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Review

Advances and challenges in stem cell culture

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ABSTRACT

Stem cells (SCs) hold great promise for cell therapy, tissue engineering, and regenerative medicine as well as pharmaceutical and biotechnological applications. They have the capacity to self-renew and the ability to differentiate into specialized cell types depending upon their source of isolation. However, use of SCs for clinical applications requires a high quality and quantity of cells. This necessitates large-scale expansion of SCs followed by efficient and homogeneous differentiation into functional derivatives. Traditional methods for maintenance and expansion of cells rely on two-dimensional (2-D) culturing techniques using plastic culture plates and xenogenic media. These methods provide limited expansion and cells tend to lose clonal and differentiation capacity upon long-term passaging. Recently, new approaches for the expansion of SCs have emphasized three-dimensional (3-D) cell growth to mimic the *in vivo* environment. This review provides a comprehensive compendium of recent advancements in culturing SCs using 2-D and 3-D techniques involving spheroids, biomaterials, and bioreactors. In addition, potential challenges to achieve billion-fold expansion of cells are discussed.

Contents

Abbreviations: SCs, stem cells; 2-D, two-dimensional; 3-D, three-dimensional; ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells; ECM, extracellular matrix; MEF, mouse embryonic fibroblast; LIF, leukemia inhibitory factor; MSCs, mesenchymal stem/stromal cells; HA, hyaluronic acid; PEG, polyethelene glycol; PLL, poly-L-lysine; PLA, poly-lactic acid; PGA, poly-glycolic acid; PCL, polycaprolactone; PGLA, poly-dl-lactic acid-co-glycolic acid; Dex-SH, thiol-functionalized dextran; PEG-4-Acr, PEG functionalized with four-arm acrylate; PDMS, polydimethylsiloxane.

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1. Introduction

Stem cells (SCs) have the capacity to self-renew and differentiate into specialized cells and are defined by their origin and degree of potency [1]. Pluripotent SCs are capable of unlimited self-renewal and differentiation into any of the over 200 types of cells in the body [2,3]. There are two sources of pluripotent SCs. First, embryonic stem cells (ESCs) are derived from the inner cell mass of a pre-implantation blastocyst [4] and pluripotency is controlled by an intrinsic regulatory network of core transcription factors, octamer-binding transcription factor 4 (OCT4), sex determining region Y-box 2 (SOX2), and Nanog homeobox (NANOG) [5]. Second, induced pluripotent stem cells (iPSCs) are derived by the ectopic or elevated expression of four transcription factors, OCT4, SOX2, Kruppel like factor 4 (KLF4), and MYC proto-oncogene (C-MYC) essential for induction of pluripotency in somatic cells [3].

Another type of SCs, mesenchymal stem/stromal cells (MSCs) are isolated from adult sources such as bone marrow and adipose tissues [6] or perinatal tissues, such as umbilical cord, cord blood, placenta and amniotic fluid [7,8]. MSCs are characterized by adherent growth to plastic culture plates, exhibiting clonal growth. They are positive for mesenchymal surface markers, CD90, CD73, CD29, and CD105 and negative for hematopoietic lineage markers, CD45, CD34, and HLA-DR [9]. Unlike pluripotent SCs, MSCs are multipotent and differentiate into only limited cell types such as osteogenic, chondrogenic, and adipogenic cells [10]. Also, their self-renewal and differentiation potential is dependent upon the source of isolation [11]. In addition, MSCs derived from adult tissues are also affected by aging and exposure to environmental stresses, which could alter genetic stability [8]. In comparison to adult MSCs, MSCs obtained from perinatal tissues exhibit higher growth and stemness potential [12].

As such, the study of SCs has contributed to the elucidation of basic biochemical and developmental processes [13–17]. They have also shown tremendous potential for *in vitro* disease models, tissue engineering, regenerative medicine, and cell therapy as well as pharmaceutical applications [18–21]. Furthermore, they can be used for drug discovery and development [22,23]. All these uses require large-scale production of high quality cells [24].

Traditionally, SCs are propagated as a monolayer in two-dimensional (2-D) plastic culture plates and often require undefined or xenogenic materials including but not limited to attachment substrates, cytokines and growth factors, as well as serum. Use of xenogenic or animal derived media can potentially transmit pathogens and limit reproducibility between cultures due to lot-to-lot fluctuation of the material used [25]. Monolayer culture necessitates routine passaging to maintain self-renewal and potency of cells, which is highly inefficient for large-scale expansion of cells. In addition, 2-D attachment alters cell shape and geometry [26], leading to cell flattening and changes in the internal cytoskeleton and nuclear shape [27], which in turn modifies gene and protein expression [28,29]. Furthermore, studies have shown that the composition and organization of the extracellular matrix (ECM) can also send biochemical and mechanical signals for cell differentiation [25,30,31].

2-D culture techniques and applications have been practiced for the majority of primary and established cell lines and standardized for analytical assays ranging from microscopy and counting cells to the study of disease processes and drug testing [32]. Notably, 2-D culture has been used for directed differentiation of SCs into many specialized cells, including chondrocytes, osteocytes, adipocytes, cardiomyocytes, smooth muscle cells and hepatocytes [33,34]. However, 2-D culture often results in a lack of functional derivatives [35]. Studies have also shown that 2-D monolayer culture fails to accurately reproduce animal physiology [36], and proves insufficient to validate drug discovery [37].

Overall, 2-D culture conditions lack the intricacy necessary to mimic the SC niche, dynamic and specialized three-dimensional (3-D) microenvironments, which are responsible for the regulation of SC fate *in vivo*. Native 3-D niches allow for complex spatial interactions between cells, ECM components, and gradients of nutrients, oxygen, and waste [38]. The effect of the 3-D microenvironment can be easily demonstrated in pluripotent SCs, which retain their pluripotency when injected into the inner cell mass of another embryo, but spontaneously differentiate into all three germ layers when injected elsewhere in animals due to external cues [39]. Furthermore, supportive niches for multipotent SCs, have been identified in the gonads, intestinal crypts, hair follicles, and bone marrow *in vivo,* whereby anchored cells release signals that govern continued self-renewal or lineage-specific differentiation [40]. Transplantation of multipotent SCs outside the niche results in differentiation into various cell lineages dependent upon the cues in the surrounding *in vivo* microenvironment [41].

To address the various problems facing 2-D culture of SCs, 3-D culture methods have been developed to control cell fate by incorporating physiologically relevant and biomimetic microenvironments by mimicking the ECM composition and stiffness of the SC niche *in vitro* [27,38,42,43]. However, 3-D culture for SC maintenance and expansion still remains a challenge [44], primarily, due to need to optimize culture conditions for different types of cells and analysis. Therefore, is it necessary to elucidate the mechanisms important for SC maintenance, including biomaterial signaling and mechanical forces that would aid in the uniform and reproducible expansion of SCs without loss of genetic stability or differentiation potential.

This review is focused on the recent progress in 3-D culture for expansion of SCs, both in quality and quantity, acceptable for clinical, pharmaceutical and biotechnological applications. In addition, we will report some important recent advancements in 2-D culture, since these methods have historically led to significant developments in SC biology and early studies have been reviewed previously [22,45]. Overall, this review assesses the latest advances in proliferation and differentiation of pluripotent and multipotent SCs.

2. Two-dimensional culture techniques

2.1. Maintenance and differentiation of pluripotent SCs

2-D culture studies have focused on the maintenance of stemness, a self-renewing state characteristic of undifferentiated SCs, by extrinsic factors such as mechanical forces between both cells and their 3-D microenvironment, which are transduced to biological cues that can control cell shape and function [25,46–49].

Usually, pluripotent SCs are grown on plastic culture dishes coated with ECM components (such as gelatin, Matrigel, or collagen) or a mouse embryonic fibroblast (MEF) feeder layer to aid in

attachment [50]. Mouse ESCs can be maintained in their pluripotent state either by culturing them on MEF feeder layers or in medium containing leukemia inhibitory factor (LIF), a cytokine released from MEFs [48,51]. However, supplementation of LIF is insufficient to maintain the pluripotency of human ESCs, which requires the addition of fibroblast growth factor 2 (FGF2) and transforming growth factor β1 (TGFβ1) in the culture medium [52,53]. Recent evidence suggests that these differences are not species specific but rather developmental [54]. For instance, human ESCs and mouse epiblast SCs display flat morphology, and poor clonogenicity, whereas mouse ESCs exhibit a 3-D dome morphology and good clonal growth from single cells. Recent studies have shown that addition of ROCK inhibitors to the culture medium improved their propagation by protecting against apoptosis of single cells [55]. It has been proposed that there are two consecutive states of pluripotency in embryonic development, an early naïve state followed by a primed state, from which mouse ESCs and human ESCs are derived, respectively [56]. Naïve pluripotent SCs are early totipotent cells capable of differentiation into both extraembryonic and embryonic tissues, whereas primed cells are derived from a more advanced state of development and differentiate into only embryonic tissues [57].

In fact, recent studies have demonstrated the ability of human ESCs to revert from the primed to the naïve state of pluripotency [56,58], which may greatly improve the proliferative capacity of human ESCs in culture. In addition, 2-D expansion of ESCs has been improved using fully defined and xeno-free culture media and attachment substrates such as E8 media and humanized recombinant protein vitronectin, respectively [45,53,59]. However, uniform expansion of pluripotent SCs is still difficult to achieve as 2-D culture methods for propagation of pluripotent SCs are laborious, expensive and require a high level of expertise.

Pluripotent SCs can be differentiated into cell types from all three germ layers [4]. In general, 2-D culture conditions favor non-specific differentiation of pluripotent SCs. However, differentiation can be directed to specific lineages by controlling the composition and organization of the ECM, growth factors, and attachment substrates, which can send mechanical signals for cell fate determination [26]. Both ESCs and iPSCs can be differentiated using similar protocols, however, iPSCs exhibit higher variability due to the nature of somatic cells and the method used for generating iPSCs [60]. ESCs have been directed to differentiate in monolayer culture using specific induction media into ectodermal, mesodermal, and endodermal lineages, including mature neurons and glial cells [61], osteocytes [62], cardiomyocytes [63], and hepatocytes [64]. One prodigious advantage of monolayer culture is that it allows for uniform treatment for differentiation of cells [65]. As such, there has been some progress in 2-D culture using synthetic substrates and stepwise protocols for controlled differentiation of human ESCs [60,63,65]. For instance, ESCs were previously differentiated into mesoderm cell types including cardiomyocytes in monolayer culture with approximately 30% efficiency using MEF conditioned medium, followed by treatment of Activin A and BMP4 [63]. However, addition of GSK3 and Wnt inhibitors to the medium resulted in approximately 80–90% differentiation of ESCs into cardiomyocytes [60]. Similarly, stepwise strategies have been applied to differentiate ESCs first into endoderm, hepatic progenitors, and then into hepatocytes using specific growth factors [64]. Likewise, mouse ESCs were differentiated into ectoderm lineage using neural differentiation medium supplemented with a sonic hedgehog antagonist to form neocortical-like neurons [66]. However, the differentiated neurons were heterogeneous and failed to display specific neuronal characteristics *in vitro* [66]. Despite great strides, differentiation of ESCs using 2-D culture often results in mixed populations of differentiated cells [67].

2.2. Maintenance and differentiation of MSCs

Unlike pluripotent SCs, MSCs are naturally adherent and do not require xenogenic substrates for attachment to culture plates, although they are typically cultured in the media containing animal serum. However, xenogenic-free defined media have recently become available for expansion of MSCs [68]. While supplementation of media with soluble factors is known to maintain stemness of ESCs, the exact mechanism of MSC self-renewal is not well known [69]. Nevertheless, mimicking the *in vivo* microenvironment such as hypoxia (2% O_2) has been shown to enhance stemness in MSCs, resulting in a significant proliferation, without the loss of multipotent differentiation potential over a 6 week culture period [70].

Expansion of MSCs in 2-D culture is highly inefficient. Usually expansion consists of growth in multiple tissue culture flasks to increase the surface area for large-scale attachment and propagation of MSCs [71,72]. Uniform distribution, growth, and harvesting processes are important to minimize heterogeneity and improve cell yield [73]. Successful *in vitro* culture of MSCs requires an understanding of the signaling pathways that influence both the proliferation and guided differentiation of these cells.

Numerous studies have shown MSC differentiation into osteocytes, adipocytes, and chondrocytes [12,74,75] as well as cardiomyocytes [76] in 2-D cultures. This is often achieved by using cell-specific differentiation media and assessed by translational and transcriptional gene expression, as well as ECM deposition. However, expansion of MSCs in monolayers leads to phenotypic changes, whereby spindle shaped MSCs exhibit a broad and flattened morphology upon passaging, which in turn alters cell fate and differentiation potential [12,77,78]. In particular, chondrogenic differentiation of MSCs in 2-D culture is less efficient when compared to high-density cell culture methods, utilizing pellet culture or spheroids [79]. Despite the limitations of 2-D culture, MSCs have been reported to differentiate into numerous cell types of not only mesodermal but also ectodermal lineages, including retinal progenitors and photoreceptors [80,81], and neural cells [82,83], as well as endodermal lineages, such as hepatocyte-like cells [84,85], and pancreatic islet-like cells [86,87]. However, *in vitro* induction of differentiation is often not sufficient to yield functionally competent cells [88]. For instance, transient differentiation of MSCs into retinal ganglion precursor-like cells was reported [89], suggesting that MSCs may de-differentiate during extended culture. While MSCs are a promising source for cell replacement and immune-modulation, improvements in differentiation protocols are necessary for clinical applications.

3. Three-dimensional culture techniques

Due to the inherent problems with the 2-D culture techniques attempts have been made to design 3-D culture methods to better recapitulate native microenvironments. Therefore, several approaches have been explored to maintain, expand, and differentiate SCs utilizing various 3-D culture systems with or without biomaterials under static and dynamic conditions as summarized in Fig. 1.

3.1. Static three-dimensional culture

3.1.1. Spheroids

One of the simplest methods of 3-D culture was achieved by formation of multicellular aggregates, or spheroids, which allow 3-D interactions with cells and the ECM in the absence of additional substrates [90]. These spheroids have been utilized with a wide range of adherent cell types, formed by forced or spontaneous aggregation techniques including hanging drop, rotating

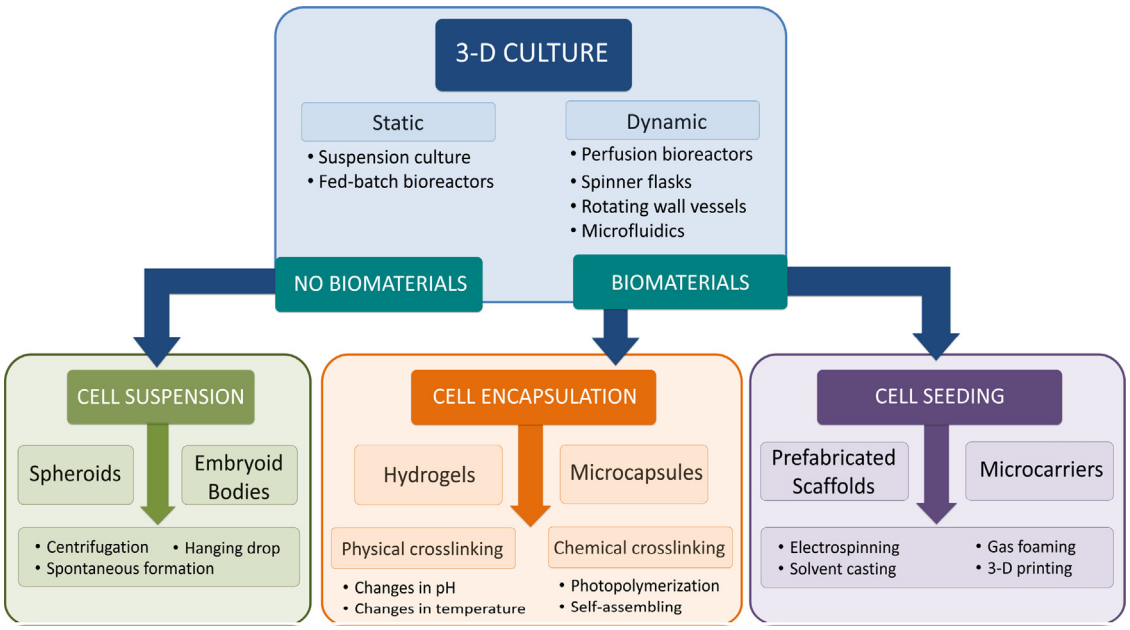

Fig. 1. Schematic of approaches used for three-dimensional culture of stem cells.

culture, or low-adhesion culture plates in suspension culture [91–93]. Spheroids are comprised of both highly proliferative, non-proliferative, and apoptotic cells with limited diffusion of oxygen and nutrients to the center of the spheroid, leading to an increasing hypoxic environment [94]. Due to their heterogeneous nature, spheroids have been more successfully employed to study complex 3-D cell structures, cell differentiation and cancer biology [90,95] in comparison with homogenous cell proliferation.

Typically, pluripotent SCs are induced to differentiate by formation of 3-D spheroids called embryoid bodies (EBs) capable of spontaneous differentiation into all three germ layers [96]. This mimics embryonic development and promotes heterogeneous differentiation, whereby induction into various specific cell types can be achieved by addition of cytokines or growth factors to the medium [97–99]. However, the inherent heterogeneity complicates both the reproducibility of directed differentiation as well as high-throughput screening techniques of EBs [100,101]. The size and number of EBs has been shown to affect differentiation efficiency [102] as observed in cardiomyocyte [103], and hematopoietic differentiation [100]. Studies have indicated that differentiation can also be controlled by varying the size of the EBs. Since EBs are comprised of varied microenvironments with differential availability to oxygen and other soluble factors, cell position in the EB can differentially regulate cell fate determination [104]. Furthermore, large EBs favored differentiation into endoderm and mesoderm cell types, while smaller EBs tended to differentiate into ectoderm [104,105].

In comparison to 2-D culture conditions, 3-D spheroid culture improved hepatocyte differentiation of ESCs based on phenotypic analysis, expression of hepatocyte genes and proteins, and the ability to excrete biliary metabolic products [106,107]. 3-D spheroid culture of ESCs also displayed improvement in endothelial cell tube formation [108], functional bile ductal differentiation [109], engraftment potential of hepatic progenitor cells [110], and paracrine signaling in hematopoietic differentiation [100]. Overall, 3-D spheroid culture generated functional derivatives of ESCs. However, long-term suspension culture of spheroids often results in agglomeration of cells, leading to necrotic centers due to limited diffusion of nutrients and oxygen into and waste out of the aggregate [94].

In contrast to ESCs, short-term spheroid culture has been employed for maintenance and expansion of MSCs [90]. When subcultured back to 2-D culture conditions, spheroid grown MSCs displayed an undifferentiated morphology, and enhanced differentiation potential *via* increased ECM deposition when compared to adherent grown MSCs [111]. MSCs cultured in 3-D spheroids exhibited increased clonal growth and multipotency [112], as well as altered miRNA expression, and increased acetylation in the promoter regions of pluripotency genes, OCT4, SOX2, and NANOG [113].

Additionally, MSC spheroids grown in short-term culture for 3 days exhibited higher expression of anti-cancer and anti-inflammatory factors including, IL-24, TNFα-related apoptosis inducing ligand, and CD82 as well as TNFα stimulated gene/protein 6 (TSG-6) and stanniocalcin-1, respectively [114]. These studies suggested that 3-D spheroid culture improves the therapeutic properties of MSCs. Another study showed that spheroid cultured MSCs had improved retention, survival, and integration following transplantation in a porcine model [115]. However, long-term culture of MSCs in spheroid culture has been shown to effectively induce differentiation [116]. In comparison with monolayer culture, MSCs grown in 3-D spheroids displayed increased chondrogenic differentiation [117], as well as osteogenic differentiation *in vitro* and *in vivo*, exhibiting enhanced bone regeneration in calvarial defects in rats [118].

Large-scale expansion of SCs using these methods is difficult due to the inability to control aggregate size, leading to agglomeration, necrosis/apoptosis in spheroids, and inhibition of cell proliferation [114]. This requires minimization of spheroid agglomeration by stirring or incorporation of biomaterials, which will be discussed in Section 3.2.1 and 3.2.2.

3.1.2. Biomaterials

Natural and synthetic biocompatible and biodegradable materials can provide various biological signals and differing degrees of mechanical strength. Biomaterials have been increasingly integrated into *in vitro* culture to mimic the biochemical and biophysical properties of SC niches to help direct self-renewal or differentiation of cells into specific lineages [119]. Incorporation of natural biomaterials may result in varying levels of inductive sig-

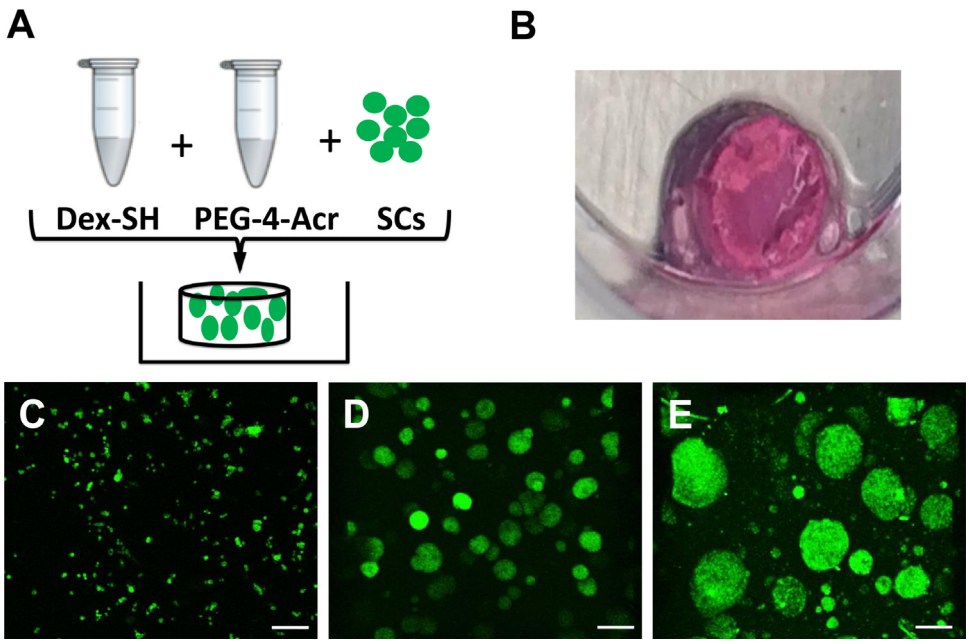

Fig. 2. Three-dimensional growth of green fluorescent protein (GFP) labeled mouse ESCs in self-assembling scaffolds. (A) Schematic of self-assembling scaffold formation and encapsulation of mouse ESCs (B) Image of the self-assembled scaffold, and (C–E) representative composite confocal images of fluorescent green labeled ESCs grown in Dex-SH/PEG-4-Acr self-assembling hydrogels at 2, 7 and 21 days, respectively. 3-D cultured ESCs exhibited undifferentiated clonal growth. Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nals, cell attachment, and scaffold integrity. Natural biomaterials, including agarose, fibronectin, collagen type 1 (Col1), hyaluronic acid (HA), chitosan, and alginate, can also be functionalized but are more difficult to control *in vitro* as they often transduce biological signals to cells [120]. As a result, biomaterials can aid in cell maintenance and differentiation. However, problems such as the lot-to-lot variability and the potential of xenogenic media components to cause disease limit their use [120]. Synthetic polymers such as polyethelene glycol (PEG), poly-L-lysine (PLL), poly-lactic acid (PLA), poly-glycolic acid (PGA), polycaprolactone (PCL), and poly-dl-lactic acid-co-glycolic acid (PGLA), are often end-functionalized with the addition of crosslinking groups [48]. PEG polymers are arguably the most commonly studied since they are non-toxic, easily functionalized, and exhibit low protein adsorption, making them safe for biomedical applications in humans [121].

Natural biomaterials and synthetic polymers are increasingly being used in 3-D scaffolds for maintenance, expansion and differentiation of SCs. Scaffolds with tunable mechanical properties allow modulation of cell viability, proliferation and differentiation [122,123]. The scaffold constructs are able to mitigate cell aggregation that negatively affects SC viability and growth in suspension culture, by limiting the availability of oxygen and nutrients to cells at the center of aggregates resulting in quiescence and ultimately necrosis/apoptosis [22,124,125]. Scaffolds can also incorporate soluble factors such as cytokines or growth factors or be further functionalized with adhesive ligands for anchorage dependent proliferation, providing a framework for ECM production for SC self-renewal and differentiation [120]. They are formed using various techniques such as electrospinning, solvent casting, gas foaming, 3-D printing, and self-assembly resulting in scaffolds with distinctly different pore size, elasticity, adhesion, and tensile strength [126,127].

Generally, two types of scaffolds are used for 3-D culturing of SCs. First, prefabricated or rigid scaffolds require cell seeding, or migration of cells into the scaffold. These scaffolds are frequently used for differentiation protocols, since substrate stiffness, and mechanical and biomaterial signaling are important for SC differentiation. The second type of scaffolds self-assemble encapsulating cells at the time of scaffold formation. The self-assembly of biomaterials is usually promoted by various crosslinking methods. Physical crosslinked scaffolds are often called reversible gels and can be formed *via* reversible changes in pH or temperature, resulting in mechanically weak scaffolds [128]. Whereas scaffolds formed *via* chemical crosslinking resulting in covalent bonds tend to yield mechanically strong scaffolds [129,130]. Hydrogel scaffolds made of hydrophilic biomaterials are often used for SC culture because they mimic the fully hydrated ECM of natural soft tissue [131]. Components of the hydrogels can also be modified with drugs, cytokines, and growth factors, to support growth and differentiation of cells *in vitro* as well as integration of cells *in vivo* [132–135]. Scale up of 3-D culture is limited by the size of hydrogel scaffolds due to nutrient diffusion in static culture.

Generally, maintenance of ESCs in 3-D hydrogel scaffolds requires routine passaging akin to 2-D cultures *via* scaffold degradation [44,136–138]. Human pluripotent SCs cultured in thermoresponsive hydrogels and passaged every 5 days for over 50 passages maintained pluripotency in about 95% of the cell population [136]. Similarly, iPSCs grown as aggregates on highly porous electrospun polystyrene 3-D scaffolds maintained their pluripotency and differentiation potential for numerous passages under xeno-free conditions [137]. However, ideal 3-D culture systems should support both the expansion of pluripotent SCs while minimizing the need for routine passaging, thus limiting cell manipulation.

HA based substrates have been frequently used to support the growth of pluripotent SCs [44,139,140], since HA is present during embryogenesis and highly expressed in undifferentiated cells [141]. In addition, modified HA hydrogels prepared at various rigidities have been used to assess the effect of mechanical strength on ESC stemness in 3-D culture; ESCs grown on softer hydrogels best supported the proliferation and pluripotency of ESCs in both feeder-free and MEF conditioned media by mimicking the microenvironment of the inner cell mass of the pre-implantation blastocyst [140,142]. Prolonged 3-D culture of human ESCs in photopolymerized HA hydrogels maintained the pluripotency and self-renewal potential without passaging for 20 days [139] signifying that the 3-

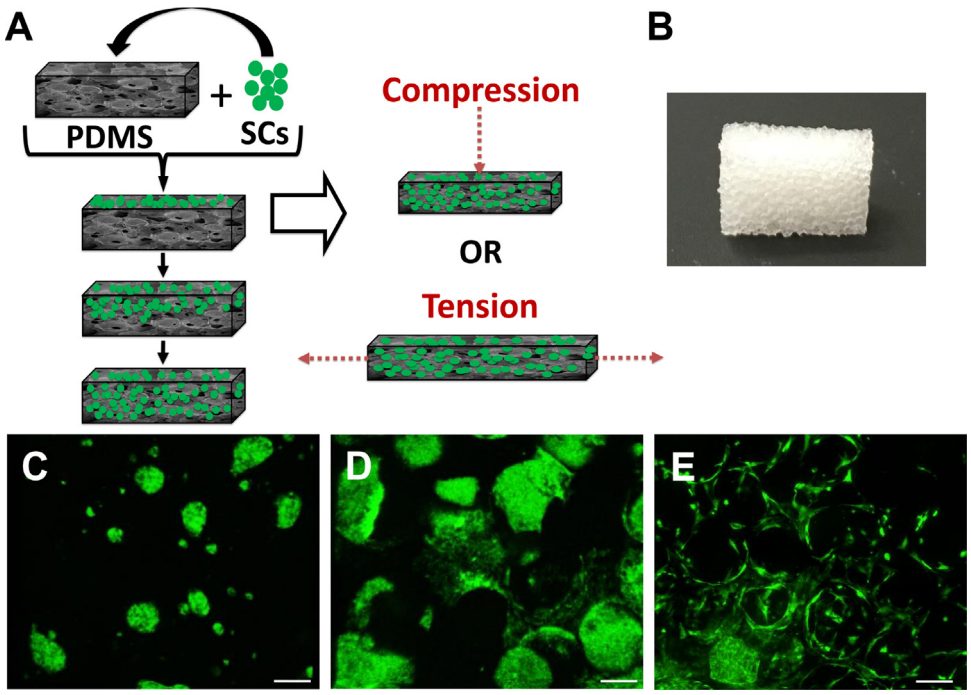

Fig. 3. Three-dimensional growth of GFP labeled mouse ESCs in prefabricated PDMS scaffolds. (A) Schematic of ESCs seeding in prefabricated PDMS scaffolds subjected to mechanical stress (compression or tension) (B) Macroscopic images of the PDMS scaffolds, and (C–E) representative confocal images of green fluorescent labelled ESCs grown in prefabricated PDMS scaffolds at 2, 7 and 21 days, respectively. ESC seeded scaffolds were compressed at 0.05 MPa for 24 h on day 7 and further cultured for 2 weeks. Compression of the PDMS scaffolds induced differentiation of ESCs into chondrogenic lineage as evident from the fibroblastoid cell morphology. Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

D hydrogels provided a biomimetic microenvironment supportive of compact clonal growth of ESCs.

We recently reported that self-assembling 3-D hydrogels, made of thiol-functionalized dextran (Dex-SH) and PEG functionalized with tetra-acrylate (PEG-4-Acr), maintained mouse ESCs for over 6 weeks without passaging [143]. The schematic of the formation of self-assembling scaffolds with simultaneous encapsulation of ESCs is depicted in Fig. 2A and the clonal growth of the encapsulated cells is shown in Fig. 2C–E. Interestingly, growth of ESCs was anchor independent since the scaffolds lack adhesive ligands. Furthermore, expression of three pluripotency markers, OCT4, NANOG and KLF4, was enhanced in ESCs grown in 3-D hydrogels during an extended period of culture. Similar observations have been reported in several studies suggesting that both dimensionality and inductive biomaterial signaling affects the mechanism of self-renewal in ESCs [144,145]. For instance, differential regulation of stemness genes was observed in ESCs cultured in three different scaffolds prepared using PGLA, collagen, and chitosan, resulting in upregulation of OCT4 expression but downregulation of SOX2; whereas NANOG was only highly upregulated in chitosan scaffolds [144,145]. Taken together, it can be speculated that the scaffold microenvironments play an important role in influencing cell-matrix communication. The mechanism of upregulation of pluripotent genes in ESCs grown in 3-D culture warrants future investigation. Although 3-D culture of ESCs in hydrogel scaffolds holds great promise, further optimization incorporating adhesion ligands and growth factors, might aid in the proliferation of human ESCs.

Differentiation of ESC derivatives using 3-D scaffolds have been shown to improve the processes of chondrogenesis [146,147], osteogenesis [148], hematopoiesis [149], neurogenesis [150], cardiomyocyte differentiation [151] and hepatocyte proliferation [152] by mimicking the biochemical and mechanical signaling *in vivo* [153]. For instance, Col1 scaffolds combined with fibronectin induced vascularization and endothelial differentiation [151], whereas incorporation of laminin resulted in cardiomyocyte differentiation [151]. 3-D culture in synthetic self-assembling peptide scaffolds [154] and nanofibrous scaffolds [155] promoted ESC differentiation into osteoblast-like cells. While nanofibrous PCL scaffolds supplemented with gelatin supported chondrogenic differentiation of iPSCs [156]. PEG hydrogels conjugated with N-Cadherin derived peptides (His-Ala-Val-Asp-Lle) induced neural differentiation of mouse ESCs [157]. Modification of alginate scaffolds with adhesive cue, RGD (Arg-Gly-Asp) sequences supported retinal differentiation of ESCs and derivatization of retinal tissue [158].

To determine the effects of mechanical properties on differentiation, ESCs were seeded on prefabricated polydimethylsiloxane (PDMS) scaffolds, capable of transducing mechanical signals to cells, due to their high elasticity [30]. As such PDMS scaffolds can be used to study both the effects of matrix elasticity as well as the application of compressive or tensile forces (Fig. 3). 3-D culture of mouse ESCs in 3-D PDMS scaffolds resulted in selective chondrogenic differentiation due to both PDMS matrix elasticity and transduction of compressive stress. PDMS grown ESCs expressed decreased levels of pluripotent markers genes, concurrent with an increase in chondroprogenitor gene markers [147]. We assessed the effect of 3-D culture of mouse ESCs grown in PDMS scaffolds for 7 days, after which a compressive stress (0.05 MPa) was applied for 24 h. PDMS grown ESCs exhibited differentiated fibroblastoid morphology upon compressive stress (Fig. 3C–E). As such biomimetic scaffolds are useful for both the study of differentiation processes and the generation of ESC derivatives for tissue engineering and transplantation applications.

MSC: fibroblastoid morphology strongly correlates with the organization of actin cytoskeleton, and distribution of focal adhesion [159]. Although one of the characteristic features of MSCs is their adherent growth in 2-D culture conditions, there have been conflicting reports on the necessity of attachment in 3-D culture. MSCs photoencapsulated in nondegradable PEG hydrogels showed improved viability when scaffolds were conjugated with adhe-

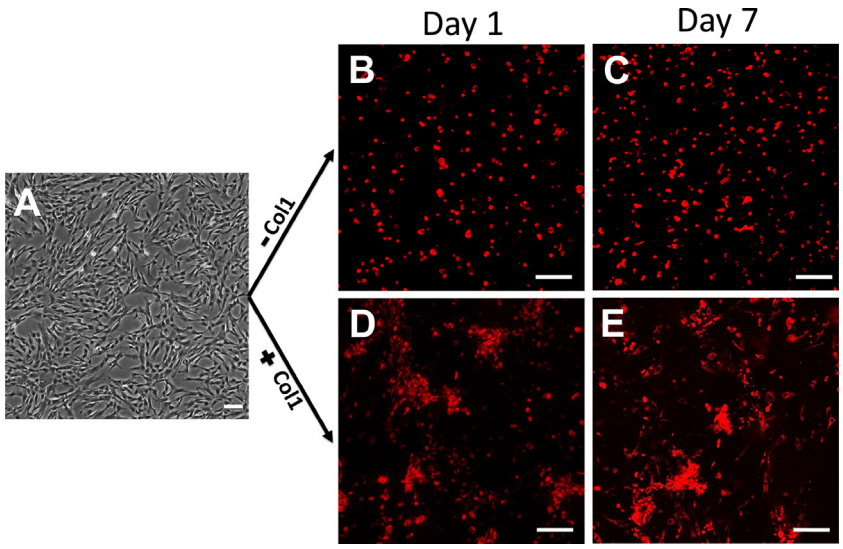

Fig. 4. Human umbilical cord derived Cy3 labeled MSCs grown in three-dimensional self-assembling scaffolds. (A) Light micrograph of 2-D cultured MSCs. (B-E) Representative confocal images of red fluorescent labeled MSCs in Dex-SH/PEG-4-Acr self-assembling scaffolds in the absence and presence of collagen type 1 (Col1) fibers grown for 1 (B and D) and 7 (C and E) days, respectively. MSCs exhibited fibroblastoid morphology when cultured in 3-D scaffolds supplemented with Col1. Scale bars = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sive ligands, RGDSP (Arg-Gly-Asp-Ser-Pro), and to a lesser extent IKVAV (Ile-Lys-Val-Ala-Val) sequences, derived from fibronectin and laminin respectively, similar to findings on MSC adhesion in 2-D culture [42]. The effect of cell spreading and proliferation of MSCs in hydrogel scaffolds was studied using 3-D HA hydrogels supplemented with and without RGD sequences. MSCs proliferated in both hydrogels; however, cell spreading was only observed in scaffolds modified with RGD sequences [160]. Proliferation was limited by matrix stiffness, crosslinking density, RGD concentration, and the rate scaffold degradation in a concentration dependent manner [160,161]. However, results indicated that hydrogel degradation significantly increased MSC viability, even in the absence of cell adhesion ligands [42].

Similarly, we observed that umbilical cord derived MSCs (Fig. 4A) in Dex-SH/PEG-4-Acr self-assembling scaffolds did not show significant proliferation or MSC fibroblastoid morphology (Fig. 4B–C). When Dex-SH/PEG-4-Acr self-assembling scaffolds were prepared with Col1 fibers, Cy3-labeled MSCs adhered, displayed fibroblastoid morphology, and proliferated as visualized by confocal microscopy (Fig. 4D-E).

3-D culture of MSCs may improve adaptability upon transplantation *in vivo* and enhance the secretion of indoleamine 2,3-dioxygenase, IL-6, M-CSF, IL-10, TGFβ, HGF, and PGE2 factors that are involved in immunosuppression [162,163].

3-D scaffold grown MSCs have been reported to improve the outcome of cell implantation [164–166]. For instance, MSCs grown in 3-D collagen-pullulan hydrogels induced secretion of angiogenic cytokines, vascular endothelial growth factor-a (Vegf-a) and monocyte chemoattractant protein-1 (Mcp-1). Hydrogel grown MSC grafts accelerated wound healing and neovascularlization by differentiating into fibroblasts and endothelial cells [165]. Co-culture of encapsulated MSCs in 3-D HA hydrogels with macrophages, resulted in lower CD16 and HLA-DR, and higher CD206 expression, suggesting that they could be more suitable for transplantation [167]. Interestingly, 3-D collagen-pullulan hydrogels grown bone-marrow MSCs displayed enhanced self-renewal as well as expression of pluripotent genes, OCT4, SOX2, and KLF4, when compared to MSCs grown in 2-D culture conditions [165].

3-D culture conditions mimicking *in vivo* microenvironments have also been investigated to differentiate MSCs. Mechanical stiffness and loading properties of the scaffolds promoted differentiation of MSCs into various specific lineages [168]. For instance, softer substrates induced MSC differentiation into neural and beta islet cells [169], as well as differentiation into chondrocytes and adipocytes [170–172], whereas increase in substrate stiffness supported MSC differentiation into myoblasts, and osteoblasts [173]. Stiffer substrates also enhanced integrin binding and induced osteogenic differentiation [170,172]. Compressive forces mimicking joint action also induced chondrogenic differentiation of MSCs *via* mechanotransductive scaffolds increasing chondrogenic gene expression in MSCs [174–176].

Scaffolds incorporating collagen, a principal component of connective tissue ECM, have been utilized to differentiate MSCs into mesodermal cell lineages including osteoblasts [177,178], chondrocytes [179], and cardiomyocytes [180]. MSCs differentiated into chondrogenic lineage when cultured in 3-D scaffolds composed of HA to mimic cartilage ECM [181,182]. MSCs cultured in 3-D scaffolds composed of chitosan, a mechanically strong polysaccharide, promoted osteogenic differentiation [183,184]. In contrast, umbilical cord derived MSCs differentiated into neurons in soft 3-D alginate scaffolds [185]. Therefore, it is important to optimize the composition and mechanical properties of 3-D scaffolds to direct MSC differentiation into specific cell lineages.

Overall, 3-D culture for maintenance and expansion of MSCs could improve upon current culture techniques by utilizing mechanical signals, spatial gradients of soluble factors, and cell-cell as well as cell-ECM interactions. These studies can shed light on basic cell biology processes and mechanisms. Large-scale cell expansion using individual scaffold constructs in static culture remains challenging. If scaffold size is increased, cell growth in the center of the scaffold may be limited due to diffusion of oxygen, nutrients, and waste into and out of the scaffold. Additional methods of dynamic culture, including agitation and flow perfusion systems, are currently being utilized to control culture conditions for more uniform growth *in vitro*.

3.2. Dynamic 3-D culture

Large quantities of homogeneous high quality cells are needed for therapeutic, pharmaceutical, and biotechnological applications,

which is not feasible by traditional methods using 2-D culturing techniques. Dynamic bioreactors are helpful in culturing cells at high densities, allowing reproducible cell growth and minimizing variability witnessed with expansion of cells in 2-D culture [72]. Large scale expansion of cells in bioreactors requires well defined and regulated culture conditions, including pH, temperature, and dissolved oxygen concentrations as well as removal of metabolite and waste products [36]. Agglomeration of cells may occur in static culture when cells are seeded at high concentration, reducing oxygen and nutrients transfer and increasing cell death in large cell aggregates [69].

Several designs of bioreactors exist and can be used for a large range of applications, from expansion or differentiation of single cells to whole tissue culture [36]. Spinner flasks are most commonly employed due to their low cost and ease of use, whereby an internal impeller allows for continuous agitation of suspended cells by varying the rate of stirring [186]. This method results in hydrodynamic shear stress, mixing the cells with oxygen and nutrients and leading to a more homogenous environment, however, excessive agitation leads to cell death [187]. For this reason, rotating wall vessels were designed to limit shear stress by rotating horizontally, allowing for culture mixing without an internal stirring mechanism [188]. Culture parameters can be controlled by flow perfusion systems to allow continuous exchange of nutrients and waste [189], in contrast to fed-batch bioreactor systems which are limited both in scale and length of culture due to a buildup of metabolites and waste that occurs over time [190]. As such media must then be changed at regular intervals to limit the inhibitory effect of waste accumulation or cells would have to be inoculated in multiple bioreactors to achieve large scale cell expansion. Lastly, mechanical force bioreactors have been developed to mimic tissue physiology, utilizing compressive and tensile forces, and are increasingly being used in conjuncture with tissue engineered constructs for cell differentiation [146,191]. These techniques can be further modified by the use of biomaterials (*i.e.* microcarriers and microcapsules) allowing for growth of cells at high concentration of cells in suspension culture, as stated below [69]. Overall, cell growth and fate is dependent on culture conditions, including cell seeding density, media composition, and incorporation of biomaterials.

3.2.1. Microcarriers

Microcarriers, small beads approximately 100–300 μm, are composed of various materials, including polystyrene, gelatin, dextran, and collagen, synthesized with varying porosities, and topographies [192]. They provide an adhesive surface for anchorage-dependent cells in suspension culture [193,194]. Adhesion of cells on microcarriers can be optimized to promote expansion or differentiation. Microcarriers also limit aggregation of cells and provide a large surface area for cell growth to high densities [192], whereby the concentrated cells can then be collected by enzymatic dissociation [195].

Dynamic culture techniques involving spinner flasks and microcarriers have been shown to support human ESC proliferation to high densities [196–198]. Attachment of pluripotent SCs has been achieved by coating the surface of microcarriers with adhesive proteins [72,199]. Cellulose microcarriers coated with Matrigel, an assortment of ECM proteins, have been used for long-term expansion and maintenance of human ESCs [200]. Microcarriers coated with ECM proteins such as vitronectin, laminin or PLL, have been shown to promote pluripotent SC growth [201]. In fact, a 12-fold increase in the growth of human ESCs adherent to microcarriers was observed in continuous perfusion bioreactor systems under xeno-free conditions, lacking animal derived materials [198].

One of the main advantages of 3-D culture using microcarriers allows for expansion and subsequent differentiation into specific cell lineages using differentiation media. Higher quality of human ESCs expanded using microcarriers was demonstrated by their ability to form EBs 10 times more efficiently than cells grown under 2-D conditions [202]. Microcarriers were also found to support differentiation of EBs into hemangioblasts, which were capable of differentiating into hematopoietic and endothelial cells [203]. Both expansion and differentiation of ESCs into cardiomyocytes were achieved using the microcarrier spinner culture technique [201]. The ESC derived cardiomyocytes expressed cell-specific transcription factors, ion channel genes, and sarcomeric proteins. Likewise, ESCs were first expanded to 45-fold using collagen coated microcarriers and then differentiated into an endoderm lineage, specifically pancreatic islets and liver cells, using differentiation media [204]. However, it is not always clear if ESCs differentiated using microcarriers are functional. In one of the studies, hepatic cells derived from ESCs grown using microcarriers in stirred bioreactor secreted proteins at lower levels than those of primary hepatocyte [205].

Microcarriers have also been investigated to scale up MSC proliferation in suspension culture. MSCs derived from bone marrow and placenta have been expanded on microcarriers grown in dynamic bioreactors. However, they exhibited lower cell viability and proliferation compared to expansion in static culture flasks [206,207]. MSCs isolated from different bone marrow donors showed various rates of proliferation when grown in dynamic bioreactors and expanded on Cytodex 3, collagen coated dextran based microcarriers, despite exhibiting similar growth characteristics in 2-D culture [72]. Similar studies were performed testing a variety of microcarriers, and reproducible growth of MSCs derived from different bone marrow samples was observed when cultured on SoloHill plastic beads, implying that biomaterial signaling played a large role in MSC proliferation [192]. Therefore, determination of a suitable microcarrier is important for MSC expansion. In addition, various culture parameters, including oxygen concentration, agitation rate, and nutrient exchange, are important consideration to achieve improved proliferation of MSCs.

Considerable interest has been shown in the differentiation of MSCs using microcarriers [195,208–210]. Several reports demonstrated that microcarriers aided in MSC differentiation into osteogenic [195], and chondrogenic [208,209] lineages. In fact, biodegradable PCL microcarriers supported differentiation of fetal MSCs into osteogenic cells *in vitro*, expressing higher levels of osteogenic genes and calcium deposition and equivalent bone formation upon transplantation in comparison to 2-D cultured cells [210]. Microcarriers, which co-released TGFβ3, were shown to improve chondrogenic differentiation of MSCs [208]. In another study, MSCs seeded on microcarriers showed improved chondrogenic regeneration upon transplantation in a mouse model of osteoarthritis [209]. Despite some success in the use of microcarriers for growth and differentiation of MSCs, the functional status of the differentiated cells should be further investigated.

3.2.2. Microencapsulation

In contrast to microcarriers, which primarily aid in attachment of cells to the surface of beads, microencapsulation is a process in which cells are immobilized within a semi-permeable material or membrane that allows the diffusion of nutrients, oxygen, and growth factors essential for cell growth. The process of microencapsulation of cells inside spherical capsules can be used not only to protect against agglomeration and shear forces in suspension culture, but also to prevent immune reactions upon transplantation [211]. The microcapsule microenvironment can be modified to maintain stemness or induce differentiation of SCs dependent on the composition of the biomaterials used and the incorporation of growth factors [212]. Cells are often encapsulated by emulsification or extrusion processes forming protective capsules around the cells and allowing growth within the capsule [213]. Alginate and agarose are two of the most commonly used biomolecules for encapsu-

lation of cells by photo-crosslinking and temperature dependent gelation, respectively [214,215]. Soft alginate capsules are often layered with mechanically strong polycations (*i.e.* chitosan, PEG, PLL) to improve capsule integrity [69]. Additionally, since agarose capsules lack adhesive properties, they can be modified by incorporation of additional biomaterials, such as collagen or gelatin, to promote cell attachment and growth [214].

Maintenance of viability and pluripotency as well as homogeneity of cell populations are essential for large-scale expansion of ESCs. It is important to prevent cell aggregation and ameliorate conditions that allow diffusion of nutrients, oxygen, and growth factors. Microencapsulation in agarose prevented aggregation of cells and allowed higher growth of ESCs without necrosis [216]. In general, encapsulation of pluripotent SCs have been shown to protect against chemical and mechanical stresses, including hydrodynamic shear forces that negatively affect cell viability in suspension culture [22,124,125]. ESCs encapsulated in calcium-alginate hydrogel microcapsules grew for an extended culture period in xeno-free conditions of without affecting cell viability or pluripotency [217].

ESCs encapsulated in alginate beads differentiated into beta cells capable of producing insulin [218]. Furthermore, ESCs were also differentiated into hepatocytes capable of secreting albumin and urea [219], suggesting that microencapsulation aided ESC differentiation into functional endodermal derivatives. Similarly, mesodermal differentiation of ESCs was facilitated by microencapsulation. ESCs grown in PLL coated alginate capsules, differentiated into cardiomyocytes expressing high levels of Nkx2.5 and GATA4 [220]. Whereas, ESCs encapsulated in PEGDA microcapsules, modified with RGD sequences [221], and HA hydrogel microcapsules [222] supported differentiation into chondrogenic cells. Osteogenic differentiation of ESCs was promoted by culture in calcium phosphate cement microcapsules, enhancing bone regeneration [223]. Ectodermal differentiation was facilitated by 3-D culture in alginate and alginate modified with HA to improve differentiation of ESCs into neural cells expressing synaptic markers [224,225]. Overall, 3-D culture by microencapsulation of ESCs for amplification and differentiation may prove to be integral for tissue engineering applications.

Microencapsulation of MSCs is often used for immune modulation [226], resulting in decreased fibrosis [227], and inflammation [228] upon transplantation. Since expansion of MSCs is more reliant on adhesion, modification of microcapsules with adhesive ligands and or ECM components improved cell expansion and differentiation [229]. Dynamic culture improved proliferation of MSCs encapsulated in alginate microcapsules at when compared to static culture [230]. However, MSCs encapsulated in alginate supplemented with adherence ligands, remained viable but did not proliferate [231], suggesting that attachment alone is not sufficient for cell growth. When compressive but not sheer stress was applied to the alginate encapsulated MSCs, they differentiated into chondrogenic progenitors [230]. Alginate microcapsules modified with RGD sequences, allowed for sustained release of TGFβ1 and induce MSC differentiation with increased chondrogenic gene expression and matrix deposition *in vitro* and cartilage regeneration *in vivo* [232]. Preferential MSC differentiation into chondrogenic lineage with enhanced ECM production was also observed in 3-D culture in calcium modified alginate microcapsules [233]. Microencapsulation techniques have also been utilized to promote osteogenic differentiation of MSCs in dynamic 3-D culture *via* mineralized alginate [234] and PLL coated alginate [235], as well as collagen [236], and collagen modified with agarose [237] and chitosan [238] microcapsules. In addition to differentiation, microencapsulation of MSCs could prove to be an advantageous method for cell delivery of MSC derivatives for repair of degenerative tissues *in vivo*, while minimizing immune response.

Dynamic bioreactors used in conjuncture with microcarriers and microcapsules have shown potential to improve the quality and quantity of cells. However, many challenges still persist including the potential for differentiation upon agglomeration of cells, altered gene expression, production of hydrodynamic shear forces, and variability in culture. Therefore, further research is needed, combining dynamic bioreactors with biological and engineered components for optimal expansion and differentiation of SCs.

3.2.3. Microfluidics

Microfluidics involving small-scale systems, both in terms of volume and size, are increasingly being investigated for culturing cells. They can be used to mimic the *in vivo* microenvironment *via* perfusion media exchange and chemical gradients of soluble factors for the analysis of single cells [239]. They have been used to study the effects of cell-secreted signals and microenvironment on SC fate [240]. These advancements have led to the development of devices for whole organ systems such as 'organ-on-chips', which mimics the body's microenvironments [32].

Microfluidics can control the soluble microenvironment, which in turn can modulate autocrine/paracrine signaling between cells, and shear stress due to hydrodynamic flow rates [240]. Mouse ESCs cultured in microfluidic perfusion for 4 days, proliferated at higher flow rates but not under the slowest flow rate [241]. When cultured in microfluidic chambers in the absence of feeder layers, mouse ESCs formed colonies and exhibited increased LIF expression, nearly 140 times than that secreted in macro-culture conditions, suggesting that endogenous signaling may play a large role in SC fate determination [242]. When LIF secretion was inhibited, ESCs preferentially differentiated into mesodermal lineages [242]. Microfluidic culture systems can also be used to induce differentiation of ESCs by varying culture conditions, flow rate, and augmentation of exogenous growth factors [243]. Slow-flow microfluidics supported differentiation of mouse ESCs into neuron-like and Schwann-like cells [244]. Additionally, variation in the flow rate in discontinuous perfusion culture allowed for direct differentiation of ESCs into cardiomyocytes and hepatocytes, showing functional phenotypes and responses to drug treatments in microfluidic culture systems [245].

Similar to pluripotent SCs, microfluidic culture systems can be used for small scale proliferation and controlled differentiation of MSCs. Continuous perfusion using microfluidic micromass culture allowed for 34-fold proliferation of MSCs [246]. Subsequent addition of inductive growth factors resulted in homogenous chondrogenic differentiation of MSCs [246]. Constant microfluidic perfusion culture differentiated MSCs into hepatocyte-like cells that expressed cell-specific genes and were capable of low-density lipoprotein uptake, suggestive of functional cells [247]. The application of extremely low fluidic shear stress in microfluidic devices induced cell migration and osteogenic differentiation in MSCs [248,249]. Mimicking the vascular mechanobiology *in vivo via* dynamic stretch and circumferential strain in microfluidic devices, resulted in differentiation of MSCs into blood vessel-like cells [250]. MSCs subjected to both IBMX (3-isobutyl-1-methylxanthine) and fluid flow shear stress resulted in a threefold increase in the neuronal cell differentiation [251]. Microfluidic culture techniques are still in their infancy but are promising for elucidation of biological processes, tissue engineering, and pharmaceutical applications.

In summary, numerous types of 3-D culture techniques show improvement in both proliferation and differentiation of SCs. There are advantages and disadvantages for each 3-D culture system based on the desired application as summarized in Fig. 5. In general, these techniques differ in both complexity and controllability. For instance, spheroid formation is a very simple technique that allows for cell-ECM interaction in 3-D, without the incorporation of biomaterials. However, spheroid culture results in heterogeneous

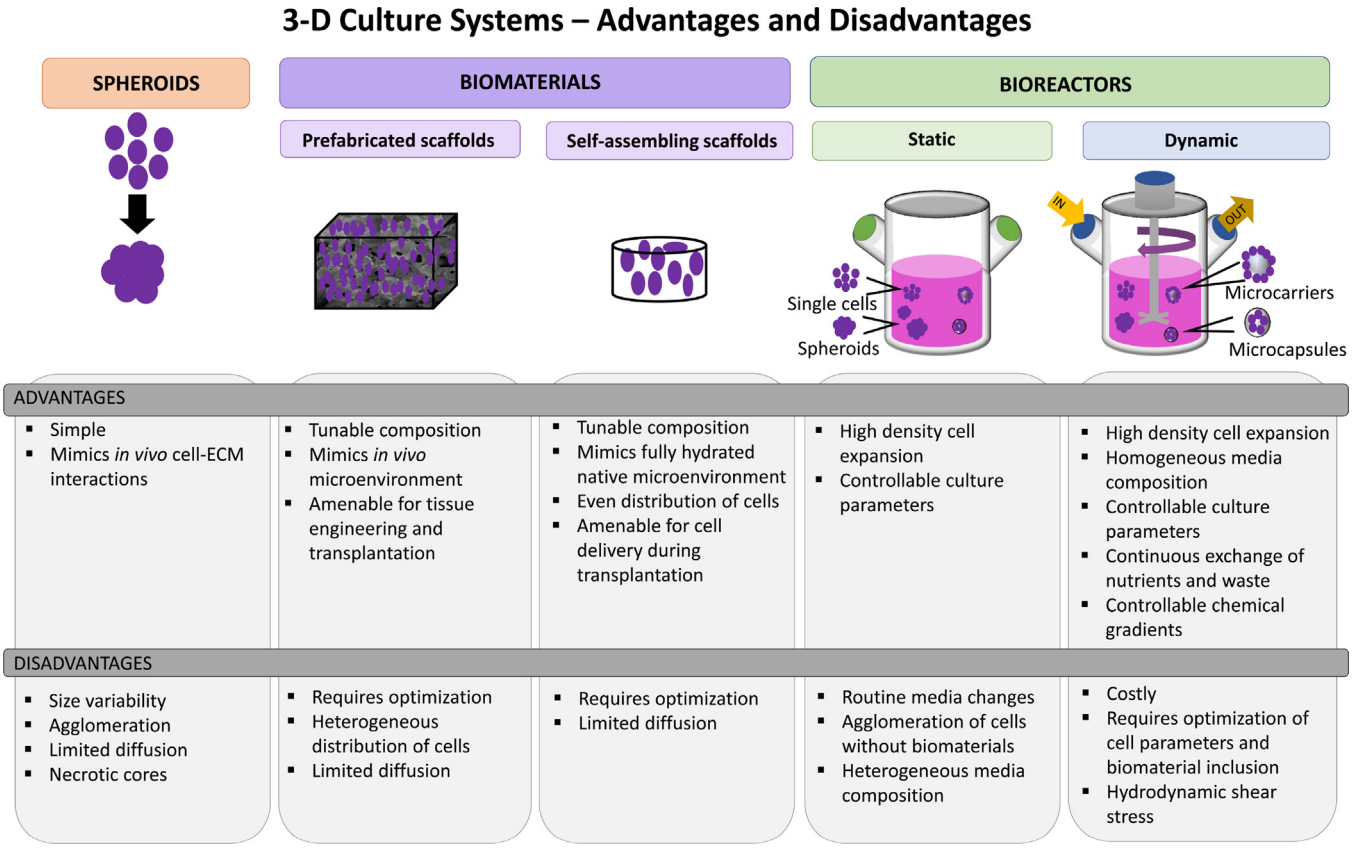

Fig. 5. Advantages and disadvantages of various 3-D culture systems.

cell populations and microenvironments, including the formation of necrotic/hypoxic cores. The inclusion of biomaterials can improve cell growth and differentiation *via* inductive biochemical and mechanical signaling by mimicking the *in vivo* ECM. Determination of the appropriate biomaterials and mechanical properties of 3-D scaffolds must be optimized for each cell type and application. Additionally, scaffolds derived from biological materials may result in poor reproducibility due to lot-to-lot variability. Bioreactors support large-scale growth and/or differentiation of SCs. Static bioreactors are limited by agglomeration of cells in the absence of biomaterials (either microcarriers or microcapsules) and by need for batch media changes. In contrast, dynamic bioreactors can be highly controllable, *via* agitation and media perfusion allowing for complex homogenous media and cell dispersion. However, these methods are costly and are also affected by the introduction of hydrodynamic stress on cells, which can be limited by the use of biomaterials. Additional optimization is needed to determine the ideal culture parameters, and means of cell analysis during large-scale culture.

4. Challenges

Since the isolation of human ESCs [4], significant progress has been made in understanding the mechanisms of self-renewal, pluripotency, and differentiation using selective media. Likewise, MSCs have been isolated from various tissues and cultured as well as differentiated into mesodermal lineages. Furthermore, new xeno-free media have been developed to culture SCs that can be used for clinical purposes. However, progress regarding the propagation of other SCs such as hematopoietic SCs and progenitors remains sparse. Even the expansion and differentiation of ESCs and MSCs face a number of challenges. These challenges include:

4.1. Heterogeneity of cultured cells

2-D culture techniques are inherently inefficient, labor intensive and expensive, and yield limited expansion of SCs. Moreover, 2-D cultures yield heterogeneous populations of SCs and derivatives. Both ESCs and MSCs tend to undergo non-specific differentiation under 2-D culture conditions. Even directed differentiation of SCs into specific lineages is often only partially achieved and the differentiated derivatives lack functionality. Furthermore, survival, integration, and function of augmented cells grown in 2-D culture conditions are limited. Overall, scale-up of 2-D cultures is difficult and cells cultured under 2-D conditions often fail to mimic the natural microenvironment *in vivo* for both maintenance and differentiation of SCs.

4.2. Clonal growth

Clonal growth is correlated to the self-renewal of SCs. Decreased clonal growth of SCs is indicative of the reduction of self-renewal capacity and limited SC expansion, particularly for adult SCs such as MSCs. Passaging of MSCs have been shown to reduce clonal growth, proliferative capacity, and self-renewal potential. Human ESCs survive poorly and lose clonogenicity after single cell dissociation, limiting their proliferative potential. Synchronized clonal growth of single cells is important to obtain a homogenous population of cells. Therefore, improvements in the culturing of MSCs and pluripotent SCs are necessary for large scale and long-term expansion of these cells.

4.3. Biomaterials

Often 3-D culture is facilitated by incorporation of biomaterials to mimic the biochemical and biophysical properties of the native

3-D microenvironment. Numerous natural and synthetic biomaterials have been used to develop suitable scaffolds to mimic *in vivo* conditions for cell growth. However, it is often difficult to determine which biomaterial would be most appropriate for the growth and differentiation of a particular SC type. For instance, different biomaterials induce variability and reduce reproducibility when used in culture. Biomaterials are also known to play role in signaling that could affect maintenance and differentiation of SCs. Therefore, further studies are needed to identify appropriate biomaterials that could improve quality and quantity of cultured cells.

4.4. Optimization of culture conditions

Despite the rapid increase in the volume of literature regarding the growth and differentiation of SCs, standardized methods for culturing cells are lacking. 2-D culturing methods are simple and commonly practiced but are less efficient. Efforts are needed to develop 3-D culture systems, which while more efficient involve a number of parameters, including media composition, biomaterials, mechanical stress, oxygen content, pH, and media perfusion, which influence both the quality and quantity of cultured cells. In addition, agglomeration of cells and diffusion of nutrient/waste exchange are also important for standardization of the culture methods. This can result in heterogeneous cell populations, necrosis, and inhibition of cell proliferation.

Recent advances in the use of various bioreactors to expand or differentiate SCs are encouraging. Dynamic bioreactors have shown greater promise compared to static bioreactors. However, dynamic bioreactors, which employ agitation or media perfusion could introduce hydrodynamic shear stress, negatively effecting cell viability.

4.5. Functional activity

One of the major considerations of any culturing method is that the cultured cells should have optimal viability and exhibit functional activity comparable to cells found *in vivo*. These cells should also be able to survive and integrate into the tissue *in vivo*. Cells exposed to hypoxic stress have been shown to be more apt to survive upon transplantation. Therefore, cell conditioning should be an important consideration prior to their use for clinical purposes. Furthermore, development of efficient methods yielding homogenously differentiated cells from SCs may help to yield reproducible results but are challenging. Future studies should be focused on investigating the temporal factors that emulate *in vivo* conditions to devise microenvironments using appropriate biomaterials specific for growth and differentiation of SCs.

4.6. Culture scale up

There is a great need to produce large quantities of high quality cells for various biomedical applications. Current methods for cell expansion yield millions of cells, which have limited autologous usage for individual patients. Even that may not be effective as recent studies have shown that higher concentrations of transplanted cells are more efficacious. Allogenic cell therapy for multi-center clinical trials requires billions to trillions of cells. Scale up expansion of cells to such levels would require substantial advancements in the media, culturing devices and techniques. One advantage of large scale culturing of cells is that they can be used for cell therapy for multiple patients in a clinical trial and results can be correlated. However, large-scale production systems involving bioreactors and GMP facilities necessitate collaboration between biomedical researchers and engineers. In addition, a combination of techniques, including biomaterials modified with growth factors or ligands as well as other growth parameters to optimally grow and differentiate SCs into specific cell types would be essential to further advance the fields of cell therapy, tissue engineering and regenerative medicine.

5. Conclusion

Recently, significant advancements in the culturing of SCs, particularly using biomaterials and bioreactors, have been reported. These 3-D culture methods not only overcome the limitations associated with 2-D culture but also have the potential to expand cells to billion fold as would be needed for clinical trials, pharmaceutical or biotechnology applications. 3-D culturing of cells using bioinductive, biodegradable and biocompatible polymers mimicking the ECM, dimensionality and spatial gradients found in the native microenvironments is not only helpful in expansion and improving functionality but are also amenable for specific differentiation of SCs. Scale-up production of cells requiring bioreactors and GMP facilities would benefit from the multidisciplinary collaborations among the biomedical researchers and engineers. Despite challenges, advances in 3-D culture systems will provide impetus for accelerating the therapeutic uses of SCs to treat many diseases and disorders that have no cure.

Disclosure statement

No competing financial interests exist.

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References

[1] S.J. Morrison, N.M. Shah, D.J. Anderson, Regulatory mechanisms in stem cell biology, Cell 88 (1997) 287–298.
[2] B.E. Reubinoff, M.F. Pera, C.-Y. Fong, A. Trounson, A. Bongso, Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro, Nat. Biotechnol. 18 (2000) 399–404.
[3] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[4] J.A. Thomson, J. Itskovitz-Eldor, S.S. Shapiro, M.A. Waknitz, J.J. Swiergiel, V.S. Marshall, et al., Embryonic stem cell lines derived from human blastocysts, Science 282 (1998) 1145–1147.
[5] I. Chambers, S.R. Tomlinson, The transcriptional foundation of pluripotency, Development 136 (2009) 2311–2322.
[6] K. Bieback, S. Kern, A. Kocaomer, K. Ferlik, P. Bugert, Comparing mesenchymal stromal cells from different human tissues: bone marrow, adipose tissue and umbilical cord blood, Bio-Med. Mater. Eng. 18 (2008) S71–6.
[7] N. Beeravolu, C. McKee, A. Alamri, S. Mikhael, C. Brown, M. Perez-Cruet, et al., Isolation and characterization of mesenchymal stromal cells from human umbilical cord and fetal placenta, J. Vis. Exp. 122 (2017) e55224.
[8] S. Mikhael, N. Beeravolu, G.R. Chaudhry, Umbilical cord derivatives for intervertebral disc regeneration: advances and challenges, Cell Gene Ther. Insights 2 (2016) 629–634.
[9] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, et al., Minimal criteria for defining multipotent mesenchymal stromal cells: The International Society for Cellular Therapy position statement, Cytotherapy 8 (2006) 315–317.
[10] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, et al., Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[11] L. Sensebé, M. Gadelorge, S. Fleury-Cappellesso, Production of mesenchymal stromal/stem cells according to good manufacturing practices: a review, Stem Cell Res. Ther. 4 (2013) 66-.
[12] N. Beeravolu, I. Khan, C. McKee, S. Dinda, B. Thibodeau, G. Wilson, et al., Isolation and comparative analysis of potential stem/progenitor cells from different regions of human umbilical cord, Stem Cell Res. 16 (2016) 696–711.

[13] J. Jung, S. Buisman, G. de Haan, Hematopoiesis during development, aging, and disease, Exp. Hematol. 44 (2016) 689–695.
[14] P. Kumar, Y. Tan, P. Cahan, Understanding development and stem cells using single cell-based analyses of gene expression, Development 144 (2017) 17–32.
[15] J. Mathieu, H. Ruohola-Baker, Regulation of stem cell populations by microRNAs, Adv. Exp. Med. Biol. 786 (2013) 329–351.
[16] K. Takahashi, S. Yamanaka, A developmental framework for induced pluripotency, Development 142 (2015) 3274–3285.
[17] J.K. Van Camp, S. Beckers, D. Zegers, W. Van Hul, Wnt signaling and the control of human stem cell fate, Stem Cell Rev. 10 (2014) 207–229.
[18] Z. Gamie, R.J. MacFarlane, A. Tomkinson, A. Moniakis, G.T. Tran, Y. Gamie, et al., Skeletal tissue engineering using mesenchymal or embryonic stem cells: clinical and experimental data, Expert Opin. Biol. Ther. 14 (2014) 1611–1639.
[19] P.J. Ho, M.L. Yen, S.F. Yet, B.L. Yen, Current applications of human pluripotent stem cells: possibilities and challenges, Cell Transplant. 21 (2012) 801–814.
[20] S.S. Hung, S. Khan, C.Y. Lo, A.W. Hewitt, R.C. Wong, Drug discovery using induced pluripotent stem cell models of neurodegenerative and ocular diseases, Pharmacol. Ther. (2017).
[21] L. Wei, Z.Z. Wei, M.Q. Jiang, O. Mohamad, S.P. Yu, Stem cell transplantation therapy for multifaceted therapeutic benefits after stroke, Prog. Neurobiol. (2017).
[22] K.G. Chen, B.S. Mallon, R.D. McKay, P.G. Robey, Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics, Cell Stem Cell 14 (2014) 13–26.
[23] George Q. Daley, The promise and perils of stem cell therapeutics, Cell Stem Cell 10 (2012) 740–749.
[24] R. Krawetz, J.T. Taiani, S. Liu, G. Meng, X. Li, M.S. Kallos, et al., Large-scale expansion of pluripotent human embryonic stem cells in stirred-suspension bioreactors, Tissue Eng. Part C Methods 16 (2010) 573–582.
[25] J.A. Burdick, G. Vunjak-Novakovic, Engineered microenvironments for controlled stem cell differentiation, Tissue Eng. Part A 15 (2009) 205–219.
[26] F. Guilak, D.M. Cohen, B.T. Estes, J.M. Gimble, W. Liedtke, C.S. Chen, Control of stem cell fate by physical interactions with the extracellular matrix, Cell Stem Cell 5 (2009) 17–26.
[27] E. Knight, S. Przyborski, Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro, J. Anat. 227 (2015) 746–756.
[28] C.H. Thomas, J.H. Collier, C.S. Sfeir, K.E. Healy, Engineering gene expression and protein synthesis by modulation of nuclear shape, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 1972–1977.
[29] L. Vergani, M. Grattarola, C. Nicolini, Modifications of chromatin structure and gene expression following induced alterations of cellular shape, Int. J. Biochem. Cell Biol. 36 (2004) 1447–1461.
[30] P.A. Janmey, R.T. Miller, Mechanisms of mechanical signaling in development and disease, J. Cell Sci. 124 (2011) 9–18.
[31] P.P. Provenzano, P.J. Keely, Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling, J. Cell Sci. 124 (2011) 1195–1205.
[32] A. Skardal, T. Shupe, A. Atala, Organoid-on-a-chip and body-on-a-chip systems for drug screening and disease modeling, Drug Discov. Today. 21 (2016) 1399–1411.
[33] S.G. Almalki, D.K. Agrawal, Key transcription factors in the differentiation of mesenchymal stem cells, Differentiation 92 (2016) 41–51.
[34] H. Yamamoto, G. Quinn, A. Asari, H. Yamanokuchi, T. Teratani, M. Terada, et al., Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application, Hepatology (Baltimore, Md) 37 (2003) 983–993.
[35] N.S. Hwang, S. Varghese, J. Elisseeff, Controlled differentiation of stem cells, Adv. Drug Deliv. Rev. 60 (2008) 199–214.
[36] J.W. Haycock, 3D cell culture: a review of current approaches and techniques, Methods Mol. Biol. 695 (2011) 1–15.
[37] K. Bhadriraju, C.S. Chen, Engineering cellular microenvironments to improve cell-based drug testing, Drug Discov. Today 7 (2002) 612–620.
[38] J. Lee, M.J. Cuddihy, N.A. Kotov, Three-dimensional cell culture matrices: state of the art, Tissue Eng. Part B Rev. 14 (2008) 61–86.
[39] E. Fuchs, T. Tumbar, G. Guasch, Socializing with the neighbors: stem cells and their niche, Cell 116 (2004) 769–778.
[40] F. Gattazzo, A. Urciuolo, P. Bonaldo, Extracellular matrix: a dynamic microenvironment for stem cell niche, Biochim. Biophys. Acta 1840 (2014) 2506–2519.
[41] P.G. Robey, S.A. Kuznetsov, M. Riminucci, P. Bianco, Bone marrow stromal cell assays: in vitro and in vivo, Methods Mol. Biol. 1130 (2014) 279–293.
[42] L. Jongpaiboonkit, W.J. King, W.L. Murphy, Screening for 3D environments that support human mesenchymal stem cell viability using hydrogel arrays, Tissue Eng. Part A 15 (2009) 343–353.
[43] C.R. Kothapalli, R.D. Kamm, 3D matrix microenvironment for targeted differentiation of embryonic stem cells into neural and glial lineages, Biomaterials 34 (2013) 5995–6007.
[44] Y. Liu, L.F. Charles, T.I. Zarembinski, K.I. Johnson, S.K. Atzet, R.L. Wesselschmidt, et al., Modified hyaluronan hydrogels support the maintenance of mouse embryonic stem cells and human induced pluripotent stem cells, Macromol. Biosci. 12 (2012) 1034–1042.
[45] L.G. Villa-Diaz, A.M. Ross, J. Lahann, P.H. Krebsbach, Concise review: the evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings, Stem Cells 31 (2013) 1–7.
[46] B.M. Baker, C.S. Chen, Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues, J. Cell Sci. 125 (2012) 3015–3024.
[47] A.W. Lund, B. Yener, J.P. Stegemann, G.E. Plopper, The natural and engineered 3D microenvironment as a regulatory cue during stem cell fate determination, Tissue Eng. Part B Rev. 15 (2009) 371–380.
[48] M.P. Lutolf, P.M. Gilbert, H.M. Blau, Designing materials to direct stem-cell fate, Nature 462 (2009) 433–441.
[49] K. Saha, J.F. Pollock, D.V. Schaffer, K.E. Healy, Designing synthetic materials to control stem cell phenotype, Curr. Opin. Chem. Biol. 11 (2007) 381–387.
[50] J. Jozefczuk, K. Drews, J. Adjaye, Preparation of mouse embryonic fibroblast cells suitable for culturing human embryonic and induced pluripotent stem cells, J. Vis. Exp. 64 (2012) 3854.
[51] C. Fecek, D. Yao, A. Kacorri, A. Vasquez, S. Iqbal, H. Sheikh, et al., Chondrogenic derivatives of embryonic stem cells seeded into 3D polycaprolactone scaffolds generated cartilage tissue in vivo, Tissue Eng. Part A 14 (2008) 1403–1413.
[52] J. Beers, D.R. Gulbranson, N. George, L.I. Siniscalchi, J. Jones, J.A. Thomson, et al., Passaging and colony expansion of human pluripotent stem cells by enzyme-free dissociation in chemically defined culture conditions, Nat. Protoc. 7 (2012) 2029–2040.
[53] G. Chen, D.R. Gulbranson, Z. Hou, J.M. Bolin, V. Ruotti, M.D. Probasco, et al., Chemically defined conditions for human iPSC derivation and culture, Nat. Methods 8 (2011) 424–429.
[54] T.W. Theunissen, J.C. Silva, Switching on pluripotency: a perspective on the biological requirement of Nanog, Philos. Trans. R Soc. Lond. B Biol. Sci. 366 (2011) 2222–2229.
[55] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, et al., A ROCK inhibitor permits survival of dissociated human embryonic stem cells, Nat. Biotechnol. 25 (2007) 681–686.
[56] G. Huang, S. Ye, X. Zhou, D. Liu, Q.L. Ying, Molecular basis of embryonic stem cell self-renewal: from signaling pathways to pluripotency network, Cell. Mol. Life Sci. 72 (2015) 1741–1757.
[57] J. Nichols, A. Smith, Naive and primed pluripotent states, Cell Stem Cell 4 (2009) 487–492.
[58] O. Gafni, L. Weinberger, A.A. Mansour, Y.S. Manor, E. Chomsky, D. Ben-Yosef, et al., Derivation of novel human ground state naive pluripotent stem cells, Nature 504 (2013) 282–286.
[59] S.M. Badenes, T.G. Fernandes, C.S.M. Cordeiro, S. Boucher, D. Kuninger, M.C. Vemuri, et al., Defined essential 8™ medium and vitronectin efficiently support scalable xeno-free expansion of human induced pluripotent stem cells in stirred microcarrier culture systems, PLoS One 11 (2016) e0151264.
[60] I. Batalov, A.W. Feinberg, Differentiation of cardiomyocytes from human pluripotent stem cells using monolayer culture, Biomark. Insights 10 (2015) 71–76.
[61] A. Trounson, Human embryonic stem cells: mother of all cell and tissue types, Reprod. Biomed. Online 4 (Suppl. 1) (2002) 58–63.
[62] L. Duplomb, M. Dagouassat, P. Jourdon, D. Heymann, Concise review: embryonic stem cells: a new tool to study osteoblast and osteoclast differentiation, Stem Cells 25 (2007) 544–552.
[63] M.A. Laflamme, K.Y. Chen, A.V. Naumova, V. Muskheli, J.A. Fugate, S.K. Dupras, et al., Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts, Nat. Biotechnol. 25 (2007) 1015–1024.
[64] B. Lucendo-Villarin, H. Rashidi, K. Cameron, D.C. Hay, Pluripotent stem cell derived hepatocytes: using materials to define cellular differentiation and tissue engineering, J. Mater. Chem. B Mater. Biol. Med. 4 (2016) 3433–3442.
[65] T. Ishii, K. Fukumitsu, K. Yasuchika, K. Adachi, E. Kawase, H. Suemori, et al., Effects of extracellular matrixes and growth factors on the hepatic differentiation of human embryonic stem cells, Am. J. Physiol. Gastrointest. Liver Physiol. 295 (2008) G313–21.
[66] C. Sadegh, J.D. Macklis, Established monolayer differentiation of mouse embryonic stem cells generates heterogeneous neocortical-like neurons stalled at a stage equivalent to midcorticogenesis, J. Comp. Neurol. 522 (2014) 2691–2706.
[67] E.T. Pineda, R.M. Nerem, T. Ahsan, Differentiation patterns of embryonic stem cells in two- versus three-dimensional culture, Cells Tissues Organs 197 (2013) 399–410.
[68] F. Santos, P.Z. Andrade, M.M. Abecasis, J.M. Gimble, L.G. Chase, A.M. Campbell, et al., Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture system under xeno-free conditions, Tissue Eng Part C Methods 17 (2011) 1201–1210.
[69] J.L. Wilson, T.C. McDevitt, Stem cell microencapsulation for phenotypic control, bioprocessing, and transplantation, Biotechnol. Bioeng. 110 (2013) 667–682.
[70] W.L. Grayson, F. Zhao, B. Bunnell, T. Ma, Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells, Biochem. Biophys. Res. Commun. 358 (2007) 948–953.
[71] C. Bartmann, E. Rohde, K. Schallmoser, P. Purstner, G. Lanzer, W. Linkesch, et al., Two steps to functional mesenchymal stromal cells for clinical application, Transfusion 47 (2007) 1426–1435.
[72] K.M. Panchalingam, S. Jung, L. Rosenberg, L.A. Behie, Bioprocessing strategies for the large-scale production of human mesenchymal stem cells: a review, Stem Cell Res. Ther. 6 (2015) 225.
[73] P.J. Hanley, Z. Mei, M. da Graca Cabreira-Hansen, M. Klis, W. Li, Y. Zhao, et al., Manufacturing mesenchymal stromal cells for phase I clinical trials, Cytotherapy 15 (2013) 416–422.

[74] A.I. Caplan, Mesenchymal stem cells, J. Orthop. Res. 9 (1991) 641–650.
[75] A.J. Friedenstein, R.K. Chailakhyan, N.V. Latsinik, A.F. Panasyuk, I.V. Keiliss-Borok, Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues: cloning in vitro and retransplantation in vivo, Transplantation 17 (1974) 331–340.
[76] K. Fukuda, Molecular characterization of regenerated cardiomyocytes derived from adult mesenchymal stem cells, Congenit. Anom. 42 (2002) 1–9.
[77] J.J. Bara, R.G. Richards, M. Alini, M.J. Stoddart, Concise review: bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic, Stem Cells 32 (2014) 1713–1723.
[78] S.P. Bruder, N. Jaiswal, S.E. Haynesworth, Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation, J. Cell Biochem. 64 (1997) 278–294.
[79] G.R. Erickson, J.M. Gimble, D.M. Franklin, H.E. Rice, H. Awad, F. Guilak, Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo, Biochem. Biophys. Res. Commun. 290 (2002) 763–769.
[80] S. Arnhold, Y. Absenger, H. Klein, K. Addicks, U. Schraermeyer, Transplantation of bone marrow-derived mesenchymal stem cells rescue photoreceptor cells in the dystrophic retina of the rhodopsin knockout mouse, Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie 245 (2007) 414–422.
[81] H. Rezanejad, Z.S. Soheili, F. Haddad, M.M. Matin, S. Samiei, A. Manafi, et al., In vitro differentiation of adipose-tissue-derived mesenchymal stem cells into neural retinal cells through expression of human PAX6 (5a) gene, Cell Tissue Res. 356 (2014) 65–75.
[82] A. Hermann, R. Gastl, S. Liebau, M.O. Popa, J. Fiedler, B.O. Boehm, et al., Efficient generation of neural stem cell-like cells from adult human bone marrow stromal cells, J. Cell Sci. 117 (2004) 4411–4422.
[83] K.L. Mung, Y.P. Tsui, E.W. Tai, Y.S. Chan, D.K. Shum, G.K. Shea, Rapid and efficient generation of neural progenitors from adult bone marrow stromal cells by hypoxic preconditioning, Stem Cell Res. Ther. 7 (2016) 146.
[84] K.D. Lee, T.K. Kuo, J. Whang-Peng, Y.F. Chung, C.T. Lin, S.H. Chou, et al., In vitro hepatic differentiation of human mesenchymal stem cells, Hepatology (Baltimore, Md) 40 (2004) 1275–1284.
[85] X. Zhou, L. Cui, X. Zhou, Q. Yang, L. Wang, G. Guo, et al., Induction of hepatocyte-like cells from human umbilical cord-derived mesenchymal stem cells by defined microRNAs, J. Cell Mol. Med. 21 (2017) 881–893.
[86] Z. Mehrfarjam, F. Esmaeili, L. Shabani, E. Ebrahimie, Induction of pancreatic beta cell gene expression in mesenchymal stem cells, Cell Biol. Int. 40 (2016) 486–500.
[87] C. Moriscot, F. de Fraipont, M.J. Richard, M. Marchand, P. Savatier, D. Bosco, et al., Human bone marrow mesenchymal stem cells can express insulin and key transcription factors of the endocrine pancreas developmental pathway upon genetic and/or microenvironmental manipulation in vitro, Stem Cells 23 (2005) 594–603.
[88] W. Xu, G.-X. Xu, Mesenchymal stem cells for retinal diseases, Int. J. Ophthalmol. 4 (2011) 413–421.
[89] X. Sun, J. Ge, R. Jiang, S. Duan, G. Song, Y. Duan, et al., Induced differentiation of bone marrow mesenchymal stem cells towards retinal ganglion precursor cells, Cell Res. 18 (2008) S76–S.
[90] P.R. Baraniak, T.C. McDevitt, Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential, Cell Tissue Res. 347 (2012) 701–711.
[91] G.M. Keller, In vitro differentiation of embryonic stem cells, Curr. Opin. Cell Biol. 7 (1995) 862–869.
[92] M.C. Lewis, B.D. MacArthur, R.S. Tare, R.O. Oreffo, C.P. Please, Extracellular matrix deposition in engineered micromass cartilage pellet cultures: measurements and modelling, PLoS One 11 (2016) e0147302.
[93] Y. Wang, M.H. Kim, S.R. Tabaei, J.H. Park, K. Na, S. Chung, et al., Spheroid formation of hepatocarcinoma cells in microwells: experiments and monte carlo simulations, PLoS One 11 (2016) e0161915.
[94] R. Edmondson, J.J. Broglie, A.F. Adcock, L. Yang, Three-dimensional cell culture systems and their applications in drug discovery and cell-based biosensors, Assay Drug Dev. Technol. 12 (2014) 207–218.
[95] A. Abbott, Cell culture: biology's new dimension, Nature 424 (2003) 870–872.
[96] J. Itskovitz-Eldor, M. Schuldiner, D. Karsenti, A. Eden, O. Yanuka, M. Amit, et al., Differentiation of human embryonic stem cells into embryoid bodies compromising the three embryonic germ layers, Mol. Med. (Cambridge, Mass) 6 (2000) 88–95.
[97] M.Y. Son, H.J. Kim, M.J. Kim, Y.S. Cho, Physical passaging of embryoid bodies generated from human pluripotent stem cells, PLoS One 6 (2011) e19134.
[98] J. Stenberg, M. Elovsson, R. Strehl, E. Kilmare, J. Hyllner, A. Lindahl, Sustained embryoid body formation and culture in a non-laborious three dimensional culture system for human embryonic stem cells, Cytotechnology 63 (2011) 227–237.
[99] B. Valamehr, S.J. Jonas, J. Polleux, R. Qiao, S. Guo, E.H. Gschweng, et al., Hydrophobic surfaces for enhanced differentiation of embryonic stem cell-derived embryoid bodies, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 14459–14464.
[100] S.-H. Hong, T. Werbowetski-Ogilvie, V. Ramos-Mejia, J.B. Lee, M. Bhatia, Multiparameter comparisons of embryoid body differentiation toward human stem cell applications, Stem Cell Res. 5 (2010) 120–130.
[101] M.D. Ungrin, C. Joshi, A. Nica, C. Bauwens, P.W. Zandstra, Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates, PLoS One 3 (2008) e1565.
[102] C.L. Bauwens, R. Peerani, S. Niebruegge, K.A. Woodhouse, E. Kumacheva, M. Husain, et al., Control of human embryonic stem cell colony and aggregate size heterogeneity influences differentiation trajectories, Stem Cells 26 (2008) 2300–2310.
[103] P.W. Burridge, D. Anderson, H. Priddle, M.D. Barbadillo Munoz, S. Chamberlain, C. Allegrucci, et al., Improved human embryonic stem cell embryoid body homogeneity and cardiomyocyte differentiation from a novel V-96 plate aggregation system highlights interline variability, Stem Cells 25 (2007) 929–938.
[104] J. Park, C.H. Cho, N. Parashurama, Y. Li, F. Berthiaume, M. Toner, et al., Microfabrication-based modulation of embryonic stem cell differentiation, Lab Chip 7 (2007) 1018–1028.
[105] R. Peerani, B.M. Rao, C. Bauwens, T. Yin, G.A. Wood, A. Nagy, et al., Niche-mediated control of human embryonic stem cell self-renewal and differentiation, EMBO J. 26 (2007) 4744–4755.
[106] G. Pettinato, R. Ramanathan, R.A. Fisher, M.J. Mangino, N. Zhang, X. Wen, Scalable differentiation of human iPSCs in a multicellular spheroid-based 3D culture into hepatocyte-like cells through direct wnt/beta-catenin pathway inhibition, Sci. Rep. 6 (2016) 32888.
[107] K. Subramanian, D.J. Owens, R. Raju, M. Firpo, T.D. O'Brien, C.M. Verfaillie, et al., Spheroid culture for enhanced differentiation of human embryonic stem cells to hepatocyte-like cells, Stem Cells Dev. 23 (2014) 124–131.
[108] N. Kachamakova-Trojanowska, W. Nowak, K. Szade, J. Stepniewski, K. Bukowska-Strakova, M. Zukowska, et al., Generation of functional endothelial cells with progenitor-like features from murine induced pluripotent stem cells, Vascul. Pharmacol. 86 (2016) 94–108.
[109] L. Tian, A. Deshmukh, Z. Ye, Y.Y. Jang, Efficient and controlled generation of 2D and 3D bile duct tissue from human pluripotent stem cell-derived spheroids, Stem Cell Rev. 12 (2016) 500–508.
[110] S.E. Kim, S.Y. An, D.H. Woo, J. Han, J.H. Kim, Y.J. Jang, et al., Engraftment potential of spheroid-forming hepatic endoderm derived from human embryonic stem cells, Stem Cells Dev. 22 (2013) 1818–1829.
[111] W. Wang, K. Itaka, S. Ohba, N. Nishiyama, U.I. Chung, Y. Yamasaki, et al., 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells, Biomaterials 30 (2009) 2705–2715.
[112] Y. Li, G. Guo, L. Li, F. Chen, J. Bao, Y.J. Shi, et al., Three-dimensional spheroid culture of human umbilical cord mesenchymal stem cells promotes cell yield and stemness maintenance, Cell Tissue Res. 360 (2015) 297–307.
[113] H. Guo, N.T. Ingolia, J.S. Weissman, D.P. Bartel, Mammalian microRNAs predominantly act to decrease target mRNA levels, Nature 466 (2010) 835–840.
[114] T.J. Bartosh, J.H. Ylostalo, A. Mohammadipoor, N. Bazhanov, K. Coble, K. Claypool, et al., Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 13724–13729.
[115] M.Y. Emmert, P. Wolint, S. Winklhofer, P. Stolzmann, N. Cesarovic, T. Fleischmann, et al., Transcatheter based electromechanical mapping guided intramyocardial transplantation and in vivo tracking of human stem cell based three dimensional microtissues in the porcine heart, Biomaterials 34 (2013) 2428–2441.
[116] S. Alimperti, P. Lei, Y. Wen, J. Tian, A.M. Campbell, S.T. Andreadis, Serum-free spheroid suspension culture maintains mesenchymal stem cell proliferation and differentiation potential, Biotechnol. Progr. 30 (2014) 974–983.
[117] A. Ruedel, S. Hofmeister, A.-K. Bosserhoff, Development of a model system to analyze chondrogenic differentiation of mesenchymal stem cells, Int. J. Clin. Exp. Path. 6 (2013) 3042–3048.
[118] Y. Yamaguchi, J. Ohno, A. Sato, H. Kido, T. Fukushima, Mesenchymal stem cell spheroids exhibit enhanced in-vitro and in-vivo osteoregenerative potential, BMC Biotechnol. 14 (2014) 105.
[119] A.M. Bratt-Leal, R.L. Carpenedo, M.D. Ungrin, P.W. Zandstra, T.C. McDevitt, Incorporation of biomaterials in multicellular aggregates modulates pluripotent stem cell differentiation, Biomaterials 32 (2011) 48–56.
[120] T.P. Kraehenbuehl, R. Langer, L.S. Ferreira, Three-dimensional biomaterials for the study of human pluripotent stem cells, Nat. Methods 8 (2011) 731–736.
[121] R. Webster, E. Didier, P. Harris, N. Siegel, J. Stadler, L. Tilbury, et al., PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies, Drug Metab. Dispos: Biol. Fate Chem. 35 (2007) 9–16.
[122] A.B. Allen, L.B. Priddy, M.-T.A. Li, R.E. Guldberg, Functional augmentation of naturally-Derived materials for tissue regeneration, Ann. Biomed. Eng. 43 (2015) 555–567.
[123] L. Krishna, K. Dhamodaran, C. Jayadev, K. Chatterjee, R. Shetty, S.S. Khora, et al., Nanostructured scaffold as a determinant of stem cell fate, Stem Cell Res. Ther. 7 (2016) 188.
[124] S.M. Dellatore, A.S. Garcia, W.M. Miller, Mimicking stem cell niches to increase stem cell expansion, Curr. Opin. Biotechnol. 19 (2008) 534–540.
[125] R.C. Dutta, A.K. Dutta, Cell-interactive 3D-scaffold; advances and applications, Biotechnol. Adv. 27 (2009) 334–339.
[126] B.P. Chan, K.W. Leong, Scaffolding in tissue engineering: general approaches and tissue-specific considerations, Eur. Spine J. 17 (2008) 467–479.

[127] I.M. El-Sherbiny, M.H. Yacoub, Hydrogel scaffolds for tissue engineering: progress and challenges, Global Cardiol. Sci. Pract. 2013 (2013) 316–342.
[128] H. Wang, S.C. Heilshorn, Adaptable hydrogel networks with reversible linkages for tissue engineering, Adv. Mater. (Deerfield Beach, Fla.) 27 (2015) 3717–3736.
[129] E. Busseron, Y. Ruff, E. Moulin, N. Giuseppone, Supramolecular self-assemblies as functional nanomaterials, Nanoscale 5 (2013) 7098–7140.
[130] J. Kopeček, J. Yang, Peptide-directed self-assembly of hydrogels, Acta Biomater. 5 (2009) 805–816.
[131] O.Z. Fisher, A. Khademhosseini, R. Langer, N.A. Peppas, Bioinspired materials for controlling stem cell fate, Acc. Chem. Res. 43 (2010) 419–428.
[132] D.S. Benoit, A.R. Durney, K.S. Anseth, Manipulations in hydrogel degradation behavior enhance osteoblast function and mineralized tissue formation, Tissue Eng. 12 (2006) 1663–1673.
[133] C. Hiemstra, Z. Zhong, M.J. van Steenbergen, W.E. Hennink, J. Feijen, Release of model proteins and basic fibroblast growth factor from in situ forming degradable dextran hydrogels, J. Control. Release 122 (2007) 71–78.
[134] J.M. Jukes, S.K. Both, A. Leusink, L.M. Sterk, C.A. van Blitterswijk, J. de Boer, Endochondral bone tissue engineering using embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 6840–6845.
[135] S.R. Van Tomme, G. Storm, W.E. Hennink, In situ gelling hydrogels for pharmaceutical and biomedical applications, Int. J. Pharm. 355 (2008) 1–18.
[136] Y. Lei, D.V. Schaffer, A fully defined and scalable 3D culture system for human pluripotent stem cell expansion and differentiation, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) E5039–48.
[137] M.F. Leong, H.F. Lu, T.C. Lim, C. Du, N.K. Ma, A.C. Wan, Electrospun polystyrene scaffolds as a synthetic substrate for xeno-free expansion and differentiation of human induced pluripotent stem cells, Acta Biomater. 46 (2016) 266–277.
[138] Y.J. Li, E.H. Chung, R.T. Rodriguez, M.T. Firpo, K.E. Healy, Hydrogels as artificial matrices for human embryonic stem cell self-renewal, J. Biomed. Mater. Res. A 79 (2006) 1–5.
[139] S. Gerecht, J.A. Burdick, L.S. Ferreira, S.A. Townsend, R. Langer, G. Vunjak-Novakovic, Hyaluronic acid hydrogel for controlled self-renewal and differentiation of human embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 104 (2007) 11298–11303.
[140] K. Xu, K. Narayanan, F. Lee, K.H. Bae, S. Gao, M. Kurisawa, Enzyme-mediated hyaluronic acid-tyramine hydrogels for the propagation of human embryonic stem cells in 3D, Acta Biomater. 24 (2015) 159–171.
[141] B.P. Toole, Hyaluronan in morphogenesis, Semin. Cell Dev. Biol. 12 (2001) 79–87.
[142] A. Mammoto, T. Mammoto, D.E. Ingber, Mechanosensitive mechanisms in transcriptional regulation, J. Cell Sci. 125 (2012) 3061–3073.
[143] C. McKee, M. Perez-Cruet, F. Chavez, G.R. Chaudhry, Simplified three-dimensional culture system for long-term expansion of embryonic stem cells, World J. Stem Cells 7 (2015) 1064–1077.
[144] E.K.A. Nur, I. Ahmed, J. Kamal, M. Schindler, S. Meiners, Three-dimensional nanofibrillar surfaces promote self-renewal in mouse embryonic stem cells, Stem Cells 24 (2006) 426–433.
[145] J. Wei, J. Han, Y. Zhao, Y. Cui, B. Wang, Z. Xiao, et al., The importance of three-dimensional scaffold structure on stemness maintenance of mouse embryonic stem cells, Biomaterials 35 (2014) 7724–7733.
[146] O. Demarteau, D. Wendt, A. Braccini, M. Jakob, D. Schafer, M. Heberer, et al., Dynamic compression of cartilage constructs engineered from expanded human articular chondrocytes, Biochem. Biophys. Res. Commun. 310 (2003) 580–588.
[147] C. McKee, Y. Hong, D. Yao, G.R. Chaudhry, Compression induced chondrogenic differentiation of embryonic stem cells in 3-D PDMS scaffolds, Tissue Eng. Part A 23 (2017) 426–435.
[148] L.A. Smith, X. Liu, J. Hu, P.X. Ma, The influence of three-dimensional nanofibrous scaffolds on the osteogenic differentiation of embryonic stem cells, Biomaterials 30 (2009) 2516–2522.
[149] N. Dehdilani, K. Shamsasenjan, A. Movassaghpour, P. Akbarzadehlaleh, B. Amoughli Tabrizi, H. Parsa, et al., Improved survival and hematopoietic differentiation of murine embryonic stem cells on electrospun polycaprolactone nanofiber, Cell J. (Yakhteh) 17 (2016) 629–638.
[150] G.J. Bakeine, J. Ban, G. Grenci, A. Pozzato, S.D. Zilio, M. Prasciolu, et al., Design, fabrication and evaluation of nanoscale surface topography as a tool in directing differentiation and organisation of embryonic stem-cell-derived neural precursors, Microelectron. Eng. 86 (2009) 1435–1438.
[151] S. Battista, D. Guarnieri, C. Borselli, S. Zeppetelli, A. Borzacchiello, L. Mayol, et al., The effect of matrix composition of 3D constructs on embryonic stem cell differentiation, Biomaterials 26 (2005) 6194–6207.
[152] H. Baharvand, S.M. Hashemi, S. Kazemi Ashtiani, A. Farrokhi, Differentiation of human embryonic stem cells into hepatocytes in 2D and 3D culture systems in vitro, Int. J. Dev. Biol. 50 (2006) 645–652.
[153] L. Ghasemi-Mobarakeh, M.P. Prabhakaran, L. Tian, E. Shamirzaei-Jeshvaghani, L. Dehghani, S. Ramakrishna, Structural properties of scaffolds: crucial parameters towards stem cells differentiation, World J. Stem Cells 7 (2015) 728–744.
[154] E. Garreta, E. Genove, S. Borros, C.E. Semino, Osteogenic differentiation of mouse embryonic stem cells and mouse embryonic fibroblasts in a three-dimensional self-assembling peptide scaffold, Tissue Eng. 12 (2006) 2215–2227.
[155] L.A. Smith, X. Liu, J. Hu, P.X. Ma, The influence of three dimensional nanofibrous scaffolds on the osteogenic differentiation of embryonic stem cells, Biomaterials 30 (2009) 2516–2522.
[156] J. Liu, H. Nie, Z. Xu, X. Niu, S. Guo, J. Yin, et al., The effect of 3D nanofibrous scaffolds on the chondrogenesis of induced pluripotent stem cells and their application in restoration of cartilage defects, PLoS One 9 (2014) e111566.
[157] H.J. Lim, M.C. Mosley, Y. Kurosu, L.A. Smith Callahan, Concentration dependent survival and neural differentiation of murine embryonic stem cells cultured on polyethylene glycol dimethacrylate hydrogels possessing a continuous concentration gradient of n-cadherin derived peptide His-Ala-Val-Asp-Lle, Acta Biomater. 56 (2017) 153–160.
[158] N.C. Hunt, D. Hallam, A. Karimi, C.B. Mellough, J. Chen, D.H. Steel, et al., 3D culture of human pluripotent stem cells in RGD-alginate hydrogel improves retinal tissue development, Acta Biomater. 49 (2017) 329–343.
[159] Y.P. Lo, Y.S. Liu, M.G. Rimando, J.H. Ho, K.H. Lin, O.K. Lee, Three-dimensional spherical spatial boundary conditions differentially regulate osteogenic differentiation of mesenchymal stromal cells, Sci. Rep. 6 (2016) 21253.
[160] Y. Lei, S. Gojgini, J. Lam, T. Segura, The spreading, migration and proliferation of mouse mesenchymal stem cells cultured inside hyaluronic acid hydrogels, Biomaterials 32 (2011) 39–47.
[161] N. Mehrban, E. Abelardo, A. Wasmuth, K.L. Hudson, L.M. Mullen, A.R. Thomson, et al., Assessing cellular response to functionalized α-helical peptide hydrogels, Adv. Healthcare Mater. 3 (2014) 1387–1391.
[162] S. Aggarwal, M.F. Pittenger, Human mesenchymal stem cells modulate allogeneic immune cell responses, Blood 105 (2005) 1815–1822.
[163] S. Meirelles Lda, A.M. Fontes, D.T. Covas, A.I. Caplan, Mechanisms involved in the therapeutic properties of mesenchymal stem cells, Cytokine Growth Factor Rev. 20 (2009) 419–427.
[164] A.E. Ropper, D.K. Thakor, I. Han, D. Yu, X. Zeng, J.E. Anderson, et al., Defining recovery neurobiology of injured spinal cord by synthetic matrix-assisted hMSC implantation, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E820–E829.
[165] K.C. Rustad, V.W. Wong, M. Sorkin, J.P. Glotzbach, M.R. Major, J. Rajadas, et al., Enhancement of mesenchymal stem cell angiogenic capacity and stemness by a biomimetic hydrogel scaffold, Biomaterials 33 (2012) 80–90.
[166] B. Sadlik, G. Jaroslawski, D. Gladysz, M. Puszkarz, M. Markowska, K. Pawelec, et al., Knee cartilage regeneration with umbilical cord mesenchymal stem cells embedded in collagen scaffold using dry arthroscopy technique, Adv. Exp. Med. Biol. (2017).
[167] S.E. Hanson, S.N. King, J. Kim, X. Chen, S.L. Thibeault, P. Hematti, The effect of mesenchymal stromal cell-hyaluronic acid hydrogel constructs on immunophenotype of macrophages, Tissue Eng. Part A 17 (2011) 2463–2471.
[168] H. Lv, L. Li, M. Sun, Y. Zhang, L. Chen, Y. Rong, et al., Mechanism of regulation of stem cell differentiation by matrix stiffness, Stem Cell Res. Ther. 6 (2015) 103.
[169] G.J. Her, H.C. Wu, M.H. Chen, M.Y. Chen, S.C. Chang, T.W. Wang, Control of three-dimensional substrate stiffness to manipulate mesenchymal stem cell fate toward neuronal or glial lineages, Acta Biomater. 9 (2013) 5170–5180.
[170] N. Huebsch, P.R. Arany, A.S. Mao, D. Shvartsman, O.A. Ali, S.A. Bencherif, et al., Harnessing traction-mediated manipulation of the cell/matrix interface to control stem-cell fate, Nat. Mater. 9 (2010) 518–526.
[171] J.S. Park, J.S. Chu, A.D. Tsou, R. Diop, Z. Tang, A. Wang, et al., The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-beta, Biomaterials 32 (2011) 3921–3930.
[172] A.J. Steward, D.J. Kelly, Mechanical regulation of mesenchymal stem cell differentiation, J. Anat. 227 (2015) 717–731.
[173] Y.S. Pek, A.C. Wan, J.Y. Ying, The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel, Biomaterials 31 (2010) 385–391.
[174] L. Bian, D.Y. Zhai, E.C. Zhang, R.L. Mauck, J.A. Burdick, Dynamic compressive loading enhances cartilage matrix synthesis and distribution and suppresses hypertrophy in hMSC-laden hyaluronic acid hydrogels, Tissue Eng. Part A 18 (2012) 715–724.
[175] C.B. Horner, K. Hirota, J. Liu, M. Maldonado, B.H. Park, Nam J. Magnitude-Dependent, Inversely-related osteogenic/chondrogenic differentiation of human mesenchymal stem cells under dynamic compressive strain, J. Tissue Eng. Regen. Med. (2016).
[176] E. Michalopoulos, R.L. Knight, S. Korossis, J.N. Kearney, J. Fisher, E. Ingham, Development of methods for studying the differentiation of human mesenchymal stem cells under cyclic compressive strain, Tissue Eng. Part C Methods 18 (2012) 252–262.
[177] Y. Cheng, D. Ramos, P. Lee, D. Liang, X. Yu, S.G. Kumbar, Collagen functionalized bioactive nanofiber matrices for osteogenic differentiation of mesenchymal stem cells: bone tissue engineering, J. Biomed. Nanotechnol. 10 (2014) 287–298.
[178] J. George, Y. Kuboki, T. Miyata, Differentiation of mesenchymal stem cells into osteoblasts on honeycomb collagen scaffolds, Biotechnol. Bioeng. 95 (2006) 404–411.
[179] F. Fensky, J.C. Reichert, A. Traube, L. Rackwitz, S. Siebenlist, U. Noth, Chondrogenic predifferentiation of human mesenchymal stem cells in collagen type I hydrogels, Biomedizinische Technik Biomed. Eng. 59 (2014) 375–383.
[180] Y.L. Lin, C.P. Chen, C.M. Lo, H.S. Wang, Stiffness-controlled three-dimensional collagen scaffolds for differentiation of human Wharton's jelly mesenchymal stem cells into cardiac progenitor cells, J. Biomed. Mater. Res. A 104 (2016) 2234–2242.

[181] C. Chung, J.A. Burdick, Influence of 3D hyaluronic acid microenvironments on mesenchymal stem cell chondrogenesis, Tissue Eng. Part A 15 (2009) 243–254.
[182] I.L. Kim, S. Khetan, B.M. Baker, C.S. Chen, J.A. Burdick, Fibrous hyaluronic acid hydrogels that direct MSC chondrogenesis through mechanical and adhesive cues, Biomaterials 34 (2013) 5571–5580.
[183] Y. He, Y. Dong, X. Chen, R. Lin, Ectopic osteogenesis and scaffold biodegradation of tissue engineering bone composed of chitosan and osteo-induced bone marrow mesenchymal stem cells in vivo, Chin. Med. J. 127 (2014) 322–328.
[184] K.G. Jain, S. Mohanty, A.R. Ray, R. Malhotra, B. Airan, Culture & differentiation of mesenchymal stem cell into osteoblast on degradable biomedical composite scaffold: in vitro study, Indian J. Med. Res. 142 (2015) 747–758.
[185] S.M. Hosseini, A. Vasaghi, N. Nakhlparvar, R. Roshanravan, T. Talaei-khozani, Z. Razi, Differentiation of Wharton's jelly mesenchymal stem cells into neurons in alginate scaffold, Neural Regener. Res. 10 (2015) 1312–1316.
[186] M.-Z. Ismadi, P. Gupta, A. Fouras, P. Verma, S. Jadhav, J. Bellare, et al., Flow characterization of a spinner flask for induced pluripotent stem cell culture application, PLoS One 9 (2014) e106493.
[187] J.A. King, W.M. Miller, Bioreactor development for stem cell expansion and controlled differentiation, Curr. Opin. Chem. Biol. 11 (2007) 394–398.
[188] G. Mazzoleni, D. Di Lorenzo, N. Steimberg, Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? Genes Nutr. 4 (2009) 13–22.
[189] J.F. Alvarez-Barreto, S.M. Linehan, R.L. Shambaugh, V.I. Sikavitsas, Flow perfusion improves seeding of tissue engineering scaffolds with different architectures, Ann. Biomed. Eng. 35 (2007) 429–442.
[190] J.D. Berry, P. Godara, P. Liovic, D.N. Haylock, Predictions for optimal mitigation of paracrine inhibitory signalling in haemopoietic stem cell cultures, Stem Cell Res. Ther. 6 (2015) 58.
[191] I. Martin, D. Wendt, M. Heberer, The role of bioreactors in tissue engineering, Trends Biotechnol. 22 (2004) 80–86.
[192] Q.A. Rafiq, K. Coopman, A.W. Nienow, C.J. Hewitt, Systematic microcarrier screening and agitated culture conditions improves human mesenchymal stem cell yield in bioreactors, Biotechnol. J. 11 (2016) 473–486.
[193] O.-W. Merten, Advances in cell culture: anchorage dependence, Phil. Trans. R. Soc. B : Biol. Sci. 370 (2015), 20140040.
[194] A.L. van Wezel, Growth of cell-strains and primary cells on micro-carriers in homogeneous culture, Nature 216 (1967) 64–65.
[195] A. Shekaran, E. Sim, K.Y. Tan, J.K. Chan, M. Choolani, S. Reuveny, et al., Enhanced in vitro osteogenic differentiation of human fetal MSCs attached to 3D microcarriers versus harvested from 2D monolayers, BMC Biotechnol. 15 (2015) 102.
[196] D.E. Kehoe, D. Jing, L.T. Lock, E.S. Tzanakakis, Scalable stirred-suspension bioreactor culture of human pluripotent stem cells, Tissue Eng. Part A 16 (2010) 405–421.
[197] Y. Nie, V. Bergendahl, D.J. Hei, J.M. Jones, S.P. Palecek, Scalable culture and cryopreservation of human embryonic stem cells on microcarriers, Biotechnol. Progr. 25 (2009) 20–31.
[198] M. Serra, C. Brito, M.F. Sousa, J. Jensen, R. Tostoes, J. Clemente, et al., Improving expansion of pluripotent human embryonic stem cells in perfused bioreactors through oxygen control, J. Biotechnol. 148 (2010) 208–215.
[199] C. Szpalski, M. Barbaro, F. Sagebin, S.M. Warren, Bone tissue engineering: current strategies and techniques–part II: cell types, Tissue Eng. Part B Rev. 18 (2012) 258–269.
[200] A.K. Chen, X. Chen, A.B. Choo, S. Reuveny, S.K. Oh, Expansion of human embryonic stem cells on cellulose microcarriers, Curr. Protoc. Stem Cell Biol. (2010), Chapter 1:Unit 1C.11.
[201] A.T. Lam, A.K. Chen, J. Li, W.R. Birch, S. Reuveny, S.K. Oh, Conjoint propagation and differentiation of human embryonic stem cells to cardiomyocytes in a defined microcarrier spinner culture, Stem Cell Res. Ther. 5 (2014) 110.
[202] A.T.-L. Lam, A.K.-L. Chen, S.Q.-P. Ting, S. Reuveny, S.K.-W. Oh, Integrated processes for expansion and differentiation of human pluripotent stem cells in suspended microcarriers cultures, Biochem. Biophys. Res. Commun. 473 (2016) 764–768.
[203] S.J. Lu, T. Kelley, Q. Feng, A. Chen, S. Reuveny, R. Lanza, et al., 3D microcarrier system for efficient differentiation of human pluripotent stem cells into hematopoietic cells without feeders and serum [corrected], Regen. Med. 8 (2013) 413–424.
[204] L.T. Lock, E.S. Tzanakakis, Expansion and differentiation of human embryonic stem cells to endoderm progeny in a microcarrier stirred-suspension culture, Tissue Eng. Part A 15 (2009) 2051–2063.
[205] Y. Park, Y. Chen, L. Ordovas, C.M. Verfaillie, Hepatic differentiation of human embryonic stem cells on microcarriers, J. Biotechnol. 174 (2014) 39–48.
[206] S. Sart, Y.J. Schneider, S.N. Agathos, Influence of culture parameters on ear mesenchymal stem cells expanded on microcarriers, J. Biotechnol. 150 (2010) 149–160.
[207] D. Schop, F.W. Janssen, E. Borgart, J.D. de Bruijn, R. van Dijkhuizen-Radersma, Expansion of mesenchymal stem cells using a microcarrier-based cultivation system: growth and metabolism, J. Tissue Eng. Regen. Med. 2 (2008) 126–135.
[208] Y.M. Lin, J.F. Lim, J. Lee, M. Choolani, J.K. Chan, S. Reuveny, et al., Expansion in microcarrier-spinner cultures improves the chondrogenic potential of human early mesenchymal stromal cells, Cytotherapy 18 (2016) 740–753.
[209] M. Morille, K. Toupet, C.N. Montero-Menei, C. Jorgensen, D. Noël, PLGA-based microcarriers induce mesenchymal stem cell chondrogenesis and stimulate cartilage repair in osteoarthritis, Biomaterials 88 (2016) 60–69.
[210] A. Shekaran, A. Lam, E. Sim, L. Jialing, L. Jian, J.T. Wen, et al., Biodegradable ECM-coated PCL microcarriers support scalable human early MSC expansion and in vivo bone formation, Cytotherapy 18 (2016) 1332–1344.
[211] R.M. Hernandez, G. Orive, A. Murua, J.L. Pedraz, Microcapsules and microcarriers for in situ cell delivery, Adv. Drug Deliv. Rev. 62 (2010) 711–730.
[212] T. Maguire, E. Novik, R. Schloss, M. Yarmush, Alginate-PLL microencapsulation: effect on the differentiation of embryonic stem cells into hepatocytes, Biotechnol. Bioeng. 93 (2006) 581–591.
[213] Š. Selimović, J. Oh, H. Bae, M. Dokmeci, A. Khademhosseini, Microscale strategies for generating cell-encapsulating hydrogels, Polymers 4 (2012) 1554-.
[214] L. Gasperini, J.F. Mano, R.L. Reis, Natural polymers for the microencapsulation of cells, J. R. Soc. Interface 11 (2014) 20140817.
[215] M.S. Shoichet, R.H. Li, M.L. White, S.R. Winn, Stability of hydrogels used in cell encapsulation: an in vitro comparison of alginate and agarose, Biotechnol. Bioeng. 50 (1996) 374–381.
[216] S.M. Dang, S. Gerecht-Nir, J. Chen, J. Itskovitz-Eldor, P.W. Zandstra, Controlled, scalable embryonic stem cell differentiation culture, Stem Cells 22 (2004) 275–282.
[217] N. Siti-Ismail, A.E. Bishop, J.M. Polak, A. Mantalaris, The benefit of human embryonic stem cell encapsulation for prolonged feeder-free maintenance, Biomaterials 29 (2008) 3946–3952.
[218] N. Wang, G. Adams, L. Buttery, F.H. Falcone, S. Stolnik, Alginate encapsulation technology supports embryonic stem cells differentiation into insulin-producing cells, J. Biotechnol. 144 (2009) 304–312.
[219] S. Fang, Qiu Y.-d, L. Mao, Shi X.-l, D.-c. Yu, Y.-t. Ding, Differentiation of embryoid-body cells derived from embryonic stem cells into hepatocytes in alginate microbeads in vitro, Acta Pharmacol. Sin. 28 (2007) 1924–1930.
[220] D. Jing, A. Parikh, E.S. Tzanakakis, Cardiac cell generation from encapsulated embryonic stem cells in static and scalable culture systems, Cell Transplant. 19 (2010) 1397–1412.
[221] N.S. Hwang, S. Varghese, Z. Zhang, J. Elisseeff, Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine-glycine-aspartate-modified hydrogels, Tissue Eng. 12 (2006) 2695–2706.
[222] W.S. Toh, E.H. Lee, X.M. Guo, J.K. Chan, C.H. Yeow, A.B. Choo, et al., Cartilage repair using hyaluronan hydrogel-encapsulated human embryonic stem cell-derived chondrogenic cells, Biomaterials 31 (2010) 6968–6980.
[223] M. Tang, W. Chen, M.D. Weir, W. Thein-Han, H.H. Xu, Human embryonic stem cell encapsulation in alginate microbeads in macroporous calcium phosphate cement for bone tissue engineering, Acta Biomater. 8 (2012) 3436–3445.
[224] A. Bozza, E.E. Coates, T. Incitti, K.M. Ferlin, A. Messina, E. Menna, et al., Neural differentiation of pluripotent cells in 3D alginate-based cultures, Biomaterials 35 (2014) 4636–4645.
[225] L. Li, A.E. Davidovich, J.M. Schloss, U. Chippada, R.R. Schloss, N.A. Langrana, et al., Neural lineage differentiation of embryonic stem cells within alginate microbeads, Biomaterials 32 (2011) 4489–4497.
[226] J. Barminko, J.H. Kim, S. Otsuka, A. Gray, R. Schloss, M. Grumet, et al., Encapsulated mesenchymal stromal cells for in-vivo transplantation, Biotechnol. Bioeng. 108 (2011) 2747–2758.
[227] R.P. Meier, R. Mahou, P. Morel, J. Meyer, E. Montanari, Y.D. Muller, et al., Microencapsulated human mesenchymal stem cells decrease liver fibrosis in mice, J. Hepatol. 62 (2015) 634–641.
[228] E.C. Stucky, R.S. Schloss, M.L. Yarmush, D.I. Shreiber, Alginate micro-encapsulation of mesenchymal stromal cells enhances modulation of the neuro-inflammatory response, Cytotherapy 17 (2015) 1353–1364.
[229] A. Batorsky, J. Liao, A.W. Lund, G.E. Plopper, J.P. Stegemann, Encapsulation of adult human mesenchymal stem cells within collagen-agarose microenvironments, Biotechnol. Bioeng. 92 (2005) 492–500.
[230] T. Guo, L. Yu, C.G. Lim, A.S. Goodley, X. Xiao, J.K. Placone, et al., Effect of dynamic culture and periodic compression on human mesenchymal stem cell proliferation and chondrogenesis, Ann. Biomed. Eng. 44 (2016) 2103–2113.
[231] J.F. Markusen, C. Mason, D.A. Hull, M.A. Town, A.B. Tabor, M. Clements, et al., Behavior of adult human mesenchymal stem cells entrapped in alginate-GRGDY beads, Tissue Eng. 12 (2006) 821–830.
[232] A. Moshaverinia, X. Xu, C. Chen, K. Akiyama, M.L. Snead, S. Shi, Dental mesenchymal stem cells encapsulated in alginate hydrogel co-delivery microencapsulation system for cartilage regeneration, Acta Biomater. 9 (2013), http://dx.doi.org/10.1016/j.actbio.2013.07.023.
[233] M. Endres, N. Wenda, H. Woehlecke, K. Neumann, J. Ringe, C. Erggelet, et al., Microencapsulation and chondrogenic differentiation of human mesenchymal progenitor cells from subchondral bone marrow in Ca-alginate for cell injection, Acta Biomater. 6 (2010) 436–444.
[234] M. Westhrin, M. Xie, M.Ø. Olderøy, P. Sikorski, B.L. Strand, T. Standal, Osteogenic differentiation of human mesenchymal stem cells in mineralized alginate matrices, PLoS One 10 (2015) e0120374.
[235] S.A. Abbah, W.W. Lu, D. Chan, K.M. Cheung, W.G. Liu, F. Zhao, et al., Osteogenic behavior of alginate encapsulated bone marrow stromal cells: an in vitro study, J. Mater. Sci. Mater. Med. 19 (2008) 2113–2119.

[236] B.P. Chan, T.Y. Hui, M.Y. Wong, K.H. Yip, G.C. Chan, Mesenchymal stem cell-encapsulated collagen microspheres for bone tissue engineering, Tissue Eng. Part C Methods 16 (2010) 225–235.
[237] A.W. Lund, J.A. Bush, G.E. Plopper, J.P. Stegemann, Osteogenic differentiation of mesenchymal stem cells in defined protein beads, J. Biomed. Mater. Res. Part B Appl. Biomater. 87 (2008) 213–221.
[238] J.K. Wise, A.I. Alford, S.A. Goldstein, J.P. Stegemann, Synergistic enhancement of ectopic bone formation by supplementation of freshly isolated marrow cells with purified MSC in collagen-chitosan hydrogel microbeads, Connect. Tissue Res. 57 (2016) 516–525.
[239] S. Halldorsson, E. Lucumi, R. Gomez-Sjoberg, R.M. Fleming, Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices, Biosens. Bioelectron. 63 (2015) 218–231.
[240] L.M. Przybyla, J. Voldman, Attenuation of extrinsic signaling reveals the importance of matrix remodeling on maintenance of embryonic stem cell self-renewal, Proc. Natl. Acad. Sci. U. S. A. 109 (2012) 835–840.
[241] L. Kim, M.D. Vahey, H.Y. Lee, J. Voldman, Microfluidic arrays for logarithmically perfused embryonic stem cell culture, Lab Chip 6 (2006) 394–406.
[242] J. Guild, A. Haque, P. Gheibi, Y. Gao, K.J. Son, E. Foster, et al., Embryonic stem cells cultured in microfluidic chambers take control of their fate by producing endogenous signals including LIF, Stem Cells 34 (2016) 1501–1512.
[243] O. Gagliano, N. Elvassore, C. Luni, Microfluidic technology enhances the potential of human pluripotent stem cells, Biochem. Biophys. Res. Commun. 473 (2016) 683–687.
[244] P. Ramamurthy, J.B. White, J. Yull Park, R.I. Hume, F. Ebisu, F. Mendez, et al., Concomitant differentiation of a population of mouse embryonic stem cells into neuron-like cells and schwann cell-like cells in a slow-flow microfluidic device, Dev. Dyn. 246 (2017) 7–27.
[245] G.G. Giobbe, F. Michielin, C. Luni, S. Giulitti, S. Martewicz, S. Dupont, et al., Functional differentiation of human pluripotent stem cells on a chip, Nat. Meth. 12 (2015) 637–640.
[246] P. Occhetta, M. Centola, B. Tonnarelli, A. Redaelli, I. Martin, M. Rasponi, High-throughput microfluidic platform for 3D cultures of mesenchymal stem cells, towards engineering developmental processes, Sci. Rep. 5 (2015) 10288.
[247] X. Ju, D. Li, N. Gao, Q. Shi, H. Hou, Hepatogenic differentiation of mesenchymal stem cells using microfluidic chips, Biotechnol. J. 3 (2008) 383–391.
[248] X. Gao, X. Zhang, H. Xu, B. Zhou, W. Wen, J. Qin, Regulation of cell migration and osteogenic differentiation in mesenchymal stem cells under extremely low fluidic shear stress, Biomicrofluidics 8 (2014) 052008.
[249] K.M. Kim, Y.J. Choi, J.H. Hwang, A.R. Kim, H.J. Cho, E.S. Hwang, et al., Shear stress induced by an interstitial level of slow flow increases the osteogenic differentiation of mesenchymal stem cells through TAZ activation, PLoS One 9 (2014) e92427.
[250] J. Zhou, L.E. Niklason, Microfluidic artificial vessels for dynamic mechanical stimulation of mesenchymal stem cells, Integr. Biol. (Camb.) 4 (2012) 1487–1497.
[251] C.-W. Tsao, Y.-C. Cheng, J.-H. Cheng, Fluid flow shear stress stimulation on a multiplex microfluidic device for rat bone marrow stromal cell differentiation enhancement, Micromachines 6 (2015) 1470.