. 2010; Damanhuri et al. 2011; Biedermann et al. 2013; Varkey et al. 2015a, b). The main approach in skin substitutes engineering is to culture primary skin cells like stem cells, fibroblasts, keratinocytes, melanocytes, and Langerhans in a natural or biosynthetic scaffold mimicking the 3 dimensional (3D) structure of normal skin and able to promote adhesion and proliferation processes similar to the molecular signaling pathways involved in wound healing cascade (Böttcher-Haberzeth et al. 2010; Catalano et al. 2013; Sheikholeslam et al. 2017; Vig et al. 2017).

Although there are a wide variety of commercially available tissue-engineered skin substitutes, almost none of them could meet all qualifications needed for a real skin which especially include deep skin appendages, appropriate vascularization and normal pigmentation (Gurtner et al. 2008; Damanhuri et al. 2011; Catalano et al. 2013; Varkey et al. 2015a, b). The current review gives an overview of human skin structure and its function in wound healing, traditional surgical based skin grafting and their complications. Following that we focus exclusively on components of tissue engineered skin substitutes, address the most common commercially available engineered skin constructs, and potential use of stem cells in this field.

2 Human Skin Structure

Skin is the largest organ of our body and serves as a barrier to outer harmful factors present in surrounding environment. In terms of anatomy, skin composed of three different layers: avascular epidermis, highly vascularized thicker dermis and well vascularized hypodermis illustrated in Fig. 1

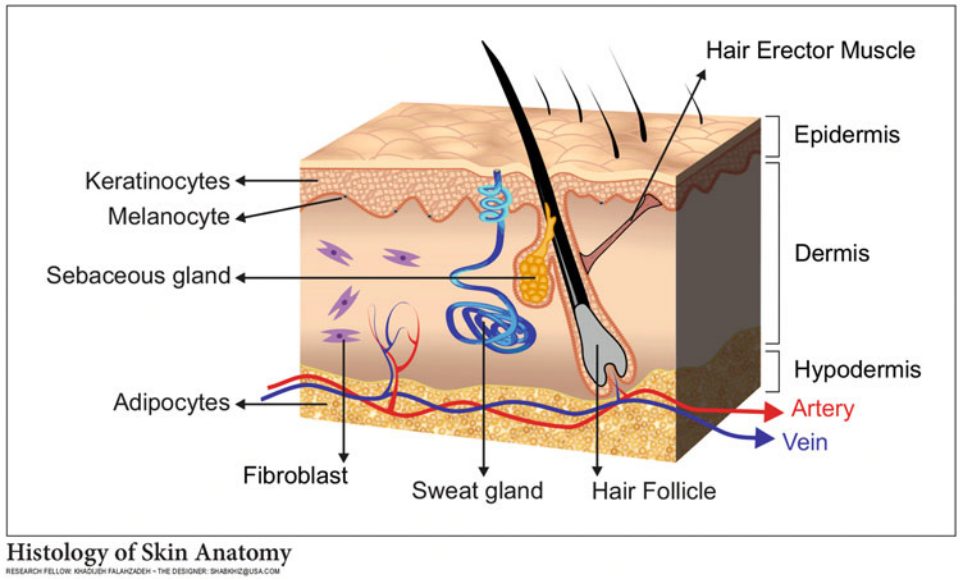

Fig. 1 Three layers of human skin named as dermis, epidermis, and hypodermis. Keratinocytes and melanocytes are abundant in epidermis while fibroblasts mainly present in dermis. Adipocytes are the main cells of deepest skin layer

(Supp and Boyce 2005a, b; Groeber et al. 2011a, b; Takeo et al. 2015; Varkey et al. 2015a, b)

Epidermis layer consists of the following cell types 1. keratinocytes which are arranged in 4 layers including strata basale, spinosum, granulosum, and corneum acting as a protective layer against various pathogens, chemical and mechanical agents, and also responsible for skin fluid balance maintenance, 2. Melanocytes that are key players in pigmentation, 3. Langerhans' cells as a member of dendritic cells family involved in skin immune response and 4. a deep layer include basal cells with high proliferation potency which helps to form a stratified epithelium allowing skin to be an efficient protection for body surface (Badylak 2002; Metcalfe and Ferguson 2007; Takeo et al. 2015).

Dermis layer is thicker than epidermis and fibroblasts are its major cell type. These fibroblasts are mainly categorized into superficial and deep ones called anti-fibrotic and pro-fibrotic, respectively (Varkey et al. 2015a, b). They function in wound healing process by secreting and remodeling of the ECM components such as collagen and fibronectin. The ECM is made of GAGs, elastin, and collagen surrounded by dermal cells like fibroblasts, smooth muscle cells, endothelial cells and mast cells is a complex 3D context composed of various structural and functional proteins that support them as a scaffold to bind and also controls the growth through binding to various growth factors (Supp and Boyce 2005a, b; Varkey et al. 2015a, b; Chua et al. 2016). ECM with a fibrous property is the main reason for skin mechanical strength and elasticity that provide blood vessels for skin nourishment. Thanks to GAGs linking with connective tissue proteins to make proteoglycans, the great integrity of human skin is guaranteed (Ashkenas et al. 1996; Badylak 2002; Kalluri 2003; Hay 2005; Varkey et al. 2015a, b). Collagen is the main protein of ECM and more than 20 different types of collagen have been identified until now. The primary structural collagen in dermal tissue is collagen type I (Van der Rest and Garrone 1991).

Fibronectin is another abundant protein in ECM of submucosal structures and basement membrane. Fibronectin can be found in two forms: soluble and tissue isoforms. Due to its structure it is a desirable scaffold for adhesion of cells during wound healing process

(Schwarzbauer 1991; Miyamoto et al. 1998). The next one, is an adhesion protein called laminin found in the ECM of basement membrane (Schwarzbauer 1999). Dermis comprises various receptors for pain, temperature, and touch. Moreover, various appendages like hair follicles, sweat and sebaceous glands that are responsible for temperature control and skin lubrication pass through epidermal and dermal layers to reach the surface. Sweat glands are coated with a layer of keratinocytes and play roles in epidermal healing (Sorrell et al. 2008; Breitkreutz et al. 2009; Tracy et al. 2016).

One of the most important parts of the skin that is essential for its integrity is a specialized zoon beneath the epidermis called basement membrane separating the epidermis from the underlying dermis layer. Different types of collagen and non-collagen fibers including collagen types IV and VII, elastin, nidogen, laminin, especially type 5 laminin, tenascin, fibrillin-1 and also GAGs containing hyaluronic acid (HA), chondroitin sulfate, and proteoglycans like perlecan are found in basement membrane (Timpl and Brown 1996; Aumailley and Rousselle 1999; Schwarzbauer 1999; LeBleu et al. 2007).

They give a functional structure to physically separate from epidermis and contribute to differentiation of keratinocytes to form the not living corneum layer (Parkinson et al. 2011; Varkey et al. 2015a, b; Viswanathan et al. 2016) Cross-linked collagen and elastin fibers have an fundamental role for providing elasticity for dermis (Ushiki 2002; Supp and Boyce 2005a, b). HA, and chondroitin sulfate make it possible to form a negatively charged hydrophilic space allows dermis to take part in absorbing stresses and catch the full force of mechanical disturbances (Amini-Nik et al. 2011; Amini-Nik et al. 2014; Tracy et al. 2016; Shah and Amini-Nik 2017). Furthermore, entactin, collagen type XVIII and VII are also other important fibers found in the basement membrane zone. Laminin in addition to collagen types IV and VII secreted by dermal fibroblasts are able to make hemidesmosomes that is an anchoring fibrils in matrix. This complex ultrastructure is considered as an essential factor for secure attachment of keratinocytes to the dermis (Delvoye et al. 1988; MacNeil 2008).

Hypodermis (subcutis) layer is skin deepest layer mostly consisted of adipocytes and play critical roles in thermoregulation, energy supply, mechanical properties, and insulation. It also act as a pool for regulatory factors like anti-inflammatory adipokines and adiponectin which affect dermis and stimulate fibroblasts to produce HA, respectively. Three layers of human skin behave as a well-coordinated system that is a matter of great importance to its proper structure and function (Metcalfe and Ferguson 2007; MacNeil 2008; Cardinal et al. 2009; Böttcher-Haberzeth et al. 2010; Biedermann et al. 2013; Varkey et al. 2015a, b).

3 Skin Injury

Although skin is a widely spread organ in the human body with a variety of aforementioned functions, some of its roles are still unknown due to difficulties in defining scales for measurements(Shakespeare 2001). However, we know that skin integrity and its barrier functions can be corrupted by several harmful agents contributing to different types of injuries such as acute burn wounds caused by heat, cold, friction, radiation, chemicals, and electricity, and chronic injuries like pressure sores and leg ulcers (Tiwari 2012; Varkey et al. 2015a, b).

Injuries can be mainly classified according to their depth into following categories 1. superficial or epidermal (first degree) 2. superficial and deep dermal or superficial/deep partial thickness (second degree) c. full thickness (third degree). This classification is applicable in any skin defect but mostly in burn injuries. For instance, in ulcers that has a wide range from epidermal to full thickness, grade IV pressure ulcers are as severe as full thickness burns. Deep wounds including dermal and hypodermal injuries can lead to fluid imbalance, bacterial infections, immune-defficiency, and loss of thermoregulation and disability in some cases (Damanhuri et al. 2011; Kahn et al. 2011; Varkey et al. 2015a, b; Dixit et al. 2017a, b). Acute wounds caused by

traumas are prone to form scar under delayed and inappropriate wound care conditions (Carter et al. 2014). Chronic wounds or inappropriate wound repair as a result of increased pro-inflammatory cytokines, diabetes mellitus, aging, insufficient arterial supply of the lower limbs, and pressure effects could lead to abnormal wound healing processes (Falanga 1993; Mustoe et al. 2006; Moore et al. 2015). Due to lack of functional interactions between cells and ECM, many cellular and molecular aberrations have been found in chronic wounds (Schultz and Wysocki 2009). In the most widespread chronic wounds including pressure and leg ulcers, patients undergo a long-term treatment with limited movements that expose them to a higher risk of ischemia and necrosis development (Falanga 1993; Phillips 1994). Epidermolysis bullosa (EB) is another complication which patient's basement membrane proteins are genetically impaired and they fail to produce an efficient barrier in wound healing process (Uitto and Pulkkinen 2001). Conventional and safe treatments for these serious wounds is autologous skin transplantation along with its own advantages and disadvantages that will be discussed in detail in a subsequent sections.

In patients with background disease like giant congenital nevi which the injured area cover more than 50% TBSA and patients confront lack of autologous skin donor site, there is no sufficient available skin area for autologous grafting. Also, congenital nevi can ultimately develop melanoma if it remains untreated (Bittencourt et al. 2000). Common interventions for chronic wounds owning to insufficient autologous skin to cover wounds with larger surface area, a great deal of pain, and slow healing process, do not seem an effective strategy (Phillips and Gilchrest 1990).

Unlike chronic wound, acute wounds often undergo a systematic and ordered healing steps containing inflammatory reaction, proliferation, and tissue remodeling or scar formation (Niessen et al. 1999; Demidova-Rice et al. 2012). These three phases are far different from chronic wounds repair that show no regular wound surface, regular proliferation and remodeling. High economic burden of chronic wound caring (e.g. approximately $20 billion each year in the US), long-term healing process which commonly lasting more than one month, graft loss, and high infectious rate made it a challenging issue for specialties and patients (Braddock et al. 1999; Boyce and Warden 2002; Demidova-Rice et al. 2012; Järbrink et al. 2017). Recent advancements in medical care of skin injuries can improve infection control of wounded site and bringing back the fluid balance which culminate in a decreased rate of mortality and morbidity especially among who suffering from severe burn injuries or chronic non-healing wounds (Rose and Herndon 1997; Association, A. B 2016)

4 Wound Healing

After an injury occurred by different reasons like genetics or trauma, there will be a cascade of signaling pathway to initiate wound healing process. In order to have a better understanding of this complicated mechanism it has been divided into four distinct phases as 1. damage limitation stage, 2. inflammatory stage, 3. proliferative stage, and 4. remodeling or maturation stage (Midwood et al. 2004; Wu et al. 2007; Wynn 2008; Biedermann et al. 2013). Hemorrhage and clot formation via blood coagulation to limit the bleeding and make a temporary barrier to fluid loss and pathogens colonization are main characteristics of the first step. This phase is accompanied by entrance of healing signal sensitive cells to the site of wound so as to proceed the battle (R, R 1969; Shakespeare 2001; Vig et al. 2017).

Second phase that lasting 4 days is described by inflammatory responses like increased blood flow and vascular permeability leading to redness and swelling, local edema, a provisional ECM formation composed of fibrin, fibrinogen, and fibronectin, and also diapedesis of leukocytes to invade wound bed (Rhett et al. 2008; Xue and Jackson 2015). This is mostly because of increasing the production of vasoactive substances like histamine and activating the complement system. Leukocytes like monocytes and neutrophils as professional phagocytic cells involved in innate immunity, penetrate to the wounded area and

contribute to removal of necrotic tissue and wound debris, as well as reduction and control of infectious agents by means of their anti-infectious roles (Shakespeare 2001; Harvey 2005; Wynn 2008; Catalano et al. 2013; Vig et al. 2017). Dermal fibroblasts undergo proliferation in response to neutrophils and macrophages secreted cytokines in wound site (Xue and Jackson 2015).

Following this step, proliferation of fibroblasts and vascular endothelial cells under the stimulation of growth factors produced by cells involved in inflammatory reactions occurs. This step known as proliferative phase. Fibroblasts can produce collagen and fibronectin which displace with fibrin provisional matrix formed in previous phase. Numbers of the fibroblasts differentiate into myofibroblasts helping to the contraction of wounded area. Also initiation of angiogenesis and capillary formation lead to forming of granulation tissue. Proliferation of epithelial cells in deep dermal appendages in the borders of the injury leads the closure of wound. (Shakespeare 2001; Harvey 2005; Wynn 2008; Catalano et al. 2013; Takeo et al. 2015; Vig et al. 2017).

The last phase that may last several months include re-epithelialization characterized by the migration of keratinocytes from the marginal parts of the wound to the surface of granulation tissue beneath the blood clot, formation of a mature epithelium and scar formation by fibroblasts. Re-epithelialization help to restoration of the elasticity and strength of dermis and lack of proper re-epithelialize leads to disruption of the skin barrier function causing dehydration and external infection. This great potency of epidermis for wound healing originates from stem cells and committed progenitor cells present in this layer (Blanpain et al. 2004; Rhett et al. 2008; De Rosa and De Luca 2012). However, in deep injuries wound healing process undergoes a negative change that remains a chronic wound in which re-epithelialization can only occur in margins of the wound. This may increase the risk for developing excessive scar formation and skin grafts should be used in order to prevent unwanted results. The final stage of wound repair lasting months or years after wound closure and includes modifications in fibrous tissue due to fibroblastic enzymes and functions of fibroblasts to regulate connective tissue integrity by deposition and remodeling of ECM (Diegelmann and Evans 2004; Shevchenko et al. 2009). These four phase end in healing of the wound are shown schematically in Fig. 2.

Scar is the final result of wound healing and can be classified into three main groups including atrophic, hypertrophic, and keloid scars. Atrophic scar described as indented sunken skin area mostly pitted with sharply defined edges. Hypertrophic are swollen, pruritic, and erythematous scars usually seen in burn injuries caused by overproduction of collagen fibers of connective tissue. Keloid scar also called overgrowing scars outgrow beyond the wound boundary and spread to normal skin surrounding the lesion (Shakespeare 2001; Harvey 2005; Bock et al. 2006; Takeo et al. 2015; Vig et al. 2017).

Tissue oxygen level varies in different parts of lesion owning to the diffusion of oxygen from vessels to wound edges. This gradient act as a vital factor required in wound healing which allows fibroblasts to produce collagen fibers in a high-oxygen level microenvironment. At the

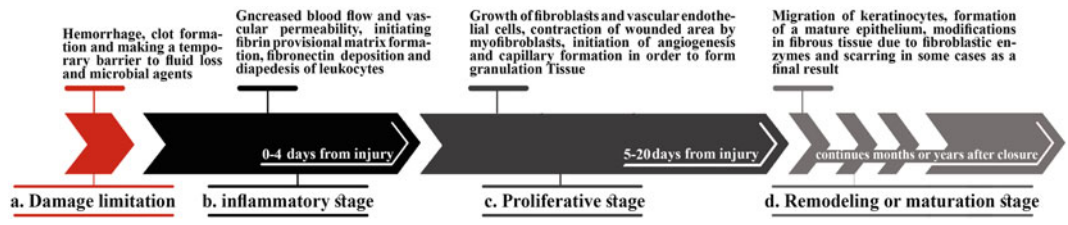

Fig. 2 Four phase of wound healing process include damage limitation, inflammation, proliferation and remodeling or maturation

center of wound, low-oxygen level provide a perfect condition for macrophages to cytokine secretion essential for induction of a variety of reactions such as chemotaxis and proliferation relevant to wound repair (Niccole et al. 1977; Priya et al. 2008).

Determination of injury's depth is of great significance for clinicians to choose the best medical intervention suitable for the patient's situation (Shakespeare 2001). Thanks to the function of a population of epidermal keratinocyte stem cells, epidermal and superficial dermal injuries will be healed with no need for surgical intervention. Without repository of keratinocyte stem cells in epidermis, epithelial stem cells from dermal appendages (hair follicles and sweat glands) will be responsible for wound healing and dermal regeneration (Damanhuri et al. 2011; Biedermann et al. 2013). In patients encounter deep dermal and hypodermal injuries usually there is an urgent need for surgical excision of epidermis and superficial dermis as so to transplant keratinocyte stem cells present in epidermis of donor to the lesion site of the recipient. In this cases epithelium regeneration is limited to edges of the wound because sources of keratinocyte or epithelial stem cells has been wiped out. Even in gold standard intervention which is split thickness skin grafts, scar formation is an unwanted outcome due to the actions of myofibroblasts as critical actors of fibrosis (Enoch et al. 2009; Böttcher-Haberzeth et al. 2010).

Dermal fibroblasts resident at the edges of the lesion could become activated under the stimulation of transforming growth factor β (TGFβ) or its downstream signaling molecules like connective tissue growth factor (CTGF) produced by lymphocytes and macrophages or pathogenic molecular patterns that lead to gain a smooth muscle cell-like phenotype and take part in tissue repair. Additionally, they express α-smooth muscle actin ordered in bundles of microfilaments which confirm their highly contractile nature that is the main cause of wound contraction and irreversible ECM remodeling in scar formation. (Shakespeare 2001; Tomasek et al. 2002; Wynn 2008; Enoch et al. 2009; Micallef et al. 2012). It is noteworthy that scarless embryonic wound healing occur due to the absence of myofibroblasts and action of especial embryonic fibroblast applying tractional forces in order to wound closure (Adzick and Lorenz 1994; Nodder and Martin 1997; Gurtner et al. 2008).

Wound healing proceeds through two different mechanisms including regeneration or fibrosis. Regeneration results in tissue repair with the natural skin structure as it outcome while fibrosis lead to overgrowth of connective tissue, excessive collagen production, dysfunctional skin, and scar formation (Gurtner et al. 2008). Chronic fibrotic diseases have no clinically effective treatment and cause considerable burden in developed countries (Gurtner et al. 2008; Wynn 2008; Hold et al. 2009). In fibrosis, the balance between synthesis and degradation of collagen is shifted to synthesis and end in excessive amounts of collagen, hypercellularity, and fibroblasts patches (Bock et al. 2006). Making a local anti-fibrotic microenvironment could help to reduce myofibroblasts-mediated fibrotic remodeling and improve regeneration of cells (Varkey et al. 2015a, b). In the next section, we will discuss the primarily developed methods for wound repair.

5 Different Methods of Wound Healing

Different approaches are being applied for wound treatment including autologous skin grafting, skin allografts, and xenografts (Dixit et al. 2017a, b; Vig et al. 2017). Autologous skin grafting (either meshed or unmeshed) as a therapeutic approach dating from nineteenth century can be employed in patients with deep dermal and hypodermal injuries, especially in burn wounds where there is a need for keratinocytes to heal the wound. In order to provide qualified cells, the graft must include the full epidermal layer and superficial dermis or STSG as the gold standard for autologous defect dressing. STG is achieved by using dermatome to facilitate skin harvesting from normal skin area of the patient body and transplanted in their lesions. Thickness of dermis layer is determinant for healing quality and can lower the scar formation and unwanted cosmetic

problems. Re-harvesting of donor site to maintain the thickness can be done for a few times (Akan et al. 2003; Janeway et al. 2005; Böttcher-Haberzeth et al. 2013; Vig et al. 2017). From the advent of autologous skin grafting, great developments allow rapid progress in this area which include advancements in instrumentation and processing of autograft e.g. using electric dermatomes for harvesting donor site or mesher processing system to spared excised skin in order to stretched it for covering larger area of the body (Phillips 2001; Boyce and Warden 2002; Shimizu and Kishi 2012). Cultured epithelial autograft (CEA) is a new therapeutic method for severely burned patients and seems a very effective strategy. CEA could be composed of keratinocytes obtained from small area of normal skin biopsy which undergo proliferation in laboratory to make sheets of CEA. Combination of CEA and dermis-like substitute improves healing process efficacy and reduce delayed wound closure (Rheinwald and Green 1975; Lataillade et al. 2017a, b).

Contrary to autologous methods, skin allografts obtained from frozen cadavers in skin banks or living donors are not affected by limitation for donor availability. The use of this alternative strategy dating as far back as the 1503 (Vig et al. 2017). Allografts can establish a functional barrier, stimulate production of growth factors and cytokines, and increase angiogenesis. Allograft doesn't interfere with normal healing process and granulation forming. Allografts can promote inflammation at the lesion site because of the rejection induced by immune system of host and this can be useful for providing a suitable barrier. Autologous skin grafts may be employed in later stages of treatment (Bello et al. 2001a, b; Rockwell et al. 2003; Janeway et al. 2005; Cardinal et al. 2009; Halim et al. 2010a, b; Vig et al. 2017).

Temporary grafts obtained from other species, which can actively encourage dermal regeneration by deposition of animal derived collagen to injured site, are called xenograft. The origin of their applications dates back to the fifteenth century BC. The same as skin allograft these xenogenic grafts provide a temporary cover for human lesions. Porcine and bovine skin analogs considered as the most prevalent xenografts (Shores et al. 2007; Halim et al. 2010a, b; Nathoo et al. 2014a, b; Vig et al. 2017).

5.1 Advantage and Disadvantages

Autologous skin grafts have no risk of immune reaction and rejection duo to the fact that donor and recipient are the same. This method overcome limitations for donor availability and immediate need for sufficient amount of skin graft. But some of its drawbacks are long-term hospitalization, terrible pain, availability of donor site in wide surface area injuries, and increased risk of infection (Jeschke et al. 2004; Cardinal et al. 2009; Halim et al. 2010a, b). Allografts and xenografts solved the problem of limited donor site while risk of inflammation at the wound site, immune rejection, and viral transmission remains as the biggest disadvantages of these strategies (Rockwell et al. 2003; Cardinal et al. 2009). Allografts and xenografts are a good option for using as a temporary barrier, but they can't be long-lasting due to host immunogenic rejection. Furthermore, there are a limited number of tissue banks supplying clinically approved allograft (Janeway Jr et al. 2001; Rockwell et al. 2003; Cardinal et al. 2009; Catalano et al. 2013). Although CEA seems a more beneficial strategy, there are some downsides such as long term preparation time, fragility, and low success rate that hamper its progress to wide clinical applications (Bargues et al. 2011; Vig et al. 2017). According to aforementioned limitations it was essential to develop a skin graft which overcome obstacles associated with a successful transplantation technique.

6 Clinical Demands for Engineered Skin Substitutes

Skin disintegration can lead to adverse effects in patients. Dehydration, necrosis, increased wound depth, and shock are some serious consequences of direct fluid losses through the surface of injury. Microbial infections and necrosis of cells can be

seen by progression of injury. Sepsis or systemic inflammation can result from recipient immune system response to infectious agents (Sheridan 2009). Excessive plasma protein loss, mainly albumin, induce low colloid oncotic pressure and edema that can disrupt normal functions of lung and gut. Due to these points, there is an urgent need for mediate wound closure, especially in the case of full-thickness injuries (Herndon and Parks 1986; Herndon et al. 1989; Sheridan 2009; Biedermann et al. 2013). Our body itself is not capable of wound closure of deep injuries or large burns without surgical interventions. The best method to treat these sever lesions would be autografts, if could provide large surface area (Sheridan 2009).

In full-thickness skin wound, treatment starts with the immediate excision of damaged tissue to prevent inflammation, infection, and scar formation (Trottier et al. 2008). In sever lesions this contains two stages: the first step is applying a material to provide a dermis-like structure that should be vascularized and the second one is placing an epidermal material on top of it which can be skin grafts or tissue-engineered skin equivalents. However, the most common method in wound healing is autologous skin graft which gives the patient a permanent barrier (Burke et al. 1981; Herndon and Parks 1986; Herndon et al. 1989; Pouliot et al. 2002; Larouche et al. 2016). As mentioned in former section, autografts are not able to supply large skin surface area and allograft could be a suitable alternative in this situation (Vermette et al. 2007; Gómez et al. 2011). Despite generally positive results, due to scar formation their clinical outcomes are not wholly satisfactory in all cases (Munster et al. 1990; Debels et al. 2015). Furthermore, these techniques require surgical skill, large amount of high-value care, and numbers of experienced resources to provide high quality care for patients, especially who suffering from chronic wounds (Shakespeare 2001).

Keeping the chronic wounds sterile and moist is an important point which could be achieved by topical wound dressings like povidone-iodine solutions or cotton gauze dressings. But this conventional method may not be efficient in elderly patients or patients that have comorbidities such as diabetes mellitus or dysfunctional venous valves leading to hypoxia and wound bed (Nicholas and Yeung 2017a, b). There are some alternative treatments such as negative-pressure wound therapy using a vacuum dressing, hyperbaric oxygen therapy by inhaling oxygen under pressure, electrical stimulation, and shock wave therapy via audible, low-energy sound waves (Valencia et al. 2001; Bilker 2002; Gürsoy et al. 2014; Farsaei et al. 2015; Frykberg and Banks 2015). All kinds of these methods require the patient's own cells to proliferate and wound repair. Therapy resistant ulcers are another example for chronic wounds which require at least 12 weeks to be treated under standard treatments and impose a large economic burden on the health care organizations (Langer and Rogowski 2009; Campbell and Parish 2010; González-Consuegra and Verdú 2011).

Low success rate of conventional methods of wound management necessitate the need for manufacturing of skin replacements including a layer of keratinocytes seeded on a biocompatible carrier. This can assist to make a microenvironment suitable for both fibroblast and epithelial cells to repair the wound and reduce unwanted outcomes of aforementioned methods (Shakespeare 2001; Langer and Rogowski 2009). A multidisciplinary field called tissue engineering has emerged by cooperation between biomedical and biomaterial engineers, cell and molecular scientists, and physician to develop viable and advanced medical devices to restore normal functions of damaged tissue. With the aid of this interdisciplinary field, a great deal of bioengineered skin substitutes have been innovated to apply as a proper dressing over the injured site to heal treatment resistant wounds which could be equal or even more effective than conventional wound healing methods (Langer and Rogowski 2009; Debels et al. 2015).

7 Development of Skin Substitutes

The first use of skin grafts dates back to India at 2500 BC when they were used to treat heavily injured limbs and this method was forgotten until the European Renaissance. (Chick 1988) Reverdin used small autologous epidermal grafts for the first time in spite of scar formation in the nineteenth century (Horch et al. 2005). Thiersch's grafts, however, contained some dermis. Following that, STSG became more common due to improvements in skin harvesting instruments in the 1930s (Herndon 2007). Until today, numerous skin grafting techniques have been invented and successfully used (Lee 2000; Alrubaiy and Al-Rubaiy 2009).

The use of allograft skin as an easier method of skin transplantation in comparison with autologous grafting was emerged from 1870s. Before that, skin xenografts were developed in 1804 (Girdner 1881; Gallico 3rd and O'connor 1985). These reports revealed that the nineteenth century considered as a turning point in conventional skin grafting technology. interestingly, during World War II and even before, skin allografts were frequently used to heal injured soldiers (Brown and McDowell 1942).

Nowadays human skin has got many clinical uses. Also, skin is processed by skin banks. The American Association of Tissue Banks (AATB) and the US Food and Drug Administration (FDA) are responsible for regulations and standardize of skin processing and banking (Pearson et al. 2008). An increase in the availability of cadaver allografts and a remarkable decrease in infectious disease transmission was seen under strict control of these organizations (Saffle 2009).

In spite of clinical uses of skin grafts, they may not be available to replace an extensive skin loss. They may cause pain, scarring, slow healing and infection and an allogenic graft may be rejected (Lee 2000; Horch et al. 2005). In order to solve these problems, scientist were motivated to look for synthetic skin grafts (Alrubaiy and Al-Rubaiy 2009). These kinds of skin substitutes were first described by Joseph Gamgee in 1880 (Ho 2002). His skin substitute was made of cotton wool covered with layers of gauze on each side. 15 years later, Mangoldt invented "epithelial cell seeding", in which epithelial cells were obtained from superficial epithelium and were transplanted on to chronic wounds (Horch et al. 2005).

In 1975, Rheinwald grew human keratinocytes on cytoplasmic fluid of murine fibroblasts. Then O'Conner and his colleagues where the first team to report autologous cultured epithelial cells in order to treat burned patients in 1981 (Leigh and Watt 1994). CEAs and bioengineered skin substitutes started to become useful in wound healing in 1970s (O'Connor et al. 1981).

These efforts led to successful engineering of skin dermal analogs composed of type I collagen seeded by fibroblasts. When an epidermal substitute is used in combination with a dermal layer, the manufactured equivalent is called composite substitutes which are suitable alternatives for healing deeper wounds. These composite skin substitutes have emerged since 1990s (Boyce et al. 1995). Langer and Vacanti described tissue engineering in 1993 and finally in 2005 genetically modified substitutes were introduced (Herndon and Parks 1986; Langer and Vacanti 1993; Damanhuri et al. 2011).

In spite of major improvements and the use of several skin equivalents, we have not yet been able to regenerate all features of human skin (Damanhuri et al. 2011). Some new methods and materials used in tissue engineering to regenerate wounds with the minimal side effects will be discussed in following sections:

7.1 Skin Substitutes' Characteristics

Skin substitutes were developed as an effective therapy to reduce complications associated with conventional skin transplantation. In order to fulfill this goal, skin equivalents should meet some essential criteria to be considered as an optimal skin substitute (Nicholas et al. 2016a, b). Some important characteristics of skin substitutes are mentioned below.

7.1.1 Protective Functions

As the most fundamental attribute, a sterile skin substitute must be capable of protecting wound area from infectious agents, especially in burns and chronic wounds (Laurie et al. 2010; Siddiqui and Bernstein 2010; Mann et al. 2012). It should also prevent both fluid loss and accumulation by transmitting water in a way similar to normal skin (Woodroof 2009; Namdar et al. 2010; Damanhuri et al. 2011). Skin substitutes act as temporary or permanent which show supportive and mechanical strength and are made to bear mechanical tensions and sheer forces (Damanhuri et al. 2011; Biedermann et al. 2013; Dixit et al. 2017a, b).

7.1.2 Suitable Surface for Adherence and Biological Functions

Skin substitutes must provide an ideal environment for cell proliferation, differentiation, migration, and proper functions according to their porosity and morphological features (Wang et al. 2005). They should also support angiogenesis (Vig et al. 2017). Moreover, to be a protective dressing their rapid adhesion to the wound surface is also an important feature essential for cell differentiation (Jensen and Wheelock 1996; MacNeil 2007).

7.1.3 Minimal Adverse Reactions

The inflammatory response of immune system following skin transplantation may lead to fibrosis and scarring which could end in graft rejection. Immune mediated graft rejection has not been seen in autografts while is prevalent in allogeneic and xenogeneic tissue grafts (Varkey et al. 2015a, b; Nicholas et al. 2016a, b). Synthetic skin grafts may induce different degrees of immune reaction according to their toxicity and immune effects. Obviously, lack of antigenicity, toxicity, immunogenicity, and the minimal risk of disease transmission make a skin equivalent more suitable for clinical applications (MacNeil 2007; Damanhuri et al. 2011; Vig et al. 2017). Therefore, scientists endeavor to manufacture safe engineered skin substitutes in a way that have long-term survival (Dixit et al. 2017a, b).

7.1.4 Pliable and Clinician Friendly

To be easily used even on irregular wound surfaces such as knees, hips, and hands, skin constructs must be durable, malleable, and flexible. They should be easy to handle and comfortable for dressing the lesions and not to be fragile (Damanhuri et al. 2011; Varkey et al. 2015a, b; Nicholas et al. 2016a, b; Vig et al. 2017).

7.1.5 Stable and Biodegradable

Skin substitutes should eventually be replaced with the patient's skin, so they should be biocompatible and biodegradable with well-controlled degradation rate. They not only should not interfere dermis vascularization but also maintain their structure until complete vascularization that usually take a week (MacNeil 2007; Biedermann et al. 2013; Nicholas et al. 2016a, b; Vig et al. 2017).

7.1.6 Long Term Storage and Cost-effectiveness

As healthcare costs are rising, cheaper than today's life-saving treatments such as skin substitutes should become available for patients in need of skin grafts (Yildirimer et al. 2012). These substitutes should have long shelf life, be easy to store and widely available (Damanhuri et al. 2011; Varkey et al. 2015a, b; Vig et al. 2017).

7.2 Skin Substitutes' Composition

The main components of skin substitutes include scaffolds, growth factors, and cells as depicted in Fig. 3 (Nicholas et al. 2016a, b). Each of these components with their varieties are discussed in details in following sections.

7.2.1 Scaffold

Scaffolds are 3D ECM analogs which contribute to cell adhesion, proliferation, and differentiation and are also compatible with neovascularization process essential for keeping dermal and epidermal cells alive. Different polymers used as scaffolds give each one specific physical and

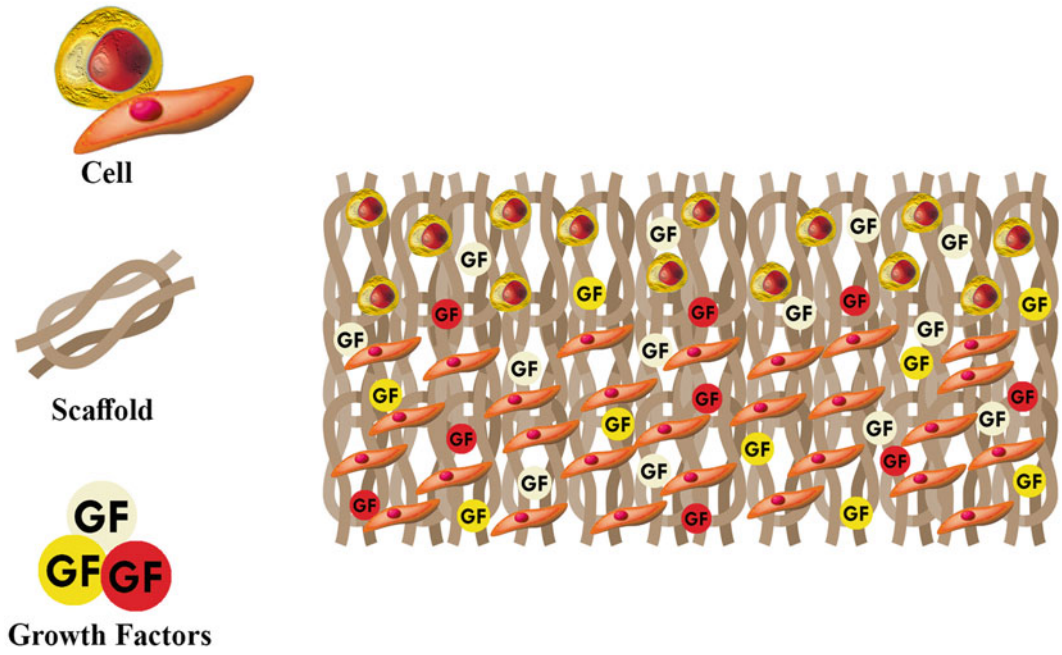

Fig. 3 Three main component of a tissue engineered skin substitutes are cells, scaffolds, and growth factors. Desired cells which are mostly epidermal keratinocytes or dermal fibroblasts. Scaffolds that may be natural, synthetic or their combinations. Different types of growth factors such as FGFs, EGF, VEGF, and TGF β

chemical features (Nicholas et al. 2016a, b). Three main types of biomaterial including natural, synthetic, and composite (the combination of natural and synthetic) which used as scaffolds in skin substitute manufacturing are discussed below.

7.2.1.1 Natural Biomaterials

Due to the fact that scaffolds are intended to have similar characteristics as natural ECM in tissue engineering, structural and functional ECM components such as collagen, gelatin, elastin, and HA are common biocompatible material suitable applying as scaffolds in skin tissue engineering (Sheikholeslam et al. 2017). We focus on some of the most commonly used natural biomaterials containing collagen, chitosan, HA, gelatin, fibronectin, fibrin, elastin, pullulan, alginate, and laminin.

Collagen

Collagen with a triple helix structure made of the repetitive amino acid sequences is one of the most important proteins in ECM that plays a fundamental role in healing process and is considered as the main source of skin's tensile strength (Cen et al. 2008; Glowacki and Mizuno 2008; Groeber et al. 2011a, b). It is the most frequent biomaterial used in skin equivalents manufacturing which 29 different types of it has been found until now (Larouche et al. 2016; Nicholas et al. 2016a, b). Collagen is formed by three polypeptide fibers of about 1000 amino acids length cross-linked to each other (Sheikholeslam et al. 2017). Collagens can give various characteristics to skin analogs according to their species origin and relevant tissues. Most common sources of collagens for skin substitutes are bovine skin and tendons, porcine skin, intestine, or bladder mucosa, and rat tail (Badylak 2004; Chattopadhyay and Raines 2014). Because of the risk of disease transmission, there is a tendency towards producing synthetic collagen (Olsen et al. 2003). Collagen form approximately 70% of dermal matrix dry weight which mainly composed of type I collagen (Metcalfe and Ferguson 2007; Annabi et al. 2010). Other types of dermal collagen include collagen types III and

V (Cen et al. 2008). Among a wide variety of collagen types, type I and III are key players of dermis and types IV and VII are frequently found in basement membrane (Tsuji and Sawabe 1987; Price et al. 2007; Rnjak et al. 2009).

Owning to leading role of type I collagen in dermis, it is by far the most abundant biomaterial to produce collagen-based scaffolds which could be used in combination with types III and V collagen (Nicholas et al. 2016a, b). In spite of salient features of collagen that made it the most prevalent biomaterial suitable for tissue engineering, including elegant structural motifs, high degree of biocompatibility, biodegradability, its ability to improve adhesion, proliferation, and migration, its mechanical strength is weaker than a normal skin (Lee et al. 2001; O'brien 2011). To solve this problem collagen fiber should be cross-linked with other types of biomaterials such as chitosan, GAGs, HA, fibrin, gelatin, elastin, pullulan, alginate, laminin, poly-L-lactic acid (PLLA), poly glycolide-co-L-lactide (PLGA), polyethylene glycol (PEG), and poly-ε-caprolactone (PCL) (Nicholas et al. 2016a, b).

Chitosan

Chitosan is a chitin-derived linear polysaccharide with amino and hydroxyl groups and is thought to help wound healing process in hydrogel form (Han et al. 2010; Sarkar et al. 2013; Nicholas et al. 2016a, b; Rahmani Del Bakhshayesh et al. 2017). The main sources of chitosan are different crustacean shell such as shrimp and crab, squid bone plates, and fungal cell walls (Kumar et al. 2004a). It is easily metabolized and used to growth factor delivery in a controlled manner (Koide 1998; Biedermann et al. 2013). Contrary to other natural polysaccharides, chitosan is a positively charged biopolymer due to its amino groups that allow interaction with negatively charged GAGs and red blood cell (Mao et al. 2003b; Croisier and Jérôme 2013).

Its beneficial features including mild water absorption and gel formation capacity, ideal tissue adhesiveness, increased blood coagulation, antimicrobial properties, and excellent pain relief (Koide 1998; Okamoto et al. 2003; Hayashi et al. 2012; Croisier and Jérôme 2013). But its long-term stability is problematic which could be increased and decreased by cross-linking and incubating with lysozyme solution, respectively (Croisier and Jérôme 2013; Hilmi et al. 2013). Moreover, chitosan is poorly soluble in aqueous solutions except acidic ones and to optimize its characteristics it is mostly used in combination with other biopolymers and not in a pure form (Spasova et al. 2008).

Hyaluronic Acid (HA)

HA is a linear polymer made of glucuronic acid and *N*-acetylglucosamine (Zacchi et al. 1998). It is a kind of nonsulfated negatively charged GAG able to interact with other positively charged polymers like poly-l-lysine and chitosan (Mao et al. 2003a; Khademhosseini et al. 2004). This negative charge also makes the scaffold more hydrophilic and gives the ability to receive cells and promote cell proliferation rate, especially in case of fibroblasts and keratinocytes along with angiogenesis and vessel growth. It can be easily modified by esterification to become even more hydrophilic (Zacchi et al. 1998; Price et al. 2005a, b).

Special features of HA as an appropriate biomaterial for skin tissue engineering contain HA easy production process, biodegradability, free radical scavenger, nonimmunogenic and nonadhesive properties. It induces early inflammation necessary for the beginning of healing process (Edmonds et al. 2000; Biedermann et al. 2013). Same as chitosan, HA is used in combination with other biomaterials in making mono and bilayer scaffolds (Ghosh et al. 2006; Monteiro et al. 2015). For instance, in comparison with pure collagen scaffolds its combination with collagen shows improved cell migration and division (Nimni et al. 1987).

Gelatin

Gelatin is formed by collagen denaturation (Kozlov and Burdygina 1983; Choi et al. 1999). It has got a collagen-like structure with repetitive three amino acids. Gelatin's Arg-Gly-Asp (RGD) motifs enhance its interaction with cells through

integrin receptors in cell membranes. Its lysine and arginine residues give gelatin the ability to attach to cell membrane which is negatively charged (Lee et al. 2003; Su and Wang 2015). In comparison with collagen it could be used as dressing for wounds with high infectious risk due to its lower antigenicity (Choi et al. 1999; Sheikholeslam et al. 2017). In comparison with other materials, its higher solubility and lower cost have made it a cost-effective candidate combined with other materials in mono and bilayer skin analogs intended for dermal or epidermal regeneration (Lee et al. 2005; Dainiak et al. 2010; Shevchenko et al. 2014). The same as chitosan, it shows the long-term growth factors release in a controlled manner (Nicholas et al. 2016a, b). Gelatin exhibits different types of conformational structures depend on temperature, solvent or pH, and could be used for continuous release of growth factors (Takemoto et al. 2008). Although it is capable of a large quantities of water absorbance leading to supply a suitable microenvironment for cell migration, attachment, proliferation and angiogenesis, it may decrease fibroblast migration unless it used in combination with other polymers (Suzuki et al. 1990; Boyce 2001; Nicholas et al. 2016a, b).

Fibronectin and Fibrin

Thrombin can rapidly polymerize fibrinogen to form fibrin (Ahmed et al. 2008). Fibrin is a key player in hemostasis after vascular damage. Moreover, it serves as a scaffold to promote keratinocyte, neutrophil, macrophage, and fibroblast migration to start wound healing process (Weigel et al. 1986; Marino et al. 2014; Kober et al. 2015). Moreover, it could interact with fibronectin (Han et al. 2010). Fibrin has a high affinity for proteins, so it can bind to various growth factors to enhance angiogenesis in mono and bilayer scaffolds, in addition to cell adhesion through integrin incorporation (Maheshwari et al. 2000). The ability of fibrin to induce stratified epithelium production is shown in an experiment by adding human dermal fibroblasts and keratinocytes to it (Meana et al. 1998). In addition, its modification with covalent bonds can gives it new features (Bell et al. 1981). Fibrin use is easier than fibronectin in the form of glue (Han et al. 2010). Fibrin has been used as a scaffold for both stem and primary cells in different types of tissues such as bone, ligament, tendons, liver, cardiac tissue, cartilage, nervous system, and skin (Edmonds et al. 2009). It also is considered as an important autologous pool for crucial growth factors involved in wound repair process (Mahboob Morshed et al. 2014).

Fibronectin is another important glycoprotein of skin ECM that is derived from human or bovine plasma in its soluble form. It has got a high molecular weight and is able to bind collagen, fibrin, and heparin (Barber et al. 2008). Same as fibrin, it has positive effects on cell adhesion, proliferation, and contraction involved in wound healing. Fibronectin not only increases growth factors vailability, but also increases their expression levels and can form specific mats that are used in neural regeneration (Waymack et al. 2000; Boyce et al. 2002).

Elastin

Elastin is one of the fundamental proteins of connective tissue, responsible for skin elasticity. Tropoelastin is a soluble monomer fiber which by cross-linking together form hydrophobic insoluble elastin (Almine et al. 2010). Elastin production after severe wound lasts as long as 4–5 years, and may lead to its functional and structural defects (Jones et al. 2002; Min et al. 2014). Due to the fact that collagen based scaffolds suffering from lack of proper elasticity, combined with collagen it could reduce wound contraction and make the substitute more elastic similar to normal skin (Lamme et al. 1996; Lamme et al. 1998; Rnjak et al. 2011). It has been demonstrated that elastin based scaffolds reduces scar formation and promotes skin regeneration (Lamme et al. 1996; Lamme et al. 1998; Klein et al. 2001; Rnjak et al. 2011; Attia-Vigneau et al. 2014). However, because of poor mechanical strength and availability, it should be used in combination with other biomaterials (Sheikholeslam et al. 2017).

Pullulan

Pullulan produced by a fungus called *Aureobasidium* is a linear polysaccharide mainly

used in dermal injuries because of its anti-inflammatory properties (Sheikholeslam et al. 2017). Pullulan is a natural inexpensive antioxidant, which is biodegradable and nontoxic (Wong et al. 2011; Nicholas et al. 2016a, b). It is usually used in combination with collagen or gelatin scaffolds to promote their cell interactions and skin regeneration (Wong et al. 2011, Nicholas et al. 2016a, b). Moreover, its unique anti-inflammatory properties is the main cause of infection and graft rejection prevention (Wong et al. 2010). Composites of pullulan–collagen scaffolds are available for better dermal skin regeneration (Wong et al. 2010). This polymer needs more investigations to discover its exact effects on pullulan-based skin analogs (Nicholas et al. 2016a, b; Nicholas and Yeung 2017a, b).

Alginate

Alginate is a biodegradable, biocompatible, negatively charged, nontoxic natural polysaccharide isolated from brown algae cell walls (Haslik et al. 2007; Lee and Mooney 2012). It is also used with collagen to enhance its structural features (Gaspar et al. 2011). Alginate is shown to have noticeable features like adequate water absorptivity and moisture vapor transmission rate, hemostatic capability, and effective germicidal characteristics which some of these traits could be further improved if be combined with chitosan (Cooper et al. 1991). Due to their advantages, alginate-based scaffolds are widely used in skin tissue engineering, while because of their inhibitory effects on type I collagen deposition, its application is more limited than expected (Smith et al. 2012; Yuvarani et al. 2012; Khan and Ahmad 2013).

Laminin

Laminin is an abundant protein in basement membrane that induces keratinocyte confluence and epidermis formation, improved cell adhesion, migration, and proliferation in scaffolds, if used with collagen (Rho et al. 2006; Masuda et al. 2009; Behrens et al. 2012; Damodaran et al. 2013). As a layer between dermis and epidermis layers, laminin can make a scaffold much more similar to that of seen in natural human skin due to supplying proper conditions for basement membformation (Halim et al. 2010a, b).

7.2.1.2 Synthetic Materials

Hydrocarbons are the components of synthetic biomaterials. they Surely do not show the biological characteristics of natural biopolymers, but their controllable composition and easier production process highlight them as beneficial polymers for clinical applications in wound healing procedures (Sheikholeslam et al. 2017). Some of common synthetic biomaterials are discussed below.

Polyhydroxyortho Esters (POE)

PLA, polyglycolic acid (PGA), and PLGA are subgroups of POE which are hydrogels used in tissue engineering especially for cartilage, bone, ligament, and skin substitutes (Chen et al. 2002; Moran et al. 2003). They are biodegradable but not natural like epidermis due to lower stiffness than normal epidermis and may produce acidic components cause protein damage as a consequence of decreased PH in their microenvironment (Li et al. 2012). When POE expose to aqueous media, they show rapid degeneration and negative impact on the surrounding environment. To minimize these drawbacks, it is suggested that PLA and PGA be used in combinatorial constructs called copolymers and not separately (Rahmani Del Bakhshayesh et al. 2017). These 3D polymers in combination with natural polymers like collagen are one of the most widely used strategy in skin substitutes engineering (Cheng and Lee 2009).

Polylactic Acid (PLA)

PLA is a biodegradable synthetic polyester with lactic acid as its degradation metabolic byproduct. It is a suitable material for extensive application for medical needs like tissue engineering and drug delivery systems (Santoro et al. 2016). It has got L and D isomers and owning to this trait it could be used to produce different types of materials such as crystalline poly-D-lactic acid (PDLA), hemicrystalline PLLA, amorphous poly-DL-lactic acid (PDLLA), and *meso*-PLA. Moreover, some other materials like poly-

4-hydroxybutyrate copropertiuld could be added to this polymer in order to optimize properties required for an efficient synthetic scaffold (Khalilov et al. 1993). PLA as an aliphatic fiber is not able to interact well with cells, slowly degenerates and produces acidic byproducts that may give rise to an inflammation (Cui et al. 2009). Due to the fact that its hydrophobicity leads to lack of proper cell attachments, PLA could be modified to demonstrate improved adhesion to cell (Cui et al. 2009; Kim et al. 2009a). This polymer can be used to transport cells to the target area (Cui et al. 2009, Kim et al. 2009a). It is better to use PLA in combination with other polymers or modify it in order to improve its function for its clinical applications (Sheikholeslam et al. 2017).

Poly Lactic-co-Glycolic Acid (PLGA)

PLGA is a biodegradable amorphous polyester made of lactic and glycolic acid. PLGA degradation by products are lactic acid and glycolic acid due to its ester chains hydrolysis (Sadeghi-Avalshahr 2017). Although, because of similarity to PLA it may also be proinflammatory, this polyesters shown to have a minimal inflammatory response in the body and is widely used as scaffold for skin regeneration (Duan et al. 2006; Sadeghi-Avalshahr 2017). Scientists attempt to make looser fibers with improved porosity to fulfil ideal conditions for PLGA as a well-functioned synthetic scaffold including enhance cell viability, migration, infiltration, and even collagen deposition (Zhu et al. 2008; Kim et al. 2010). The lactic acid: glycolic acid ratio has s leading role in hydrophobicity and degradation rate of PLGA that is a noteworthy factor in PLGA fabrication (Sadeghi-Avalshahr 2017). After all, like the majority of materials, it is not recommended to apply pure PLGA and the addition of other materials is required to improve its physical and mechanical characteristics (Sheikholeslam et al. 2017).

Polyethylene Glycol (PEG)

The same as most synthetic polymers, PEG, also known as polyethylene oxide (PEO) exhibits easily controllable structural and compositional properties but cannot effectively interact with cells and provide optimal condition for tissue regeneration (Zhu 2010a, b). Good biocompatibility, low rate of immunogenicity, and water absorption made PEG an interesting hydrophilic polymers in aqueous environments for 3D scaffold engineering (Alcantar et al. 2000). To improve PEG qualifications for clinical use, ECM peptide motifs could be added to its structure or be mixed with other ECM materials (Sheikholeslam et al. 2017). It can reduce cell death by stabilizing cell membrane after injury and also accelerates fibroblast growth (Mann et al. 2001; Vahidi et al. 2017). PEG is one of the most extensively used material for medical applications.

Poly-ε-caprolactone (PCL)

PCL is a relatively inexpensive, extremely elastic with low rate of toxicicity polyester which is mechanically suitable for skin substitutes. Compared to PLA and PLGA, it degenerates very slowly (Ng et al. 2001; Zeng et al. 2004). PCL contributes to faster wound closure when is radially fabricated and seeded with fibroblasts which leads to promoted cell migration along the fibers (Xie et al. 2010). PCL can also reduce wound contraction because of the slow degradation rate. It has been shown that degradable poly l-lactide*co*-ε-capro-lactone (PLCL) porous copolymer leads to decreased myofibroblast formation and wound contraction (Lorden et al. 2015). PCL is not an inert polymer and its potential to interact with human mesenchymal stem cells (MSCs) in cell-based multiphasic tissue substitutes has been proved (Li et al. 2005). Moreover, PCL combined with type I collagen supply a suitable environment for fibroblasts proliferation, that focus on combinatory use of polymers to enhance their function as a 3D scaffold (Bonvallet et al. 2015).

Poly-β-Hydroxybutyrate (PHB)

PHB the main component of the crystalline cytoplasmic granules in many bacteria, is a biodegradable and biocompatible homopolymer of (R)-b-hydroxybutiric acid. It has been used as

a supportive and protective scaffold which induces cell growth and faster wound healing process (Müller and Seebach 1993; Ljungberg et al. 1999; Philip et al. 2007).

Poly Vinyl Alcohol (PVA)

PVA is another polymer which is non-toxic, biocompatible, and biodegradable. Therefore, PVA suggested as an applicable polymer for tissue engineering (Chen et al. 1994; Allen et al. 2004). PVA and PHB are used in skin tissue engineering in the form of nanofibers (Asran et al. 2010). PVA nanofiber fabricated in combination with other material such as chitosan, PCL, and gelatin for using as skin equivalents (Gholipour-Kanani et al. 2014; Choi et al. 2015).

Polyurethane (PU)

PUs are a large number of medically favorable polymers. They are utilized in commercially available PU-based skin substitutes seeded with keratinocyte for regeneration of full thickness burns and are commonly used as wound dressing fabrications (Wright et al. 1998; Khil et al. 2003; Kim et al. 2009b). When PU is fabricated with natural biopolymers leads to improved mechanical strength of manufactured copolymer (Sheikholeslam et al. 2017).

Self-Assembling Peptides (SAPs)

With the aid of nanotechnology, specific nanobiomaterials are designed to respond to changes in pH, temperature, light, or ionic concentration and due to self-complementary peptides. These factors play significant roles in self-assembly and disassembly of synthesized SAPs. SAPs contain amino acids and could be employed in 3D scaffolds used for tissue engineering and drug-release applications (Schneider et al. 2008; Kyle et al. 2009; Wu et al. 2012; Bradshaw et al. 2014). However, SAPs are mechanically weak which this trait considered as a limiting factor in their widespread clinical use as skin substitute (Sheikholeslam et al. 2017).

7.2.1.3 Combination of Natural and Synthetic (Composite) Scaffolds

As it was discussed above, mechanical strength of synthetic polymers can be combined with the inherent biocompatibility of natural ones in order to make an ideal skin substitutes mimicking the structural and functional properties of human normal skin (Sheikholeslam et al. 2017). To this end, given the complexity involved, a wide variety of material combinations has been designed in order to optimal biodegradation rate, pore size, molecular weight, hydrophobicity, mechanical strength, stiffness, growth factor release, anti-inflammatory property, and some other determinants necessary for an ideal tissue engineered skin scaffold. Some famous composite scaffolds are PLLA–collagen, Poly ethylene oxide–chitosan, Carboxyethyl chitosan/PVA, Chitosan/collagen/PEO, and PCL–collagen (Rahmani Del Bakhshayesh et al. 2017). From a material standpoint, composites constructs composed of natural and synthetic materials are the most efficient one for 3D scaffold engineering.

7.2.2 Growth Factors

Using growth factors can trigger some essential agents in healing processes such as cell migration and neovascularization, and hinder some harmful ones like fibrosis. So, growth factors like TGF-α/TGFβ, interleukin-1 (IL-1), interleukin-6 (IL-6), and interleukin-8 (IL-8) help creating efficient wound regeneration and producing functionally advanced skin substitutes (Nicholas et al. 2016a, b). Among several growth factors required for skin repair we focus on some important ones.

7.2.2.1 Fibroblast Growth Factor (FGF)

FGFs contribute to wound healing by enhancing angiogenesis, cell migration, and proliferation. In particular, fibroblast growth factor-2 (FGF-2) or basic FGF promotes neovascularization and thickens both dermis and epidermis. It also prevents wound contraction by inhibiting α-smooth muscle actin filaments and lead to reduced fibrosis via preventing fibroblast differentiation into myofibroblasts (Spyrou and Naylor 2002; Akasaka et al. 2007; Inoue et al. 2009).

7.2.2.2 Epidermal Growth Factor (EGF)

EGF mainly affects keratinocytes and fibroblasts and increases their migration and proliferation. Moreover, it promotes angiogenesis and epithelization and could trigger growth factor secretion produced by fibroblasts thus end in wound healing acceleration (Bodnar 2013; Yamamoto et al. 2013; Kuroyanagi et al. 2014).

7.2.2.3 Transforming Growth Factor-β (TGF-β)

TGF-βs are critical growth factors needed for proper wound healing. Three major types are TGF-β1, TGF-β2, and TGF-β3. It has been demonstrated that embryonic wounds are able to scarless wound repair which is attributed to higher expression levels of TGF-β3 and lower levels of TGFβ1 and TGF-β2. This pattern was revealed promising results for scarless wound healing in animal models (Ferguson and O'Kane 2004). TGF-β1 along with CTGF and other autocrine factors can induce myofibroblast differentiation from mesenchymal, epithelial, endothelial cells, or fibrocytes. Myofibroblast formation eventually leads to scar formation and fibrosis. Therefore, lower levels of TGF-β1 is suggested to prevent myofibroblast production and regeneration induction (Varkey et al. 2015a, b).

7.2.2.4 Vascular Endothelial Growth Factor (VEGF)

VEGF is the main growth factor causing angiogenesis. But it should be noted that, its high concentration is potentially associated with several different types of cancers, microvascular defects in patients suffering diabetes, fibrosis, and scar formation (Wilgus et al. 2008; Biselli-Chicote et al. 2012).

7.2.2.5 Platelet-derived Growth Factor (PDGF)

Owning to the fact that PDGFs are key players in scarless wound healing, should be precisely control to reduce scar formation. PDGFs are initial factors released after injury and mediate fibrosis by stimulating fibroblasts to dermal protein deposition which secrete growth factors, induce dermal matrix and contribute to wound healing (Borena et al. 2015; Nicholas et al. 2016a, b). Hypertrophic scar and keloid formation could be potential consequences of excessive amount of PDGFs (Niessen et al. 2001; Kim et al. 2014).

7.2.3 Cells

Each layer of human skin include the epidermis, dermis, and hypodermis contain different cell types like keratinocytes, melanocytes, fibroblasts, endothelial cells, Langerhans cells (LCs), Merkel cells, and adipocytes that are responsible for production of ECM and a variety of growth factors. All of them are pivotal elements for the skin normal function, but not critical for making a skin substitute. Adding cells, however, increases the complexity of final product and add specific features to it, depending on the cell type used (Nolte et al. 2008; Biedermann et al. 2013). Some important cell types of human skin are mentioned in this part.

7.2.3.1 Fibroblasts

Fibroblasts are the main cells in skin dermal layer that have principal roles in wound healing process. They are responsible for a variety of functions such as secretion of ECM compounds, specially collagen and fibronectin, in addition to remodeling enzymes like proteases and collagenases (Pasparakis et al. 2014). Two main kinds of fibroblasts are present in dermis layer kwon as the papillary or superficial fibroblast resident in the basal epidermal layer and the deeper one called reticular fibroblasts (Dixit et al. 2017a, b). ECM affects cells morphology and function and is the main cause of skin integrity maintenance. In addition to ECM impacts on fibroblast migration and attachment, fibroblast proliferation and differentiation, is promoted by ECM 3D structure. Moreover, the production of growth factors and ECM proteins are affected by skin matrix architecture (Sethi et al. 2002; Clark et al. 2004; Nolte et al. 2008).

To ensure successful grafting, angiogenesis must occur as soon as possible. Fibroblasts can accelerate this process by releasing pro-angiogenic agents and stimulating endothelial cells production as observed in *in vitro*

models (Shamis et al. 2013). This can be a possible benefit of using fibroblasts in skin substitutes manufacturing. Fibroblasts are also capable of starting early inflammation, necessary for the healing process. However, this inflammation may become chronic and cause myofibroblast production which as mentioned before are the bona fide reason for fibrosis (Kendall and Feghali-Bostwick 2014).

Other than the dermal layer that is mainly affected by reticular fibroblasts, papillary fibroblasts next to the basal epidermal layer can influence the epidermis, by controlling keratinocyte migration and proliferation with keratinocyte growth factor (KGF) (Nolte et al. 2008; Dixit et al. 2017a, b). Thus, it is crystal clear that fibroblasts are the key players of normal skin foundation.

7.2.3.2 Keratinocytes

Keratinocytes are most important cells in the epithelial layer and epithelialization process. At first, they lose their adhesion with other cells and start to migrate and adhere to the injured area, that is called epithelial mesenchymal transition (Lamouille et al. 2014). Then, due to their proliferation, the basal layer of epidermis is formed and their differentiation lead to stratification of suprabasal layers named spinous, granular, and corneum (Pastar et al. 2014). Stratum corneum, possess a large quantity of keratinocyte tight and adherens junctions, responsible for skin barrier function that limits material transport into the epidermis (Niessen 2007). Fibroblasts contribute to angiogenesis by producing growth factors leading to keratinocyte proliferation. Following that, keratinocytes secrete pro-angiogenic growth factors including VEGF and PDGF which stimulate angiogenesis (Pastar et al. 2014).

7.2.3.3 Melanocytes

Melanin producing cells called melanocytes that are responsible for skin pigmentation are located in the basal layer of the epidermis. One of the challenging issue in skin equivalents production is the hypopigmentation and uneven skin color following skin grafting (Nicoletti et al. 2015). Using these cells in skin equivalents may be able to restore the normal skin color in wound area (Hachiya et al. 2005). Melanocytes along with LCs and hair follicles contribute to creating uniformly pigmented substitutes resembling a natural skin morphology (Swope et al. 1997; Zheng et al. 2005; Nicholas et al. 2016a, b).

7.2.3.4 Macrophages

Macrophages originated from monocytes are necessary for coordinated wound healing processes. they digest injured matrix and its debris, induce hypertrophic scaring, and growth factors and cytokines secretion (Leibovich and Ross 1975; Bielefeld et al. 2011; Bielefeld et al. 2013) (Polverini et al. 1977). They potentially show anti-inflammatory behavior and could promote angiogenesis (Polverini et al. 1977; Bechetoille et al. 2011). Because of anti-inflammatory cytokine secretion potential, dermal macrophages in combination with dermal fibroblasts have been widely employed in engineered skin equivalents (Bechetoille et al. 2011). Hypertrophic scars are the side effects that necessitate the caution when adding macrophages in skin substitutes (Koh and DiPietro 2011).

7.2.3.5 Langerhans Cells (LCs)

LCs are dendritic cells derived from bone marrow that are distributed in the skin epidermis and are known as professional antigen-presenting immune cells (Larouche et al. 2016). They are specialized in processing and presenting antigens to T lymphocyte of immune system (Fransson et al. 1998; Groeber et al. 2011a, b). Following the migration of LCs into local dermal lymph nodes, they become mature and activate T lymphocytes by presenting the processed antigen. According to their immunological traits. LCs are suitable candidate which could be added to skin substitutes in order to monitor skin immune response and even its rejection (Catalano et al. 2013).

7.2.3.6 Endothelial Cells

Endothelial cells are the main cells forming inner surface of blood and lymphatic vessels. Using these cells can accelerate blood and lymphatic capillary formation in the dermis both directly

after integration to the graft and indirectly by stimulation of angiogenesis (Tremblay et al. 2005; Marino et al. 2014). Therefore, using endothelial cells in 3D scaffold possibly promote grafting success rate by improving capillary-like network formation, blood supply as source of oxygen and nutrients, and lymphatic drainage that are required for a normal skin (Nicholas et al. 2016a, b).

7.3 Types of Skin Substitutes

As explained below, based on the nature of skin substitutes, different classifications associated to skin equivalents are used in this field.

A. The cell content of skin equivalents has a significant impact on their classification. Regarding to that, they could be cellular or acellular analogs which also may serve as temporary or permanent wound dressing (Biedermann et al. 2013). Acellular substitutes are mainly used as protections against environmental contamination and also fluid loss. The cellular one, are more complicated and composed of one or two layers of scaffold, seeded with autologous or allogeneic cells (Biedermann et al. 2013; Varkey et al. 2015a, b). Inspired by nature, cellular manufactured skin substitutes enhance the healing process along with long-term and complete restoration of damaged tissue in addition to the reduction of graft rejection rate (Supp and Boyce 2005a, b; Shevchenko et al. 2009).

B. Another crucial factor for classification is the material origin. It can be biological (autologous, allogeneic, or xenogeneic) or synthetic (biodegradable or non-biodegradable). In other words, skin substitutes can be divided in two distinct groups including either synthetic that means made of acellular materials or natural which contain desired cell type (Shevchenko et al. 2010; Varkey et al. 2015a, b).

C. Skin equivalents could be divided into different groups regarding their anatomical structure. The skin substitute may serve as a replacement for epidermal, dermal, or bilayer dermoepidermal tissues. The last one is also known as composite analogs (Shevchenko et al. 2010; Biedermann et al. 2013).

In the following section we focus different types of skin substitute's classifications.

7.3.1 Acellular and Cellular

Acellular tissue engineered skin substitutes are most commonly used as protective agents against contamination and fluid loss, and also as means to deliver dermal matrix components, cytokines, and growth factors to promote wound healing process in the injury site (Groeber et al. 2011a, b; Catalano et al. 2013). Their first use reported in the late 1970s and recently they are mainly applied as replacements in superficial wounds and burns. Instead of dermis and epidermis, an acellular skin substitutes usually includes a nylon mesh or collagen and a silicon membrane, respectively. They usually employ as temporary skin coverage (Vig et al. 2017).

Cellular skin substitutes produced by seeding a mesh or 3D matrix with desired cell type like fibroblasts that ideally obtained from neonatal foreskin. The cell sources added to the scaffold could be either autologous or allogeneic. Allogeneic cellular skin substitutes are temporarily used as wound dressing and are further replaced by a split skin graft or are regrafted. Autologous keratinocytes, cultured based on the method introduced by Rheinwald and Green, may be useful for a long-term wound coverage (Rheinwatd and Green 1975; Rheinwald and Green 1977). Two main types of cellular autologous skin substitutes including CEA and cultured skin substitutes (CSS) are currently available (Vig et al. 2017).

7.3.2 Natural or Synthetic

Biomaterial content of a skin substitute could be natural and synthetic (Metcalfe and Ferguson 2007). As it was discussed before, natural materials like collagen, chitosan, HA and synthetic materials such as PEG, PLA, PLGA and their combinations are commonly used in the skin analog production (Metcalfe and Ferguson 2007; Zhu 2010a, b; Zhu and Marchant 2011). The scaffold structure are designed to enhance cell-biomaterial effective interactions and increase ECM components

deposition rate, induce sufficient transport of nutrients and essential factors required for proliferation, differentiation, and cell survival of (Dixit et al. 2017a, b). Scaffolds should have a solid 3D structure able to carry out the desired functions. The pore size is a matter of great importance to scaffold engineering which ideally should be around 100 μm to support cell migration and transportation. However, it has been demonstrated that pores larger than 300 μm can contribute to capillary formation through vasculogenesis and is an optimal pore size for porous scaffolds (Karageorgiou and Kaplan 2005). Therefore, by engineering scaffold properties and origins, an ideal porous 3D scaffold with optimal supportive and regulatory functions mimicking the natural skin ECM may be accessible in future.

7.3.3 Epidermal, Dermal or DermoEpidermal

7.3.3.1 Epidermal Substitutes

Epidermal substitutes were the first ones to be produced. Rheinwald and Green were the pioneer of epidermal graft usage in 1975 which cultured keratinocytes as stratified cell sheets. In order to produce epidermal replacements, lasting approximately 3 weeks, a skin biopsy of 2–5 cm^2 is required to supply autologous keratinocytes (Biedermann et al. 2013). After biopsy, epidermis is separated from dermis and then keratinocytes are enzymatically isolated and cultured *in vitro* (Groeber et al. 2011a, b). With keratinocytes of autologous origin, the end product is CEA.

CEAs are cultured autologous keratinocytes obtained from patient's skin biopsy (Supp and Boyce 2005a, b). Keratinocytes are not able to fully heal the deeper wounds or burns, and are also are difficult to handle to the wound area. They may also induce scar formation, contraction, and hyperkeratosis when used as wound dressing (Supp and Boyce 2005a, b). The presence of collagenases in the wound area might reduce CEA uptake (Bello et al. 2001a, b; Ho 2002). Du to these drawbacks, a more advanced strategy was developed via employing cadaveric allogeneic skin grafting before graft application. Then, CEA is grafted instead of the allo-epidermis used before to acclimatize the wound site (Bello et al. 2001a, b, Ho 2002).

Another strategy to produce epidermal equivalents is surgically prepared autologous keratinocytes suspension that could be directly sprayed on the wound site (Wood et al. 2007). This method leads to accelerated rate of epithelialization and epidermal maturation (Navarro et al. 2000). But there may be some doubts about their clinical application in cases such as third degree burn wounds which is not studied yet (Biedermann et al. 2013).

7.3.3.2 Dermal Substitutes

First dermal substitute with clinical usage was reported in the 1980s. It didn't contain any cells, the same as some dermal substitutes available now, with a collagen-GAG scaffold (Biedermann et al. 2013). Wounds treated with the dermal substitute showed less contraction and better mechanical stability than CEA (Groeber et al. 2011a, b). There are different types of dermal replacements, which could be cellular or cell-free. Dermal substitutes should be used only when the wound is sterile and well-prepared. During the next 3 to 4 weeks after grafting, patient's skin cells beneath the substitute colonized and vascularized forming an autologous neodermis (Shakespeare 2005). This new layer allow the removal of silicone cover that could be replaced by a STSG by another surgical process (Kearney 2001). Despite new achievements in dermal equivalent production, this methods do not seem as an ideal strategy. Although dermal substitutes production and usage needs lengthy surgery with associated pain and complications, do not considered as a cost-effective method (Branski et al. 2007; Pham et al. 2007; Groeber et al. 2011a, b). The combination of dermal and epidermal substitutes provides a better outcome especially in full thickness skin injuries (Groeber et al. 2011a, b).

In contrast to acellular products that are used permanently, products with human allogenic cells such as fibroblasts in a polymeric mesh, are mainly used as temporary wound healing dressing (Cooper et al. 1991; Heitland et al. 2004; Biedermann et al. 2013). Fibroblasts release

pivotal growth factors involved in wound healing and deposit dermal matrix proteins such as collagen which contribute to the wound regeneration (Böttcher-Haberzeth et al. 2010). In cellular dermal substitutes, desired cell types generally provide ECM proteins, a variety of growth factors, and cytokines (Groeber et al. 2011a, b). From biomaterial point of view, dermal substitutes scaffolds could have allogeneic, xenogeneic, or synthetic origin (Shevchenko et al. 2010).

7.3.3.3 DermoEpidermal or Composite Substitutes

Dermoepidermal substitutes are the most complex one and the most expensive clinically available skin substitutes. They act as alternatives for both dermis and epidermis layers of normal skin (Jones et al. 2002; Groeber et al. 2011a, b). First tissue engineered dermoepidermal substitute was produced by Bell et al in 1981 using allogeneic human cells (Biedermann et al. 2013).

These bilayered skin analogs contains autologous or allogeneic keratinocytes and fibroblasts seeded on 3D scaffolds (Pham et al. 2007; Shevchenko et al. 2009). Autologous cells are obtained from the injury site biopsy and added to collagen-GAG based construct. Then, the substitute is transplantable after 4 weeks of cultivation (Boyce 2001; Böttcher-Haberzeth et al. 2010). An ideal dermepidermal skin substitute is described as one being easy to handle, providing a suitable skin barrier, and being non-immunogenic (Gómez et al. 2011). Given complexity involved, composite replacements are the most advanced one in terms of structure and function, but they are mostly approved for temporary use (Supp and Boyce 2005a, b). They may also cause problems like reduced elasticity, graft contraction, lack of pigmentation due to absence of melanocytes, and decreased UV radiation protection (Biedermann et al. 2013).

The main expected functions of composite substitutes are growth factors and cytokines release, ECM deposition, and pain relief according to their regulatory effect in wound healing process (Groeber et al. 2011a, b). However, best endeavors to promote this technology led to production of some permanent full thickness wound coverage that could serve as complementary therapies and reduce the need for harvesting autografts (Llames et al. 2006). Based on the cells potential it is believed that they are able to regenerate 3D ECM structure needed for optimal repair regardless of using xenogeneic or synthetic constructs. Furthermore, the self-assembly method as a newly designed approach at the Laboratoire d'Or- ganogenese Experimentale (LOEX was emerged and has led to the production of fully autologous composite equivalents. These skin analogs exhibit more similarity to natural skin and provokes less immune response (Larouche et al. 2016). CSS is another kind of autologous bilayered skin substitute suitable for permanent wound coverage. This composite is user-friendly and its dermoepidermal junctions are properly formed. A wide variety of CSS could be designed according to scaffold architecture which are capable of fibroblasts growth and migration induction, active regulation of the osmotic pressure, free radicals removal, as well as regulation of the inflammatory response (Bello et al. 2001a, b; Price et al. 2005a, b; Vig et al. 2017). However, being costly and requiring long time preparation are some of CSS drawbacks (Vig et al. 2017).

Although composite substitutes are more advanced than epidermal and dermal one and exhibit therapeutic properties of both epidermal and dermal analogs, due to their own limitations the ideal skin equivalent is just around the corner.

7.4 Commercially Available Skin Substitutes

After human keratinocytes were successfully cultured in the 1970s, the first bioengineered skin substitutes called CEAs came into practice. Dermal substitutes as wound dressing intended for more sever wounds has developed since the 1980s (Dixit et al. 2017a, b). Following that, various combinations of epidermal and dermal replacements led to composite substitutes engineering. Up to now, novel innovations in tissue engineering area resulted in a wide range of commercially available engineered skin substitutes

appropriate for clinical applications. Each of them can be permanent or temporary and with its own pros and cons (Halim et al. 2010a, b; Nathoo et al. 2014a, b). As a result, an optimized engineered product should be chosen depends on the patient's requirements and severity of wound characteristics (Nyame et al. 2014a, b). As mentioned before, there are different types for classification of commercially available skin substitutes. In this review we focus on their anatomical classification into epidermal, dermal and dermoepidermal equivalents. Some prevalent examples of each category are depicted in Fig. 4 and the characteristics of the most prevalent one are discussed in the next section (Dixit et al. 2017a, b).

7.5 Applications of Clinically Available Skin Substitutes

Each commercially available engineered skin substitute has its own characteristics based on their cell content, scaffold architecture, and biomaterial type which determine their therapeutic applications. Some of the most commonly used skin substitutes from each category are discussed below and also a more complete list of common commercially available skin substitutes and their indications is shown in Table 1.

7.5.1 Commercially Available Epidermal Substitutes

Epidermal substitutes are designed to act as an equivalent to the epidermal layer of skin. Extensive burns excised and grafted early showed improvements in structure and function. As mentioned in previous sections this method was invented by Rheinwald and Green which allogeneic keratinocytes were cultured and turned into stratified epithelium with a reasonable integrity suitable for grafting (Rheinwatd and Green 1975; Gallico III et al. 1984). Scientists attempt to optimize these equivalents in terms of healing efficacy, length of treatment, and pain relief. Here we allude two common epidermal products including Epicel and Epidex.

7.5.1.1 Epicel™

Epicel™ was the first autologous skin substitute to become commercially available (O'Connor et al. 1981). It is a type of CEA and was produced when scientists were able to culture epidermal cells *in vitro* which allow wound burns care more efficient with very low risk of rejection (Green et al. 1979; Biedermann et al. 2013). Epicel™ is composed of sheets of autologous keratinocytes lain on a petrolatum gauze support which is removed one week after Epicel™ transplantation (Carsin et al. 2000). This equivalent employ in patients with severe burns

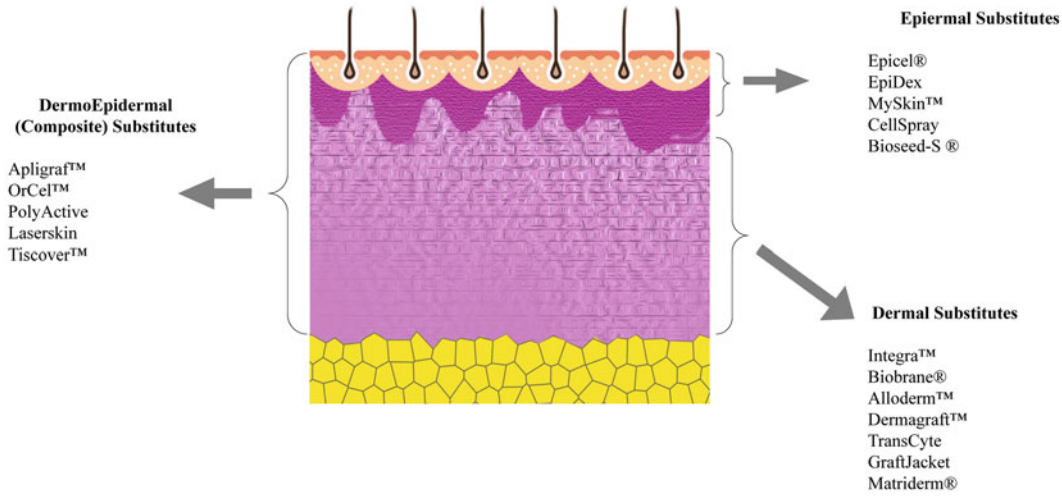

Fig. 4 The most common commercially available skin substitutes divided by their anatomical properties

Table 1 A list of common commercially available skin substitutes and their specifications

Type	Product Name	Description	Indications	References
Epidermal	Epicel®	autologous keratinocytes Sheets attached to petrolatum gauze support	Large full-thickness burns areas giant congenital nevus	Varkey et al. (2015a, b)
	EpiDex	Autologous Keratinocytes of the outer root sheath of plucked anagen hair follicles	Recalcitrant vascular leg ulcers large burn areas	Tausche et al. (2003a, b)
	MySkin™	Autologous keratinocytes seeded on a synthetic silicone polymer support layer	Neuropathic, pressure & diabetic foot ulcers	Groeber et al. (2011a, b), Dixit et al. (2017a, b))
	CellSpray	Suspension of non-cultured autologous keratinocytes	Partial- and deep partial-thickness burn	Groeber et al. (2011a, b), Esteban-Vives et al. (2016))
	Bioseed-S ®	Autologous keratinocytes re-suspended in an allogeneic fibrin sealant	Treat therapy-resistant chronic venous leg ulcers	Shevchenko et al. (2010)
Dermal	Integra™	Cell-free dermal regeneration template composed of a dermal layer made of bovine Type I collagen and shark chondroitin-6-sulphate GAG with a silicone polymer as an epidermal layer	Partial and full thickness wound, burns, Chronic ulcers, Diabetic Ulcer, Venous Ulcer, Pressure Ulcer, Arterial Insufficiency	Supp and Boyce (2005a, b); Driver et al. (2015), Dixit et al. (2017a, b)
	Biobrane®	Cell-free dermal substitute composed of an inner dermal analog 3D nylon fabric and porcine type I collagen peptides with outer ultrathin silicone film as epidermal analog	Regeneration & Wound dressing for Partial & Full thickness wounds and Chronic ulcers	Supp and Boyce (2005a, b), Groeber et al. (2011a, b), Dixit et al. (2017a, b)
	Alloderm™	Cell-free dermal substitute composed of allogeneic lyophilized cadaveric collagen as the dermis analog	Full thickness burn wounds & wound cover	Supp and Boyce (2005a, b); Nathoo et al. (2014a, b)
	Dermagraft™	Synthetic PGA/PLA, ECM and allogeneic neonatal foreskin fibroblasts	Full-thickness diabetic foot ulcers, chronic wounds, burns, venous ulcer	Groeber et al. (2011a, b), Varkey et al. (2015a, b), Nicholas and Yeung (2017a, b)
	TransCyte	Porcine dermal type I collagen coated with bio-absorbable polyglactin and with a silicone film covered nylon mesh containing allogenic neonatal foreskin fibroblasts	Partial thickness burns,	Kumar et al. (2004b), Groeber et al. (2011a, b)
	GraftJacket	Cell-free cryopreserved cadaveric dermal collagen	Diabetic ulcer, venous ulcer, pressure ulcer, arterial insufficienc	Brigido (2006); Nicholas and Yeung (2017a, b)
	Matriderm®	Cell-free bovine type I collagen matrix & α-elastin hydrolysate lyophilized dermis	Full thickness burn, chronic wounds	Halim et al. (2010a, b)
DermoEpidermal (composite)	Apligraf™	Bovine type I collagen seeded with allogeneic	Venous leg ulcers, diabetic foot ulcers,	Zaulyanov and Kirsner (2007a,

(continued)

Table 1 (continued)

Type	Product Name	Description	Indications	References
		neonatal foreskin fibroblasts and keratinocytes	partial & full thickness burns, chronic wounds	b); Nicholas and Yeung (2017a, b)
	OrCel™	Bovine Type I collagen sponge seeded with allogeneic neonatal foreskin fibroblasts and keratinocytes	Split thickness wound, mitten hand deformity after EB	Nyame et al. (2014a, b)
	PolyActive	Synthetic Polyethylene oxide terephthalate & Polybutylene terephthalate (PEO/PBT) scaffold seeded with autologous keratinocytes and fibroblasts	Partial thickness wounds	Groeber et al. (2011a, b); Dixit et al. (2017a, b)
	TissueTech Autograft System (Laserskin and Hyalograft 3D)	Recombinant HA membrane seeded with autologous fibroblasts and keratinocytes and is comprised of Hyalograft® as a dermal substitute and Laserskin as the epidermal substitute	Diabetic foot ulcers	Uccioli (2003); Groeber et al. (2011a, b)
	Tiscover™ (A-Skin)	Autologous full thickness cultured skin consisting of a pigmented epidermis on fibroblast proliferated dermis	Chronic therapy-resistant leg/foot ulcers	Varkey et al. (2015a, b)

like full-thickness burns with surface area more than 30% TBSA and also in patients with giant congenital nevus. Since donor availability is a concerning problem hindering tissue transplantation, in patient who has lost more than 60% of TBSA, the donor site availability seems a challenging issue. Thanks to Epicel™ structure, it could be choose as a temporary wound coverage in these large surface area wounds. In addition to its advantages, some pitfalls of Epicel™ restrict its widespread application including its fragility that is a troublemaker agent in the dermal-epidermal attachment, long-term preparation time, hyperkeratosis, contraction, and scar formation (Lepow et al. 2011; Varkey et al. 2015a, b).

7.5.1.2 Epidex

Epidex is another CEA substitute, but its keratinocyte sheets are produced from cells obtained from outer root sheet of patient's hair follicles and attached to silicone membranes (Tausche et al. 2003a, b; Biedermann et al. 2013). In spite of serving as a permanent substitute and being suitable for venous, diabetic ulcers, and large burn areas after *in vitro* expansion, its fragility and high cost may interfere with its widespread application (O'Connor et al. 1981; Gallico III et al. 1984; Biedermann et al. 2013).

7.5.2 Commercially Available Dermal Substitutes

Dermal replacement that was first introduced by Yannas and Burke in the 1970s, employ collagen as its scaffold. Type I collagen showed increased swelling and porosity when exposed to acid at a pH of 3. Dermal substitutes with porous scaffolds, are engineered to support cell growth, migration, revascularization, and neodermis formation which are crucial factors in skin regeneration. Best results may be achieved when adequate debridement is done prior to dermal grafting. Dermal substitutes are weak protectors against infectious agents. Therefore, their use on infected wound is accompanied by an increased risk of infection. After grafting, the scaffold is replaced by fibroblasts, endothelial cells, and inflammatory

cells. Afterwards, a thin STSG could be applied to serve as a dressing for the wound area in case of large surface area wounds. The clinical applications of dermal substitutes are not limited to extensive burns and their application is rising in other wound types (Nyame et al. 2014a, b).

7.5.2.1 Integra®

Integra™ is an acellular dermal substitute. It composed of a dermal layer contains a porous cross-linked bovine collagen and chondroitin-6-sulfate GAG and an epidermal layer that includes synthetic silicone polymer (Heimbach et al. 2003; Nyame et al. 2014a, b). The disposable silicon elastomer layer provide a temporary protection for the wound and is replaced by a thin autograft as soon as the dermis was vascularized and infiltrated by fibroblasts and other cells from the wound bed. (MacNeil 2008; Nyame et al. 2014a, b). Thus, epidermal part display a supportive barrier function and dermal part is designed to act as a scaffold. During the migration of dermal cells like fibroblasts, endogenous matrix made of collagen-GAG is synthesized by these cells and Integra™ is gradually degrades and replaces by newly formed ECM. Integra™ is mainly used when there is a need for coverage of excised burn wounds or when patient confront a limited donor site in large burns (Heimbach et al. 2003; Varkey et al. 2015a, b). It is also used for chronic diabetic foot ulcer treatment (Varkey et al. 2015a, b). Integra™ is not an immunogenic substitutes and doesn't stimulate patient's immune response (Michaeli and McPherson 1990). However, Integra™ express poor adhesive characteristics and may lead to graft loss. Additionally, it is expensive and prone to infection (Cheah et al. 2014; Chua et al. 2016; Vig et al. 2017).

7.5.2.2 Alloderm®

Alloderm® the same as Integra™ is an cell-free dermal analog includes a collagen-based scaffold that enhances tissue remodeling permanently employed into the wound bed (Sheridan et al. 1998; Catalano et al. 2013). It contains human cadaver skin that its epidermis and dermis cells are removed but contain BM (Bello et al. 2001a, b; Varkey et al. 2015a, b). To remove dermal cells, cadaver skin is treated with detergent and then goes under freeze drying processes. This contribute to keep the matrix structure more natural similar to normal dermis. Since Alloderm® is an acellular substitute and most of its antigens and allogeneic cells are removed, it has got a reduced chance of rejection and immune response induction like Integra™ (Varkey et al. 2015a, b). It provides a suitable microenvironment for patient's fibroblasts and endothelial cells migration to the scaffold in order to aid proper vascularization (Supp and Boyce 2005a, b). In spite of promoting vascularization and wound repair mechanisms, Alloderm® is expensive with a complex preparation method and could contribute to diseases transmission (MacNeil 2007; Shahrokhi et al. 2014). Alloderm® is temporarily or permanently covered by a thin layer of autologous tissue after being grafted like a dermal autograft in full thickness burn wounds (Cole et al. 2011; Varkey et al. 2015a, b).

7.5.2.3 Biobrane®

Biobrane® is a synthetic bilayer cell-free skin substitute that serves as a dermal replacement. It is mainly composed of type I porcine collagen around a 3D nylon filament and a layer of ultrathin semi-permeable silicone film as epidermal layer that controls skin fluid loss (Halim et al. 2010a, b, Varkey et al. 2015a, b). Biobrane® is one of the most prevalent skin products suitable for the pediatrics especially in partial thickness wounds, full thickness burn wounds with reduces hospitalization time, and also chronic ulcers (Halim et al. 2010a, b, Varkey et al. 2015a, b; Vig et al. 2017). Biobrane® is used as a temporary cover until the wound is repaired or autologous skin is available for grafting and is sensitive to contamination of the wound site (Varkey et al. 2015a, b, Vig et al. 2017).

7.5.3 Commercially Available DermoEpidermal or Composites Substitutes

Composite or dermoepidermal skin substitutes include both epidermis and dermis, with keratinocytes and fibroblasts obtained from allogeneic or autologous origin (Shevchenko et al. 2009). Apligraf™ and OrCel™ are discussed below.

7.5.3.1 Apligraf™

Apligraf® is a bilayer skin substitute composed of dermis and epidermis equivalents. Epidermal and dermal layers contain cultured keratinocytes and fibroblasts obtained from neonatal foreskin, respectively. Also bovine type I collagen is present in the dermal layer that promotes cell growth and differentiation (Lee 2000; Bello et al. 2001a, b; Curran and Plosker 2002). Apligraf® is used as a temporary replacement to treat partial to full thickness burns, chronic wounds, venous leg, ulcers, and diabetic foot ulcers (Shakespeare 2005; Halim et al. 2010a, b, Varkey et al. 2015a, b). Apligraf® has shown best results compared with other substitutes 4 weeks after grafting (Lepow et al. 2011). It has a beneficial effect on wound healing via providing ECM components, essential growth factors, and cytokines. Since Apligraf® do not contains antigen-presenting cells like macrophages and dendritic cells, a reduced immune system stimulations in the receiver's body is reported (Lee 2000). There is no report associated with rejection of bovine collagen or alloantigens expressed on keratinocytes or fibroblasts (Falanga et al. 1998; Zaulyanov and Kirsner 2007a, b). However, Apligraf® has a short shelf-life and treating wounds with expensive procedure (Lepow et al. 2011).

7.5.3.2 OrCel™

OrCel™ is a bilayer composite, composed of bovine type I collagen matrix seeded with cultured neonatal keratinocytes and foreskin-derived fibroblasts to form dermis (Supp and Boyce 2005a, b; Halim et al. 2010a, b; Shahrokhi et al. 2014). Its scaffold is thicker than Apligraf® and patient's cells penetrate to this 3D scaffold after transplantation. OrCel™ is employed to treat recessive dystrophic EB, partial-thickness wounds, by promoting cell migration and wound healing (MacNeil 2007). Moreover, it stimulates wound repair by cytokines and growth factors like TGFα, fibroblast growth factor-1 (FGF-1), keratinocyte growth factor-1 (KGF-1), that are released in injured site (Shevchenko et al. 2009; Varkey et al. 2015a, b). The bovine collagen, however, increases graft rejection risk and diseases transmission (Lepow et al. 2011).

Despite all efforts, owning to drawbacks associated with each of commercially available skin substitutes, their therapeutic application is hampered and none of them precisely mimics a normal skin function and structure (Varkey et al. 2015a, b).

7.6 Limitations

Some limitations associated with currently available engineered skin analogs are poor angiogenesis, uneven distribution of pigmentation, diseases transmission, host immune response, , scar formation, poor mechanical integrity, high-end price are fundamental limitations that restricted further applications of skin engineered replacements are disused below (MacNeil 2007).

7.6.1 Vascularization and Long Lasting Cell Culture

For the substitute to maintain its function and integrate into the host tissue, rapid and proper vascularization is critical. Reduced angiogenesis mainly affects the epidermal replacement and insufficient blood vessels are formed in dermal substitute (Damanhuri et al. 2011). Skin equivalents with dense or highly cross-linked matrix as well as poorly vascularized wound sites can worsen the situation and hinder vascularization (Black et al. 1998; Sahota et al. 2003; Montaño et al. 2009). As a result of poor vascularization, cells in the substitutes may die and casting off dead tissue after grafting. Even in products that allow angiogenesis, the process in not enough and further improvements are necessary (MacNeil 2007; Varkey et al. 2015a, b). In addition, cell isolation, culture, and preparation for seeding on skin scaffold takes at least 2–3 weeks before grafting on the wound bed. In case of sever and large surface area wounds, patients are in urgent need of wound care, current preparation time seems too long. To solve this issue new strategy are needed to optimized available protocols (Vig et al. 2017).

7.6.2 Pigmentation

Pigmentation is the result of melanocyte activity and distribution (Böttcher-Haberzeth et al. 2010). It contributes to recipient self-steam an skin protection from UV radiation by deposition as supra nuclear caps in keratinocytes (Böttcher-Haberzeth et al. 2013). In cultured skin replacements, abnormal persistence or lack of melanocytes can lead to hyper-or hypopigmentation, and sine the majority of skin substitutes contain only fibroblasts and keratinocytes the former is more prevalent (Boyce et al. 2002). In spite of the efforts to reach normal pigmentation in a skin substitute, this phenomenon remain an unsolved problem (Nordlund et al. 1989; Boyce et al. 1999; Supp et al. 1999). It has been demonstrated that a composite skin substitute may be able to have normal pigmentation if autologous melanocytes be added to them (Böttcher-Haberzeth et al. 2013; Nicholas and Yeung 2017a, b). However, no melanin containing engineered skin substitute is currently available for clinical use and further investigation is required for proper pigmentation in grafted sit (Biedermann et al. 2013).

7.6.3 Transmission of Diseases and Immune Response

Due to the fact that most of tissue engineered skin substitutes are derived from bovine, porcine or allogeneic human tissues, the transmission risk of infectious diseases like hepatitis B and C, human immunodeficiency virus (HIV), syphilis before, and prion-related diseases like Creutzfeldt-Jakob could not be ignored. When the final products contain viable components their complete sterilization is impossible. As a consequence, there is always a risk for disease transmission in skin grafting and there is not an acceptable test available ensuring the exact safety of transplantation process (Damanhuri et al. 2011; Groeber et al. 2011a, b; Catalano et al. 2013). Skin grafting triggers a series of immune reactions in host tissue due to foreign antigens on material or cells which may ultimately end in immune graft rejection. These reactions may initiate both tissue regeneration and inflammation, so it sounds like a double-edged sword (Remes and Williams 2006; Franz et al. 2011).

7.6.4 Skin Cells and Glands

As it was discussed before, cells like fibroblasts and keratinocytes are needed to form more differentiated structures of the normal skin. Cells such as melanocytes, LCs, adipocytes, and nerve cells are also absent in skin substitutes. Consequently, warm/hot sensation, immune regulation, pressure sensation, and proper pigmentation are lost after skin grafting in wound area (Supp and Boyce 2005a, b; Groeber et al. 2011a, b). To solve this problem extra functionally important cells such as endothelial cells and melanocytes should be added to engineered constructs (Varkey et al. 2015a, b, Vig et al. 2017). However, because of slower growth and higher apoptosis rate in endothelial cells, recent findings weren't so much impressive while there are promising outcoms encouraging the use of melanocytes, LCs, and hair follicles to tissue engineered skin substitutes (Hachiya et al. 2005; Zheng et al. 2005). Hair follicles, sebaceous glands and sweat glands especially the eccrine glands allow sweating and hair growth and also contribute to wound healing. Hair follicles in particular, accelerate the reepithelialization process (Ito and Cotsarelis 2008; Moiemen et al. 2011). Despite favorable results, there is no skin substitute currently available that includes hair follicles, sebaceous glands and sweat glands (Nicholas and Yeung 2017a, b).

7.6.5 Scar Formation

To improve mechanical features like porosity, water absorption, flexibility, and structural integrity, stiffer ECM components are needed (Nicholas et al. 2016a, b). Stiffer ECMs that are used nowadays in skin substitutes production can lead to fibrosis and exacerbate the scar formation after grafting (Meaume 2002; Humphrey et al. 2014). They have reduced protection against UV radiation and cannot provide the environment needed for sweat glands and hair follicles growth (Rue et al. 1993).

7.6.6 Cost-Effectiveness

Other obstacles that hamper widespread application of skin equivalents are the high cost and time consuming process that takes about 3 or 4 weeks to culture different cell types integrated in the skin construct. Innovations in cell tissue culture approaches and large scale production, may become able to solve these problems and produce high quality skin analogs with lower cost (Varkey et al. 2015a, b, Vig et al. 2017).

3D painters as a novel technology is rising rapidly in tissue engineering and other biomedical fields. Newly developed skin printers are becoming more applicable for manufacturing of skin substitutes (Chia and Wu 2015). Laser printing in *in vitro,* mouse models, and microfluidics are used in order to induce native-like skin regeneration and showed encouraging outcomes (Koch et al. 2009; Leng et al. 2013).

8 Stem Cells for Wound Healing

Stem cells are undifferentiated cells that display remarkable capacity to proliferate and replace themselves upon successive division. They are able to differentiate towards various cell types from a common precursor cell (Chen et al. 2009). In injuries affecting only epidermal layer, healing process is accomplished with proliferation of existing keratinocytes or keratinocyte stem cells with minimal scar formation. If the superficial dermis be injured sufficient keratinocyte stem cells are available and repair woulds in a surgical dependent manner. Epithelial stem cells present in hair follicles and/or sweat glands of the dermis can also provide an alternative sources for stem cells needed for reepithelialization process (Damanhuri et al. 2011).

Stem cells employed in skin tissue engineering could be achieved from human dermal fibroblasts, foreskin keratinocytes, epidermal keratinocyte stem cells, hair follicle stem cells, endothelial progenitor cells, MSCs derived from bone marrow, adipose tissue, umbilical cord, and amniotic membranes derived epithelial cells, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) (Li et al. 2004; Chen et al. 2009; Fortunel et al. 2010; Thangapazham et al. 2014; Lataillade et al. 2017a, b).

Using mesenchymal and ESCs in wound repair promotes healing and complete skin replacement. Additionally, it can reduce immune rejection risk caused by of B lymphocytes, T lymphocytes, and natural killer (NK) cells activation. Moreover, due to their self-renewal property, they are capable of differentiation towards different structures like sweat glands, blood vasculatures, and hair follicles (Sasai 2013). Some of the most important stem cell types used in skin tissue engineering are discussed below.

8.1 Mesenchymal Stem Cells (MSCs)

In a noble-winning study by Peter Medawar, tolerance against allogeneic skin graft was induced in recipient by injecting donor bone marrow and possibly formation of chimeric immune cells that delayed immune rejection (Widgerow 2012). This was a breakthrough in modern organ and tissue transplantation. MSCs in bone marrow are responsible for this phenomenon and may be used in situations like severe burns to solve the problem of major histocompatibility complex (MHC) incompatibility (Widgerow 2012; English 2013; Dixit et al. 2017a, b). They exhibit immunomodulatory properties like suppressing dendritic cells, T and B lymphocytes, and NK cells maturation [219] (Ankrum et al. 2014).

Multipotent MSCs as a kind of adult stem cells are capable of differentiation to different mesenchymal lineages are the origin of fibroblasts, keratinocytes, endothelial cells, and pericytes in the skin (Sasaki et al. 2008; Alt et al. 2011). In addition, MSCs can tolerate culturing and expansion processes better than regularly used cells. They could promote cell migration, differentiation, angiogenesis, skin regeneration and also secrete growth factors, collagens and cytokines contributing wound healing (Fathke et al. 2004; Akino et al. 2005; Wu et al. 2007). Moreover, due to their immunomodulatory effects they can be used in allogeneic grafts with low risk of immune

host response and promote angiogenesis necessary for successful grafting (Chua et al. 2016). Nowadays, MSCs obtained from bone marrow, adipose, amniotic membrane, cord blood, hair follicle dermal papilla and sheath, umbilical cord have raised new hopes in optimal skin regeneration (Richardson et al. 2005; Baksh et al. 2007; Miki et al. 2007; Driskell et al. 2011; Nicholas et al. 2016a, b). The ability to produce functionally important skin cells, have opened new insights in their potential therapeutic applications. Some common surces of MSCs are bone marrow, prenatal tissues, and adipose tissue.

8.1.1 Bone Marrow-Derived MSCs (BMSCs)

Most commonly used MSCs are derived from bone marrow that called BMSCs. Fibroblasts only derived from this type of MSCs produce dermal collagen III (Fathke et al. 2004). BMSCs has been successfully transplanted for skin regeneration acceleration and more frequently for therapeutic approaches (Deng et al. 2005; Basiouny et al. 2013). It has been revealed that BMSCs may be able to provide better results compared to autologous skin grafts (Badiavas and Falanga 2003). Some drawbacks of BMSCs limited number after BMSCs aspiration, intense amount of pain in aspiration process, and reduced differentiation capacity after several passages *in vitro* has made scientists look for other sources for MSCs usage in regenerative medicine (Nicholas et al. 2016a, b).

8.1.2 Umbilical Cord-Derived MSCs (UMSCs)

Different parts of umbilical cord such as cord blood, umbilical vein, and Wharton's jelly can serve as sources for prenatal UMSCs. Despite being difficult to isolate and their restricted potency after passeges, UMSCs have been used to differentiate toward fibroblasts on 3D scaffolds (Badiavas and Falanga 2003; Schneider et al. 2010; Han et al. 2011). Wharton jelly MSCs in particular, are obtained by removal of arteries and vein and are able to produce hyper-proliferating fibroblasts with lower rate of fibrosis. Moreover, they are seeded on decellularized amniotic scaffold leading to scarless wound repair in mouse models (Arno et al. 2014; Sabapathy et al. 2014). However, MSCs' wound repair potential needs more investigations to optimize their desired properties for being clinically approved in tissue engineered scaffolds.

8.1.3 Adipose-Derived MSCs (AMSCs)

Adipose tissue contains stem cells called AMSCs. Compared to BMSCs, they can undergo more divisions with less change in their potency. Furthermore, a large quantity of cells harvested from a relatively small amount of adipose tissue with less invasive approaches introduce them as a valuable alternative for BMSCs (Kern et al. 2006; Klar et al. 2017). AMSCs exhibit promising results in skin regeneration promotion when used in combination with fibroblasts leading to induction of KGF and PDGF release especially in dermal substitutes (Meruane et al. 2012; Souza et al. 2014). In spite of their superiority AMSCs need more studies to reach a standardized isolation and preparation protocol in order to guarantee their safety and quality for therapeutic applications (Nicholas et al. 2016a, b; Klar et al. 2017).

8.2 Embryonic Stem Cells (ESCs)

ESCs originated from inner cell mass within a human blastocyst 4–5 days after fertilization are pluripotent stem cells with highest stemness potential than other types of stem cells (Metallo et al. 2008). Therefore, they display more tumorigenic potential than others which highlighting that extra care should be taken for their clinical usage. ESCs promote tissue and organ regeneration and are able to differentiate into numerous cell lineages (Kim et al. 2002). Successful differentiation of ESC-derived skin revealed that they could be used for skin substitutes development (Coraux et al. 2003). However, ethical concerns are the major controversial issue hindering their clinical applications (Dixit et al. 2017a, b).

8.3 Hair Follicles Bulge Stem Cells

Hair follicle is considered as an accessible source of self-renewing stem cells that could be differentiated toward different cell types such as glial cells, neurons, smooth muscle cells, and also keratinocytes (Heidari et al. 2016). Despite all the difficulties and high cost, recent attempts have been successful in production of hair-containing skin substitutes. However, they are not available for clinical use yet and therapeutic applications of hair follicles and hair follicle stem cells may take more time (Sriwiriyanont et al. 2012; Sriwiriyanont et al. 2013; Nicholas et al. 2016a, b).

8.4 Amniotic Membranes Epithelial Cells

Epithelial cells obtained from amniotic membranes show some characteristics similar to stem cells, especially in case of cell surface marker which resemble ESCs. Therefore, these cells are suitable candidate for possible applications in wound healing and skin regeneration (Niknejad et al. 2008). In the dehydrated form, they show encouraging results for chronic wounds treatment and may be even more cost-effective than some other currently used strategy in this field (Serena et al. 2014).

8.5 Induced Pluripotent Stem Cells (iPSCs)

iPSCs are stem cells that are the outcom of changes in somatic cell nucleus which are imposed to express distinctive stemness gens innovated by Yamanaka in 2007 (Yamanaka 2007). iPSCs behave similar to ESCs and are considered as the solution for ethical problems associated with ESCs (Yu et al. 2006; Dixit et al. 2017a, b). Although they promote organ regeneration and growth, we are not aware of their long term *in vivo* compatibility. Genetic and epigenetic defects in iPSCs might lead to cancer or graft immune rejection. iPSCs are very immunogenic, but because of uncertainty of their safety, epidermal and MSCs are used more frequently. However, iPSCs may serve as a new source for autologous grafts in treatment of severe traumas and loss of extremities (Mansbridge 2009; Hanson et al. 2010; Chua et al. 2016; Dixit et al. 2017a, b).

In general, alternative methods such as using stem cells to restore skin functions after severe burn injuries have shown promising outcomes. Stem cells can be useful in skin bioengineering because they promote skin regeneration and replacement. They can be used in combination with skin substitutes currently available, or even directly (Sun et al. 2014; Dixit et al. 2017a, b). The main mechanism responsible for benefits of most of the currently available skin substitutes is protection of the injured skin from infections in addition to acceleration of wound healing (Dixit et al. 2017a, b). MSCs potentially could provide a valuable allogeneic cell source to minimize lengthy cell preparation time and offer off-the-shelf products for skin regeneration (Chua et al. 2016).

9 Conclusion

Various wound healing methods such as autologous skin grafting, skin allografts, xenografts and CEA are available for wound treatment (Nicholas and Yeung 2017a, b). Despite allografts, autografts derived from patient's own body has no immune rejection risk. Although due to limited donor site availability in large surface area injuries allografts became more preferable, allografts and xenografts a sound a temporary solution owning to their high levels of immunogenicity in patients (Erdag and Morgan 2004; Vig et al. 2017). From a clinical standpoint, painful procedures, long-term postoperative care, lack of donor site, inefficient wound regenerations, and high cost lead to the advent of engineered skin equivalents instead of conventional approaches (Janeway et al. 2005; Shores et al. 2007; Cardinal et al. 2009). Therefore, tissue engineered skin substitutes are now considered as potential replacements for damaged skin especially in

cases of severe burn. A typical tissue engineered skin substitute mainly consist of cells, growth factors and scaffolds. Three major types of these constructs are currently available that according to wound severity serve as replacements for epidermal, dermal and dermoepidermal skin layers (Biedermann et al. 2013). These substitutes should protect the injured skin against infections or mechanical stress and also provide a suitable environment for tissue repair, vascularization, and cell growth (Pham et al. 2007). They are also supposed to reduce scar and keloid formation after wound repair (Priya et al. 2008). Biomaterials employed in skin substitutes that functionally resemble skin ECM may be synthetic, natural or a combination of synthetic and natural polymers (Sheikholeslam et al. 2017). Cells intended for use in skin equivalents usually include keratinocytes and fibroblasts, while more recently, stem cells such as bone marrow derived stem cells, MSCs and iPSCs have been applied for improving skin substitutes regeneration, immunomodulation, and angiogenesis as optimized wound healing process (Damanhuri et al. 2011).

In spite of all advances achieved in tissue engineering area over recent decades, major complications such as insufficient vascularization, hypopigmentation due to lack of melanocytes, inability to produce differentiated skin structures such as hair follicles, sebaceous and sweat glands, temperature and pressure sensation, scar formation, and lack of cost-effectiveness no ideal skin substitute is currently available (Biedermann et al. 2013). With respect to recent applications of different types of stem cells containing embryonic, prenatal and adult stem cells, endothelial cells and melanocytes parallel to enormous improvements in engineering biocompatible materials like collagen, HA, elastin, PLA, PLGA, and PEG there is now a rising hope to effective therapy for incurable wounds. Recent achievements will lead to the manufacturing of skin substitutes exhibiting principle features of natural skin including hair and sweat glands, even pigmentation, and scarless healing in the future (MacNeil 2008). However, further researches and endeavors are critical to produce really natural skin-mimicking substitutes.

10 Future Perspectives

The future of skin regeneration and wound healing lies in the field of tissue engineering and regenerative medicine. In order to achieve an ideal skin substitute, different characteristics such as better vascularization by using bioreactors to help vessel formation, improved life length and integration to host tissue should be considered. Scaffold polymers, growth factors, and all cell lines should ideally mimic natural skin structure and function as possible. To this end, new cells such as melanocytes and hair follicles should be added to the 3D engineered scaffolds. Microfluidic skin printing and automatic tissue printing are emerging techniques to revolutionize tissue engineering strategies. Skin substitutes are now attracting great deal of attention and many experimental studies are required to improve the safety and efficacy of stem cells and engineered materials to respond to demands for the high quality and cost-effective products which are manufactured under standard protocols (Lovett et al. 2009; Damanhuri et al. 2011; Nicholas et al. 2016a, b; Vig et al. 2017).

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Biomaterials and Regenerative Medicine in Urology

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Abstract

Autologous gastrointestinal tissue is the gold standard biomaterial for urinary tract reconstruction despite its long-term neuromechanical and metabolic complications. Regenerative biomaterials have been proposed as alternatives; however many are limited by a poor host derived regenerative response and deficient supportive elements for effective tissue regeneration *in vivo*. Urological biomaterials are sub-classified into xenogenic extracellular matrices (ECMs) or synthetic polymers. ECMs are decellularised, biocompatible, biodegradable biomaterials derived from animal organs. Synthetic polymers vary in chemical composition but may have the benefit of being reliably reproducible from a manufacturing perspective. Urological biomaterials can be 'seeded' with regenerative stem cells *in vitro* to create composite biomaterials for grafting *in vivo*. Mesenchymal stem cells are advantageous for regenerative purposes as they self-renew, have long-term viability and possess multilineage differentiation potential. Currently, tissue-engineered biomaterials are developing rapidly in regenerative urology with many important clinical milestones achieved. To truly translate from bench to bedside, regenerative biomaterials need to provide better clinical outcomes than current urological tissue replacement strategies.

Keywords

Biomaterials · Biomedical engineering · Regenerative medicine · Stem cells · Tissue engineering

Abbreviations

ADM:	acellular dermal matrix
ADSCs:	adipose derived stem cells
BAMG:	bladder acellular matrix graft
ECM:	extracellular matrix
PGA/PLA:	polyglycolide/polylactide
PGA:	polyglycolic acid
SIS:	small intestinal submucosa
SMC:	smooth muscle cell
UBM:	urinary bladder matrix
VECM:	vascular extracellular matrix

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1 Introduction

Regenerative medicine in urology has undergone considerable progress in the last three decades with the development of urological biomaterials that are gradually integrating into urological clinical practice. Research into biomaterials in reconstructive urology began in the 1950's with the aim of creating a 'cell-scaffold' template that could support and promote urological tissue regeneration (Moore 1953). More recently, tissue-engineered biodegradable biomaterials have been proposed as templates for urinary tract reconstruction; however many are limited by a poor host derived regenerative response and deficient supportive elements for effective cellular proliferation and regeneration after *in vivo* implantation (Davis et al. 2010).

Currently, not many urological biomaterials are being implanted into patients and autologous gastrointestinal (GI) tissue remains the gold standard biomaterial for urinary tract reconstruction despite its long-term neuromechanical and metabolic complications (Flood et al. 1995). If regenerative biomaterials are to replace GI tissue for urological tissue reconstruction they should mimic the ability of the host's extracellular matrix (ECM) to regulate important cellular functions such as mitosis, proliferation, differentiation and apoptosis (Badylak 2016). These biomaterials should also possess excellent biocompatibility, have a known biodegradation profile and demonstrate favourable urological mechanical properties (Crapo et al. 2011). This narrative review aims to provide an overview of data on biomaterials and regenerative medicine in urology. We also discuss barriers that are delaying the introduction of regenerative biomaterials into daily clinical urological practice.

2 Historical Perspective

In the twentieth century synthetic biomaterials were investigated as potential alternatives to autologous gastrointestinal tissue for reconstructing the upper and lower urinary tracts. Non-biodegradable biomaterials such as polytetrafluoroethylene (PTFE), silicone, rubber, polyvinyl, and polypropylene were evaluated in the 1950s but rapidly encrusted due to prolonged contact with urine (Moore 1953). These synthetic biomaterials also predisposed to bacterial colonisation and host-derived inflammatory reactions (Kaleli and Ansell 1984). Subsequently, teflon mesh, silastic patches, gelatin sponge, collagen, and vicryl were also investigated as urological reconstructive biomaterials; however inconsistent results on animal models prevented their progression into human clinical trials (Kropp 1998; Kropp et al. 1996).

In the 1960s, mesenchymal stem cells (MSCs) were theoretically described by Becker and Till as a source of cells with multipotent regenerative potential (Becker et al. 1963). MSCs were then pioneered in a laboratory setting for regenerative purposes in 1970 by Friedenstein et al. (1970). In the late 1980s and early 1990s, tissue-engineered biomaterials were evaluated in detail for urinary tract reconstruction in animal models (Atala et al. 1993). Acellular biodegradable biomaterials were implanted into the urinary tract to provide a template for ingrowth and regeneration of native urological tissues but were limited by poorly viscoelastic mechanical properties and an inability to induce an effective tissue remodelling response (Davis et al. 2011a, b, c, d).

In 1993, the 'cell-seeded' approach was evaluated when smooth muscle cells (SMCs) and urothelial cells (UCs) were harvested from a patient's native bladder and expanded *in vitro* onto urological biomaterials (Atala et al. 1993). The *in vitro* cell-seeded scaffold evolved into a layered mass of cells after a period of weeks that would function as a urological regenerative scaffold *in vivo*. Early studies investigating this approach described promising results when cell-seeded biomaterials were implanted in rats, dogs, pigs and humans (Drewa et al. 2006; Zhang et al. 2004; Oberpenning et al. 1999; Atala et al. 2006). A limitation included its relatively invasive time-consuming nature. Furthermore, prolonged cell expansion was costly and not realistically compatible with routine clinical urological application. In the late 1990s, MSCs were

revisited in regenerative urology as stem cells have potential to differentiate into cells of endodermal and ectodermal lineage when cultured onto urological biomaterials (Pittenger 1999; Prockop 2009). Another clinical advantage with MSCs is their anti-inflammatory properties as they inhibit proinflammatory cytokines, natural killer cells (NKTCs) and T-cell expansion (Cananzi et al. 2009).

Recently, three-dimensional (3D) bioprinting technology has emerged as a novel exciting urological regenerative strategy (Moon et al. 2016). Biodegradable polymers, xenogenic extracellular matrices (ECMs), human cell lines and supportive growth factors can be constructed in a 3D format (Sullivan et al. 2012). Limitations with 3D bioprinting construction processes are an inability to consistently replicate the mechanical properties of the urinary tract with urological biomaterials and limited *in vivo* tissue integration (Song et al. 2011; Ott et al. 2008). If issues with durability and biocompatibility are addressed, 3D bioprinting may ultimately lead to whole organ development in urology in future.

3 Biomaterials for Regeneration in Urology

In urology, regenerative approaches may provide reconstructive urologists with alternative options for urinary reconstruction. Implementation of effective urological biomaterials may prevent invasive bowel surgery and its associated long-term complications. Urological biomaterials are sub-classified as either xenogenic or synthetic and are discussed in detail below (Table 1):

3.1 Xenogenic Extracellular Matrices (ECMs)

ECMs are decellularised, biocompatible, biodegradable biomaterials derived from animal organs (Crapo et al. 2011; Gilbert et al. 2006). They are decellularised by sequential mechanical, chemical and enzymatic processes to achieve a scaffold that is minimally immunogenic. After the preparation process, ECMs are preserved with glycosaminoglycans, fibronectin, laminins and growth factors that facilitate host ingrowth through a regenerative tissue remodelling response (Table 2) (Davis et al. 2010). Urological ECMs aim to provide a biologically active tissue substitute that integrates into host tissue to functionally replace or restore defective urinary tract segments (Pokrywczynska et al. 2015). Porcine small intestinal submucosa (SIS) and porcine urinary bladder matrix (UBM)/acellular bladder matrix (ABM) are favourable for urinary tract reconstruction (Table 3) (Horst et al. 2013; Gilbert et al. 2006; Pokrywczynska et al. 2015). SIS is sourced from porcine small intestine and is composed of collagen type I and smaller volumes of collagen types III, IV, V and VI (Davis et al. 2010). UBM is isolated from the porcine urinary bladder and is composed of an intact basement membrane and large volumes of collagen VII (Brown et al. 2006). During the preparation process, ECM scaffolds can be manipulated by prefabrication with collagen bioactive recognition sites and bioactive factors for directed cell interaction on the scaffold prior to *in vivo* implantation. Absorbable prefabricated surface bioactive factors include epithelial growth factor (EGF) (Yang et al. 2009), fibroblast growth factor (FGF) (Chen et al. 2010) and platelet derived growth factor (PDGF) (Lin et al. 2006).

Kanematsu et al. also developed the concept of scaffold pre-fabrication by incorporating bFGF, PDGF, insulin-like growth factor (IGF-1) and vascular endothelial growth factor (VEGF) into UBM scaffolds (Kanematsu et al. 2003). Findings demonstrated that sustained release of bioactive factors FGF and PDGF occurred in mice as the ECM degraded *in vivo* (Kanematsu et al. 2003). These regenerative characteristics make ECMs an attractive biomaterial for both urological applications. The potential disadvantages associated with incorporating bioactive factors into urological biomaterials are their short-half lives, uncontrolled diffusion and difficulties in calculating their optimal dosage volume.

There are 2 approaches for manipulating biodegradable ECM scaffolds after they are preparation. These approaches are referred to as

Table 1 Overview of *in vivo* studies that have investigated biomaterials for regenerative purposes in urology

Biomaterial	Author (year)	Cell type	Experimental model	Experimental protocol	Outcome
SIS	Kropp et al. (1996)	Acellular	Dogs	Augmentation cystoplasty	Histological evidence of tissue regeneration in augmented segments
UBM/ ABM + bioactive factors	Kanematsu et al. (2003)	Acellular	Mice	Augmentation cystoplasty	Angiogenesis in augmented segment with minimal graft shrinkage
UBM/ABM	Dorin et al. (2008)	Acellular	Rabbits	Tubularised urethral graft	Maximal urethral defect that can be repaired with acellular graft is 0.5 cm
UBM/ ABM + PGA	Atala et al. (2006)	UCs + SMCs	Humans	Augmentation cystoplasty	Decrease in ALPP and improvement in bladder compliance
PGA/PLA	Joseph et al. (2014)	UCs + SMCs	Humans	Augmentation cystoplasty	No significant improvement in bladder capacity or compliance
PGA	Kates et al. (2015)	MSCs	Humans	Ileal conduit	Regeneration of urothelium, smooth muscle and neuronal tissue on histopathology
UBM/ABM	Zhu et al. (2010)	ADSCs	Rabbits	Augmentation cystoplasty	Regeneration of urothelium, smooth muscle and neuronal tissue on histopathology
(PGA) + poly-dl-lactide-co-glycolide	Eberli et al. (2009)	UCs + SMCs	Mice	Augmentation cystoplasty	Regeneration of urothelium, smooth muscle and neuronal tissue on histopathology with good viscoelasticity
Poly (1,8-octanediol-co-citrate)	Sharma et al. (2010)	UCs + MSCs	Rats	Partial cystectomy	Regeneration of urothelium, smooth muscle and neuronal tissue on histopathology with good viscoelasticity
PGA/PLA	Jack et al. (2010)	ADSCs	Rats	Augmentation cystoplasty	Maintenance of bladder capacity and compliance with smooth muscle contractility

Abbreviations: *SIS* small intestinal submucosa, *UBM*/*ABM* urinary bladder matrix/acellular bladder matrix, *PGA* polyglycolide acid, *PLA* polylactide acid, *PGA*/*PLA* polyglycolide acid/polylactide acid, *UCs* urothelial cells, *SMCs* smooth muscle cells, *MSCs* mesenchymal stem cells, *ADSCs* adipose derived stem cells, *ALPP* abdominal leak point pressure

Table 2 Constituents of xenogenic ECM after the decellularisation process

Constituents	Function
Collagen	Abundant protein that provides mechanical support to the ECM
Glycosaminoglycans	Mucopolysaccharides that bind to act as a reservoir for storage of growth factors
Laminin	Adhesive glycoprotein that plays a role in cell differentiation and proliferation
Fibronectin	Extracellular protein that promotes biocompatibility
Growth factors	Proteins that stimulate cell growth, proliferation and differentiation

'unseeded' and 'seeded' techniques. The unseeded method involves the use of a bare ECM scaffold *in vivo* to provide a framework for ingrowth and regeneration of native tissue (Davis et al. 2010). The seeded method requires the *in vitro* culture and expansion of various cell types on an ECM scaffold to create a composite biomaterial for grafting *in vivo* (Davis et al.

Table 3 Differentiating porcine small intestinal submucosa (SIS) from porcine urinary bladder matrix (UBM)/acellular bladder matrix (ABM)

Xenogenic ECM	Characteristics
SIS	Derived from porcine jejunum and mainly composed of type 1 collagen
UBM/ABM	Derived from the porcine bladder and composed of an intact basement membrane with collagen types IV and VII

2013). Unseeded SIS and UBM are effective for regenerating small urinary tract defects in animal models through the release of stimulatory growth factors and absorbable bioactive factors whereas 'cell seeded' ECM scaffolds are more effective for regenerating larger urinary tract segments (Atala et al. 2006; Baker and Southgate 2011). Dorin et al. demonstrated that a maximum of 0.5 cm of urethra could be successfully replaced with tubularised, unseeded ECM grafts in a rabbit model (Dorin et al. 2008).

In the late 1990s, Kropp et al. reported on successful bladder augmentation with autologous smooth muscle cells and urothelium seeded onto SIS scaffolds in canine models that were neurologically intact (Kropp et al. 1996). Zhu et al. investigated the feasibility of adipose-derived stem cells (ADSCs) cultured onto UBM for augmenting rabbit bladders (Zhu et al. 2010). Findings demonstrated regeneration of smooth muscles, urothelium and nerves in the augmented tissue; however nerve bundles and smooth muscle fibres were poorly organised (Zhu et al. 2010). Encouragingly, bladders reconstructed with adipose derive stem cells (ADSCs) seeded onto UBM reached 95% of their pre-intervention bladder capacity whereas bladders that were augmented with UBM grafts alone only achieved 70% of their pre-intervention capacity (Zhu et al. 2010). More recently, phase 2 clinical trials in human patients with cell seeded ECMs are becoming prevalent with some encouraging short- and intermediate-term clinical outcomes (Atala et al. 2006; Caione et al. 2012).

3.2 Biodegradable Polymers

Synthetic biodegradable polymers have been thoroughly investigated for regenerative purposes in urology. Synthetic polymers vary in chemical composition but may have the benefit of being reliably reproducible from a manufacturing perspective (Kollhoff et al. 2011). Consistent reproducibility is a limitation with xenogenic ECMs due to variations in the composition of donor xenografts (Brown et al. 2006). Furthermore, biodegradable polymers can avoid immunological complications that are occasionally associated with xenografts when they are not thoroughly decellularised. Eberli et al. investigated biodegradable polyglycolide (PGA) and poly-dl-lactide-co-glycolide scaffolds in regenerative urology (Eberli et al. 2009). These synthetic polymers are modified to contain collagen and glycoproteins that encourage tissue ingrowth (Eberli et al. 2009). Results from benchtop and animal trials found that both scaffolds were biocompatible and possessed physical and structural characteristics that were suitable for urological tissue engineering purposes. The biodegradable polymer poly(1,8-octanediol-co-citrate) has also been investigated for regenerating the urinary tracts (Motlagh et al. 2007; Kang et al. 2006; Sharma et al. 2010). Advantages are a minimal immunogenic and inflammatory host response and favourable viscoelastic properties. Poly (1,8-octanediol-co-citrate) can also be chemically modified to allow for extended release of growth factors that stimulate angiogenesis *in vivo* in mice (Eberli et al. 2009).

The clinical progression of synthetic PGA biomaterials is exemplified by the recent development of a PGA urinary conduit scaffold as an alternative to the conventional ileal conduit for urine drainage after cystectomy (Cornu et al. 2015). The PGA tubularised conduit was seeded with autologous smooth muscle cells (SMCs), grown from ADSCs and implanted into patients undergoing radical cystectomy for bladder cancer (Sopko et al. 2015; Kates et al. 2015). Eight

patients were enrolled into this phase 2 clinical trial and short-term results demonstrated regenerated urothelium, smooth muscle and neuronal tissue on histopathology (Kates et al. 2015).

In another phase 2 clinical trial, urothelial and smooth muscle cells were cultured onto biodegradable collagen and polyglycolic acid (PGA) scaffolds in paediatric patients requiring augmentation cystoplasty for myelomeningocele (n = 7) (Atala et al. 2006). The biodegradable synthetic scaffolds were implanted with or without an omental wrap and no postoperative complications were noted after 46 months. Postoperative cystography and videourodynamic studies demonstrated an increase in bladder capacity and compliance of 1.58-fold to 2.79-fold compared to baseline values. Mean bladder leak point pressure at capacity decreased postoperatively by 56% (67 to 37.5 cmH_2O) (Atala et al. 2006). A more recent phase 2 clinical trial could not replicate these results when an autologous cell seeded polyglycolide/polylactide (PGA/PLA) composite scaffold was applied for augmentation cystoplasty in paediatric patients with spina bifida (n = 10) (Joseph et al. 2014). No improvement in bladder capacity was found on videourodynamics after 1-year or 3-years. Adverse events occurred in 4 patients with 5 patients requiring re-operation in the form of ileocystoplasty (Joseph et al. 2014).

4 Cell Sources in Regenerative Urology

Investigators can be restricted by the limited availability of host cells as a diseased urinary tract may not have sufficient quantities of healthy native cells for optimal expansion techniques. Therefore, alternative sources of cell lines are required for urological regeneration (Jack et al. 2010). Selecting the appropriate cell type for urological tissue regeneration is an important factor for *in vivo* graft survival and function (Adamowicz et al. 2013).

4.1 Stem Cells

Stem cells are an invaluable tool for promoting the host's regenerative response and modulating regrowth of diseased urinary tract tissue. Stem cells are advantageous as they self-renew and have long-term viability (Jack et al. 2010). They can be sub-categorised into embryonic stem cells and adult stem cells. Embryonic stem cells have enormous clinical potential; however they are limited by ethical considerations and cell regulation issues (Kollhoff et al. 2011). Therefore, regenerative urology has primarily focused on autologous adult MSCs. MSCs are readily available and are considered the most attractive stem cell type for tissue regeneration as they have a high proliferation rate and possess a multilineage differentiation potential (Kern et al. 2006). Bone marrow is the most studied source of mesenchymal progenitor cells and has been shown to differentiate into multiple cell types *in vitro* and *in vivo* (Pittenger 1999). Limitations with bone marrow in regenerative urology are marrow harvest morbidity, low cell yields and patient specific serum requirements (Nishida et al. 1999; Mueller and Glowacki 2001). Skeletal muscle cells, adipose stem cells and parthenogenetic stem cells have also been investigated as suitable alternatives to urological smooth muscle and urothelial cells (Jack et al. 2010). Many investigators have reported promising results with tissue engineered biomaterials using multipotent cell lineages from these different sources (Zhu et al. 2008; Rodríguez et al. 2006; Zuk et al. 2001) MSCs produce and release the angiogenic factors VEGF-A and Ang1/Ang3 which stimulate local angiogenesis. MSCs also upregulate anti-inflammatory cytokines such as IL-10 and TGF-β (Pokrywczynska et al. 2013; Kusuma et al. 2017).

Differentiation of pluripotent stem cells into urothelial and smooth muscle cells is feasible using condition defined culture media or using custom designed bioreactors (Davis et al. 2011a, b, c, d). Bioreactors replicate different physiologic environments by producing continuous or pulsatile pressures with or without compression or shear

stress forces (Shaikh et al. 2010). They have been effective in optimising the viability and proliferative potential of autologous cells seeded onto xenogenic and synthetic biomaterials. Wallis et al. assessed the feasibility of a urological bioreactor using proteins as specific phenotypic markers for differentiated urothelial cells (UCs) during a 24-h experimental period (Wallis et al. 2008). Findings demonstrated increased levels of cytokeratin 8 messenger RNA and significantly increased levels uroplakin II were expressed with RT-PCR indicated the presence of proliferating differentiated UCs *in vitro* (Wallis et al. 2008).

4.2 Voided Urine Stem Cells

Voided urine-derived stem cells derived from the upper urinary tract have also been investigated as a potential stem cell source for urological tissue-engineering (Zhang et al. 2008). Results demonstrated that urine-derived stem cells demonstrated surface markers that are associated with MSCs and pericytes. These cells differentiated into smooth muscle-like cells that expressed smooth muscle-specific gene transcripts and proteins such as α-smooth muscle actin, desmin, and myosin. Urothelial-differentiated stem cells derived from urine expressed urothelial-specific genes and proteins such as uroplakin-Ia and -III, cytokeratin (CK)-7, and CK-13 (Zhang et al. 2008). Notably, the effects of urine on urine-derived stem cells has not been investigated yet. It is hypothesised that they are more resistant to urine than other cell sources given their urological origin (Adamowicz et al. 2013).

5 Barriers for Regenerative Biomaterials in Urology

In general, regenerative biomaterials are not being implanted into urological patients on a regular basis apart from some phase 2 clinical trials. Issues with cost, regulation, manufacturing and reimbursement are delaying their progress. The cost of producing 'cell-seeded' biomaterials is estimated to be 6 times that of acellular scaffolds therefore reducing the attractiveness of this approach (Badylak 2016).

A more significant issue is that regenerative biomaterials are deficient in supportive elements that provide viability and function *in vivo*. Biodegradable biomaterials need sophisticated vascular, innervation and lymphatic networks which are not currently present for replacing larger genitourinary tract defects (Kollhoff et al. 2011). The effect of a patient's microenvironment on an implanted urological biomaterial should be fully considered prior to implanting any urological biomaterial. Surrounding mechanical forces, pH, cytotoxic agents, signalling agents and oxygen levels need to be considered for effective tissue regeneration with urological biomaterials (Jack et al. 2010). Limitations with mechanical durability and poor compliance have been persistently demonstrated with regenerative biomaterials (Davis et al. 2011a, b, c, d). One *in vitro* study compared the biomechanical properties of cell-seeded xenogenic scaffolds with normal bladder tissue (Davis et al. 2011a, b, c, d). Uni-axial tensile testing demonstrated that cell-seeded scaffolds were biomechanically inferior to the urinary bladder and the authors conclude by emphasising the importance of mechanical equivalence with biomaterials prior to *in vivo* implantation (Davis et al. 2011a, b, c, d).

Although vasculogenesis and angiogenesis occur after biomaterial implantation; both processes are currently not sufficiently efficient in terms of clinical urological applicability (Ott et al. 2008). Prefabrication of biomaterials and stimulation with pro-angiogenic bioactive factors are limited due to their inability to develop a sophisticated vascular network over a short period of time. One approach currently being investigated is to pre-vascularise the regenerative biomaterial with networks of well-formed

capillaries prior to *in vivo* implantation with 3D bioprinting technology (Baptista et al. 2011; Ability et al. 2014). Bertassoni et al. developed technology using agarose fibres to fabricate microchannel networks that are populated with endothelial cells (Bertassoni et al. 2014). This 3D template can capture the normal anatomical and vascular architecture of a urological organ (Bertassoni et al. 2014). An artificially created vascular network must integrate with the host's vasculature and remain patent long-term.

Specific urological factors that have inhibited the progression of urological biomaterials include the cytotoxic effects of urine and the presence of uropathogens in the upper and lower tracts. One *in vitro* study demonstrated that "off-the-shelf" unseeded biomaterials are clinically limited by their inability to induce tissue regeneration in the host's natural urine environment (Davis et al. 2011a, b, c, d). Cytotoxicity is attributable to cationic substances and low molecular weight products that are normally found in urine (Davis et al. 2011a, b, c, d). It appears that a pre-established impermeable urothelial layer prior to *in vivo* implantation is a prerequisite.

6 Conclusion

Biomaterials and regenerative medicine are developing rapidly in urology with many important achievements to date. Regenerative urology has evolved from the repair of small urinary tract segments to the development three-dimensional templates for fully functional organs. Despite promising *in vitro* and animal data from the 1990s, clinical implementation of urological biomaterials is not imminent in mainstream urology and further investigation is needed. To truly translate from bench to bedside, regenerative biomaterials need to provide better clinical outcomes than current urological tissue replacement strategies.

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